8th CMAPSEEC

Conference on Medicinal and Aromatic Plants of Southeast European Countries

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- **Association for Medicinal and Aromatic Plants of Southeast European Countries (AMAPSEEC)**
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Association for Medicinal and Aromatic Plants of Southeast European Countries (AMAPSEEC)

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Forward

Medicinal and aromatic plants (MAP) have been essential resources for human health from ancient times to the present day. The majority of the world's population depends on traditional medicine for primary health care needs. More than 35.000 plant species are used in herbal medicine and as spices, out of the most are of a local importance due to traditional use. Because of their increasing appliance in pharmaceutical, food, cosmetic and beverage industry, as well as use in folk and official medicine, veterinary and plant protection, herbal industry has been recognized as an important element of global economy. Together with growth in global demand for medicinal plants and in local demand for plant based traditional medicines, the pressure on the existing populations of medicinal plants has increased tremendously during the last few decades. The extinction or scarcity of these plants is not only a problem for conservation – it also results in serious problems for people's health and livelihoods. Cultivation may reduce harvesting pressure on some wild species, particularly rare and threatened species, and thus can also be an important production strategy that supports conservation. South East Europe is particularly appreciated for richness in indigenous MAP resources and long tradition in use of MAP and their products. The region is known as one of the main suppliers of MAP raw material into EU and US. In addition, medicinal plants were being the subject of a great scientific interest in the SEE region, where significant contribution to understanding of various research aspects in number of MAP species was achieved by Conferences on Medicinal and Aromatic Plants of Southeast European Countries, under organization and support of the Association for Medicinal and Aromatic Plants of Southeast European Countries (AMAPSEEC) established in 2000 by the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, R. of Serbia. We are very proud that,the 8th Conference on MAP of SEE countries took place in Durres, Albania, after the second was held in Greece, the third in Slovakia, followed by Romania, Czech Republic, Turkey and Serbia. The 8th CMAPSEEC gathers together not only the researchers from SEE region, but from all over the world. At the Conference participated over 320 researchers, experts, company's representatives and guests interested in MAP diversity, biology, conservation, ecology, phytochemistry, pharmacology, breeding, cultivation and biotechnology. Near 283 summaries of research contributions were presented, out of about 100 in the form of full papers. All contributions covered very different research areas, and were classified into to the three distinct groups: "MAP diversity at all levels and tools for its evaluation", "Pharmacology and biological effects of active MAP compounds" and "Map cultivation, breeding and biotechnology".

The full papers, categorized as review papers and original scientific papers, within this proceedings were reviewed by our Scientific Committee. In addition, a small group of papers was issued either without referring on reviewer's comments. However, we decided to include them as well, considering the subjects and research approaches interesting. We strongly believe that presented results will contribute to a general knowledge on MAP, and will encourage both young researchers and processing companies to deal with many species whose composition and biological effects were appointed as promising. Moreover, we hope that pleasant and collegial atmosphere additionally contributed to establishing of the new professional and personal contacts and to strengthening of the ones already established. Finally, strong network on scientists and professionals interested in MAP under AMAPSEEC umbrella might bring new project ideas and new value in the near future.

Editors, Prof. Dr. Zora Dajić Stevanović and Assoc. Prof. Dr. Alban Ibraliu

Foreword from the Minister of Education and Sports
Mrs. Lindita Nikolla

Ladies and Gentleman

Please Allow me to initially thank the organizers of this special event, who made possible this important conference regarding scientific research in Albania.
I also wish to express my congratulations to all the participants in the conference and to the authors of the research work to be presented today, especially our foreign guests.

Todays conference is important, because scientific research is not only an academic achievement, but also in that research and innovation determine economic growth, create new job opportunities and establish the progress of a country.

The goverment of the Republic of Albania, mandated in September 2013, has immediately initiated concrete measures to be taken in the field of reserch, while considering scientific research as a compass which can direct towards the most successful economic sectors. With an edict from the primeminister Mr.Edi Rama, an independent comission was established for the reformation of higher education and scientific research in Albania.

After several months of intensive work the commission has designed the first draft of the report on the reform, which is being discussed these days with all the interest groups and actors involved. After being finalized this report will serve as the base document for the new higher education law .
The reform will provide concrete steps toward the quality improvement of scientific research in Albania. An essential element for this improvement is the development of technical capacities and project managing, but foremost is the international cooperation at the academic level. For this reason, the ministry of education and sports has undertaken the steps for the association of albania in the EU scientific research program, Horizon 2020. This program offers good opportunities for the development of scientific research at the regional and global level.

Our goverment by being a part of the Horizon 2020 program and also Erasmus Plus, has created the opportunities that favor the internationalization of higher education and scientific research, promotion of student exchange, young researchers and academic staff.
In these programs it has been clearly stated that maximal support will be offered for the cooperation between native and foreign researchers.
Also the ministry of education and sport will support scientific research programs of quality and inovation, with structural and program financing.

Scientific research transforms every challenge into an opportunity, therefore your contribution is needed in protecting our biodiversity and promoting the application of biotechnology in creating new varieties of cultivated crops.

Honored participants, I believe that the endeavours and collaborations in the field of science, research and innovation are one of the better ways in stregthening our regional partnership and advancing our national and regional development

Thank you for your contribution, and I wish you further success in the proceeding of the conference

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TABLE OF CONTENTS

Section I

"MAP diversity at all levels and tools for its evaluation"

Introductory plenary lecture- review paper

ARE THERE STILL NEGLECTED MEDICINAL PLANTS BEYOND OFFICIAL AND TRADITIONAL CONSIDERATION?

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ABSTRACT

The use of medicinal plants and natural product drugs is embedded in a space between belief and science. By definition, 'traditional' use of herbal medicines implies substantial historical use which is factual for many products that are available as 'traditional herbal medicines'. On the other side, well acknowledged and studied herbs of verified biological effects which exhibit positive impacts on human health are listed in national, regional and world pharmacopeias, accounting for not more than 10% of those traditionally used. Modern science search for both new biological effects and interactions of already accepted medicinal herbs, and new metabolites which might show prospective bioactive effects. Metabolomic studies in plants revealed tens of thousands of such compounds. Secondary metabolites represent features that can be expressed in terms of ecological, taxonomic and biochemical differentiation and diversity. The biosynthesis and accumulation of secondary metabolites provide a basis for biochemical systematics and chemosystematics. In addition, the wide molecular diversity of secondary metabolites throughout the plant kingdom represents an extremely rich biogenic resource for the discovery of novel drugs and for developing innovative drugs. Such drugs are sometimes hidden in "ordinary" plants, i.e. in wide spread ruderal and weed species whose extracts have been recently appointed in term of respectable antioxidant and/or antimicrobial activity, as shown for *Chenopodium album*, *Convolvulus arvensis*, *Cynodon dactylon*, etc. Many of endemic plants surveyed all over the world and in Southeast Europe, started to be evaluated for their phytochemical profiles, whereas some of them were appointed as valuable source of biologically active compounds (as reported for e.g. *Achillea alexandri regis*, *Helichrysum plicatum, Micromeria dalmatica, Sideritis raeseri*, etc.). Moreover, plants occurring on specific habitats, such as serpentine, salt affected and calciferous soils for example, might be of a high interest in research of actions of the secondary metabolites, knowing that extra-optimal environmental conditions may trigger biosynthesis and accumulation of biologically active compounds. Our study aims to highlight potential of under-researched and neglected medicinal plants of Southeast Europe, including presentation of our own results on phytochemistry and biological activity in group of halophytes, endemic species and relatives of some known medicinal plants.

***Keywords**: under-researched species, halophytes, endemics, weeds, wild relatives*

INTRODUCTION

Medicinal plants have been essential resources for human health from ancient times to the present day. According to the World Health Organization (WHO), the majority of the world's population depends on traditional medicine for primary health care needs. Plants and their various remedy products can be used in prevention, alleviation of pre-ailment symptoms, and problems associated with chronic conditions, as well as for facilitation recovery and for well-being. The term "medicinal herbs" is sometimes misleading because most of them do not cure acute ailments. Moreover, the majority of medicinal plants is used in traditional human and veterinary therapy by local community and is not verified by official pharmacopeias.

Independently on the fact that a solid concept on how to define "the medicinal herb" still lacks, such species are known to contain specific secondary metabolites of favorable biological effects on human and/or animal health. Medicinal and aromatic plants (MAPs) are used as herbal remedies and food supplements. Due to their increasing appliance in pharmaceutical, food, cosmetic and beverage industry, as well as use in folk and official medicine, veterinary and plant protection, herbal industry has been recognized as important element of the global economy. The use of medicinal plants and natural product drugs is embedded in a continuum between religion and science. Apart from empirically learned medicinal and pharmacological properties, the selection of medicinal plants is dependent on cognitive features, ecological factors and cultural history [1].

In many developing countries, a large proportion of the population relies on traditional practices and long history in use medicinal plants. Although modern medicine may side-by-side accept and agree with such traditional practice, herbal medicines have often maintained their reputation for historical and cultural reasons [2].

Harvesting of medicinal plants can provide an important source of income for local people, which in turn may produce incentives for the conservation of these species and their natural habitats. Herbal industry involves a range of different activities, such as collecting of wild herbs, cultivation, processing and trade. Increased interest for use of medicinal plants and needs of modern pharmaceutical, cosmetic and food industry accelerated demand for many herbs on the global market. Great importance of world's herbal industry, as well as the increasing demand for herbs and spices and their related products, affect the genetic variability of their populations and endangerment of species under higher pressure of exploitation.

ESTIMATIONS OF NUMBER OF MEDICINAL PLANTS AND CAUSES OF THEIR JEOPARDIZING

It is thought that at least every fourth plant species among 250.000-300.000 of flowering plants is in use by man. World-wide, it is estimated that up to 70,000 species are used in folk medicine [3] out of the WHO reports over 21,000 plant taxa used for medicinal purposes [4]. Unfortunately, there is no idea how many species are used in the other areas of use, like cosmetics, spirits or aromas which makes determining exactly the number of all medicinal and aromatic plant species used worldwide impossible.

Unfortunately, many medicinal plant species are becoming scarce in the wild. Major causes include inadequate regimes over land and plants as well as the pressure of an ever-expanding market fuelled by human population growth. Wild populations of medicinal plants can be depleted by over-collecting and unsustainable management practices. The survival of medicinal plant populations and species can also be affected by loss and conversion of natural habitat, pollution and climate change, competition with invasive species, change in land use, deforestation, and other factors that influence ecosystems, species interactions and population viability and dynamics [5]. An estimated 4,000 to 10,000 species of medicinal plants face possible local, national, regional or global extinction, with serious consequences for livelihoods, economies and health care systems [6].

As stated by [7, 8], etc., global community is concerned because of jeopardizing of wild medicinal and aromatic plants (MAP) populations which resulted in sets of various directives, declarations and recommendations aiming to develop mechanisms for preservation of MAPs diversity and sustainable use of their resources. Some of a relevant international documents related to either global biodiversity (naturally targeting wild herbs as well) and in particular MAPs conservation, use, processing and trade are: CBD (Convention on Biological Diversity, 1993), CITES convention (Convention on international trade of wild flora and fauna, 1975), Millennium declaration UN and plan of Agenda 21 implementation (UN Developmental Program, 2000), Guidelines on the conservation of medicinal plants (WHO, IUCN, WWF, TRAFFIC, 2005), Cartagena protocol on biosafety (2003), EU Directive on genetic resources (2008), EU Directive 2001/83/EC on products for human health, Directive 2004/24/EC of the European parliament and of ther council of 31 march 2004 amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use.

The main general and long-term goal of conservation of target MAP species is to protect, manage and monitor the selected populations in the direction of maintenance of the natural evolutionary processes, thus allowing new variations in the gene pool allowing the species to adapt to changing environmental condition [9].

There are three main conservation strategies of MAP species: in situ (protection of their habitats), ex situ (conservation at species and germplasm level through field collections, botanical gardens and gene banks out of their natural habitats) and domestication/reintroduction and cultivation which could be conducted either in situ or ex situ, or “on domo” [10]. Because of the rapid increase in demand for MAP and situation that many species of high economic importance are in risk because of diminishing populations and/or reduction of genetic variability, there is obvious need for their conservation and implementation of standards of sustainable wild harvesting. Moreover, there is a global trend for searching of new plant drugs and new metabolites of more pronounced, improved or innovative health and well-being effects. Wide molecular diversity of secondary metabolites within the plant kingdom represents a fantastic natural source for discovery of novel drugs.

SEARCHING FOR NEW DRUGS AND METABOLOMICS TODAY

It is thought that among 250.000 known flowering plants only 6% has for biological activities and 15% for their chemical constituents [11]. Plants produce tens of thousands of different natural products known as secondary metabolites. Secondary metabolites generally show

greater individuality and diversity in their molecular structure than primary metabolites. Certain compound classes also appear to be extraordinarily rich in secondary metabolites, e.g. the structurally diverse groups of alkaloids, phenolics, acetogenins and terpenoids [12].

Although the true role of such metabolites in plants remains mostly unknown, it is evident that plants invest in synthesizing, accumulating and sorting such metabolites, often produced through complex and highly regulated biosynthetic pathways [13]. There are many reports indicating specific role of some classes of secondary metabolites, including better plant adjustment to environment conditions, pollination, protection against predators, antimicrobial, antifungal and other pathogen defense operations, as well as various parasitic, pathogenic or symbiotic interactions [14, 12, 15, 16, 17, 18]. Still, the biological roles of most products of plant secondary metabolism are not fully highlighted. Some recent reports pointed out that evolution favors means to increase biodiversity to provide novel compounds to be challenged by evolution; thus many of the compounds synthetized and accumulated by plants are part of the ‘‘screening’’ arsenal that might become important in due evolutionary time reflecting particular life strategies embedded in a particular phylogenetic framework [12, 13].

Having in mind enormous richness and diversity of secondary metabolites, out of many exhibit certain biological effects and biological interactions, it could be expected that “metabolomics” era will enable discovery of a very promising and effective bio-active plant products. Since small group of plants is up to now evaluated upon the content and activity of its secondary metabolites, it is reasonable to expect that some neglected and/or under-researched plants could be a valuable source of novel metabolites possessing prospective bio-activity.

WHERE TO LOOK FOR NEW PLANT METABOLITES?

It has been recently reported that comparison of results using regression analysis from five different floras by Moerman et al [19]. showed that holarctic peoples rely on similar plant families for their health care [1]. Furthermore, in the same survey of Leonti [1] it was stated that different studies, utilizing either regression analysis or with the binomial method, revealed that plant families belonging to the Euasterids are generally overused while families belonging to the Poales (e.g. Poaceae, Cyperaceae) as well as the family of the Orchidaceae are underrepresented in medicinal floras. Euasterids include Garryalales, Gentianales, Lamiales, Solanales, Apiales, Aquifoliales, Asterales, and Dipsacales [20]. The Euasterids are very popular in local medicines, herbals like De Materia Medica and national pharmacopeias of numerous EU and other countries. Euasterids are known to include many conspicuous species endowed with prominent organoleptic properties and the content of diverse spectrum of bioactive secondary metabolites, where the most characteristic are iridoids, idole and Asterales, pyrrolizidine alkaloids, essential oils, flavones, higher inulins, polyphenole carboxylic acids and sesquiterpenes [21]. A great part of the medicinal Euasterids are weedy species growing in or near human habitations and are therefore easy accessible. Weedy species encompass many fodders, fruits, spices and on their turn make up a considerable part of medicinal floras [22]. Probably one of the most known cases is *Artemisia annua*, widespread weed species highly appreciated for antimalarial effects [23] due to its artemisinin [24].

There are also some recent reports on novel metabolites and/or new activity of plant extracts obtained from well-known and very distributed weeds. New tropane alkaloids were found in above-ground parts of *Convolvulus arvensis* (tropine, pseudotropine, and tropinone), and are thought to cause toxic effects [25]. New sesquiterpenes of a nerolidol skeleton, named "amarantholidols" and "amarantholidosides" were isolated from *Amaranthus retroflexus* [26]. In many spread weed and/or invasive species of the genera *Cirsium* and *Carduus* numerous compounds were detected, including flavonoids, sterols and triterpenes, alkaloids, polyacetylenes, acetylenes and hydrocarbons, sesquiterpene lactones, phenolic acids, lignans, and few other compounds [27]

One of the world's most abundant weed, the *Chenopodium album* has been recently studied for total phenolic content, free phenolic acids and related antioxidant activity, where leaf methanolic extracts showed prominent radical scavenging capacity [28]. Moreover, leaf ethanolic extract of the same species exhibited significant antioxidant effect too, in addition to antibacterial and antigenotoxic activity [29]. Recent study of [30] demonstrated the DNA protective activity and immunomodulatory property of the fresh juice of the grass weed species *Cynodon dactylon*, which announced one later report on effectiveness of the *C. dactylon* aqueous extract for alleviating hyperglycemia and improving lipid profile in diabetic rats and these could be used in diabetic and coronary heart disease (CHD) management [31].

It was shown that total flavones, total phenolics, and total saponins of the invasive species *Solidago canadensis* contribute to the allelopathic effects of the species on some soilborne pathogens [32]. It is believed that secondary compounds from weed species may serve either as natural herbicides [33] produced upon allelopathy mechanisms [34] or as natural insecticides [35]. Finally, there is long evidence on tradition use of several European weed and/or wasteland species, including *Plantago* ssp., *Veronica* spp., *Chelidonium majus, Fumaria officinalis, Polygonum aviculare, Symphytum officinale, Urtica dioica, Cichorium intybus, Taraxacum officinale*, and many others.

Medicinal species need to be abundant and easily accessible, and a plant of high importance is more likely to be known to people over a wide region; therefore rare species are usually not contained in pharmacopoeias because they would easily become extinct or at best hard to find [1]. Nevertheless, many of rare or scarcely distributed plants have been evaluated for their phytochemical and pharmacological properties. This refers for both plants of specific and fragile habitats, such as saline, high-alpine, extremely wet, serpentine, etc., and for relatives of well-known and acknowledged medicinal species, mostly endemics.

It has been already reported that numerous salt tolerant species – the halophytes, exhibit certain antimicrobial, antiviral, anticancer and some other favourable biological effects, which mainly refer to *Catharanthus roseus, Cressa cretica, Tribulus terestris, Aloe barbadensis, Calotropis procera, Acanthus ilicifolius, Eryngium maritimum*, etc [36]. Some recent studies on halophytic secondary metabolites and related biological interactions pointed out that some highly salt tolerant species, such as *Crithmum maritimum, Cakile maritima, Eryngium maritimum, Atriplex halimus, Mesembryanthemum crystallinum* etc., are characterized for the presence of different phenolic compounds responsible for antioxidant effects [37]. Our own results performed on over 40 halophytes collected from different types of salt affected soils of the southern Balkan, indicate their solid capacity for free radical scavenging, which was characteristic for *Artemisia santonicum, Mentha pulegium* and, especially to *Statice gmelinii*. The highest content of the total phenolic compounds and the

flavonoids was determined for *Artemisia santonicum*, *Aster tripolium* var. *pannonicus*, *Mentha pulegium*, *Achillea collina*, *Statice gmelinii* and *Atriplex littoralis*, and *Atriplex tatarica* var. *diffusa*, *A. littoralis* and *Camphorosma annua*, respectively (article submitted, under review). It has been already accepted that halophytes evolved very efficient system of radical oxygen scavenging as adaptive mechanism linked to salt stress [38].

Endemic plants are a very special feature of flora and vegetation of any region of the world. Such plants are known to be of a limited distribution and thus are of a high biodiversity conservation concern. South East Europe, and the Balkan Peninsula, is particularly appreciated by quantitative (number of species) and qualitative (endemic, relic and internationally important species and habitats) values of biodiversity, including a great variety of mosaic habitats within the mountains, forests, grasslands, river gorges, lakes and coastline [5]. Flora of the Balkan Peninsula is one of the most diverse floras in Europe, comprising more than 8000 species of vascular plants, out of about 2600-2700 are known as endemic species [39]. Balkan and Rhodope Mountains are recognized as global Centers of Plant Diversity. The special feature of the Balkan's flora, the high endemism, also refers to MAP species.

In general, endemic MAP species of SEE are not sufficiently researched and many of them are in fact unknown for their chemical profiles and related biological activity. Knowing the importance of searching for new phytochemicals and natural sources of high biological effectiveness, much more attention should be paid on comprehensive and coordinated research of MAP endemics, out of some have already shown promising performances (Tab. 1).

Tab.1. Endemic MAP species of SEE: phytochemistry and biological activity (modified after Dajic Stevanovic et al., 2012).

Species	Active compound	Activity	Reference
Achillea alexandri-regis	Triterpenoids, flavonoids, phenolic acids, lignans	Cytotoxic , antioxidant, anti-inflammatory, anti-ulcer activity	[40]
Anthemis cylopoda	Essential oils	Antibacterial	[41]
Cinnamomosma fragrans	Essential oils	Antimicrobial	[42]
Helichrysum plicatum	Phenolic compounds, flavonoids, triterpenoids, diterpenoids, Ssteroids, phloroglucinol	Cytotoxic, antidiabetic, antiviral antimicrobial, antimutagenic antioxidant activity	[43] [44]
Centaurea kosaninii	Sesquiterpene lactones	Cytotoxic activity	[45]
Hypericum rumeliacum	Phenolic compounds and flavonoids	Antimicrobial, antioxidant, anti-inflammatory activity	[46]
Micromeria dalmatica	Phenolic compounds	Antimicrobial activity	[47]
Satureja cuneifolia	Phenolic compounds	Antimicrobial, analgesic, antioxidant activity	[47]
Sideritis raeseri	Phenols and flavonoids	Antimicrobial, anti-inflammatory, analgesic, gastroprotective, antioxidant	[48]
Sideritis condensata, Sideritis erythrantha	Phenolics	Antimicrobial	[49]
Swertia punctata	Xanthones, flavone-C-glucoside	Hypoglycemic, hepatoprotective,	[50]

		antituberculous, antimalarial, anti-inflammatory	
Thymus moroderi, Thymus piperella	Essential oils	Antibacterial, antioxidant	[51]

Finally, there is a group of under-researched species which are (relatively) frequent in flora of forests or grasslands. The good example could be widely spread clover species. The chemical profile of clovers is partly recognized. It is known that besides isoflavones, *Trifolium* plants synthesize a wide range of phenolic and polyphenolic compounds such as flavonoids, saponins, clovamides (caffeic acid esters), phenolic acids and other substances, which could serve for the production of herbal medicines, and an alternative to the conventional hormonal replacement therapy, whereas extracts obtained from various clovers have been shown to possess antioxidative and anti-inflammatory activities, inhibiting angiogenesis and displaying anti-cancer properties [52].

Our recent focus was targeted on phytochemical evaluation of natural populations of different clover species, such as *Trifolium pretense*, *T. repens, T. pannonicum, T. montanum, T. alpestre* and *T. hybridum*. The highest content of the total isoflavones, as well as daidzein, genistein, biochanin A and formononetin A was found in populations of the red clover (results in preparation for publication in collaboration with Prof. J. Cvejic, Faculty of Medicine, University of Novi Sad). Total of over 50 populations of *Trifolium* species was also evaluated for the content of the total phenolics, concentration of flavonoids and antioxidant activity. In *Trifolium pannonicum*, the highest values of the total phenolics and flavonoids of 120,08 mg GA/g extract and 351,6 mg Ru/g extract were determined, respectively, corresponding with the best antioxidant properties (31,66 IC 50 value in mg/ml). All obtained results on phenolic compounds, isoflavones and biological activity, were in accordance with consideration of Kolodziejczyk-Czepas [52] that clover species other than *T. pratense* could be very valuable source of bio-active molecules.

CONCLUSION

Medicinal and aromatic plants are a subject of many studies, aiming at discovery of new secondary metabolites and/or distinguishing the biological role of bio-active compounds in biological interactions. Many of these species are well appreciated and known throughout the world, while some others are under-researched and only recently have been target of phytochemical and pharmacological evaluation. It was shown that apart of plants indicated in national and international pharmacopeias, and those listed in local ethnobotanical/ ethnopharmacognosy studies and data-bases, there are still neglected plants which possess interesting phytochemical profiles and promising biological effects. Among them, plants of specific habitats (e.g. saline habitats, high-alpine, serpentine ground habitats, etc.,) followed by plants of a very limited distribution – the endemics could be stressed. On the other hand, some widely spread plants, such as weeds or ordinary grassland species have been also appointed as valuable sources of various phytochemicals.

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Introductory plenary lecture- Review paper

GENETIC RESOURCES OF MEDICINAL AND AROMATIC PLANTS OF ALBANIA – CURRENT STATUS OF THE NATIONAL COLLECTION OF MAPS

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ABSTRACT

Albania is a country of rich natural genetic diversity of medicinal and aromatic plants, which are grown over a wide range of ecological habitats. Medicinal and aromatic plants have a major contribution to the growth of agricultural products in all regions of Albania. National collection of medicinal and aromatic plants is represented by more than 300 species, with the ex situ status of conservation, and in situ and on farm status conservation status. The *ex situ* seed collection of medicinal and aromatic plants stored in Albanian Gene Bank is represented with 450 accessions, including 17 genera . In the last two decades several of medicinal and aromatic plants are affected by the phenomenon of genetic erosion, and 68 endangered species were included in the National Red Book List.

Key words**: Genetic diversity, medicinal and aromatic plants, conservation status.**

INTRODUCTION

Albania is a country of rich natural genetic diversity of medicinal and aromatic plants and represents one of the European countries with a very rich flora. Albanian Flora includes about 3 250 plant species or about 30 % of European Flora [15], out of 30 are endemic species and about 180 sub-endemic species [19]. This diversity is attributable to favourable climatic conditions, ranging from coastal subtropical towards inland continental climates; its geographical position in the Mediterranean region and in the Balkan Peninsula resulted in many different types of landscape [15].

Medicinal and aromatic plants are grown over a wide range of ecological habitats in the country, including forests, grasslands, alpine, coastal and many other habitats [14, 16]. Medicinal and aromatic plants have a major contribution to the growth of agricultural products value in all regions of Albania.

There is a huge number of medicinal plants in the world. In US, almost 1800 medicinal plant species are commercially available. It has been estimated that about 13,000 species of plants have been used as traditional medicines by various cultures around the world for a centuries [**19**]. In any case, , there is no other category of plants useful to man (with the possible exception of ornamental plants) that includes so many species, and the question naturally arises why such a staggering number of plants have useful medicinal properties.

Medicinal and aromatic plant collection of Albania is represented by more than 300 species; many of them are well-known by the local population, which have a long tradition in collecting them either for individual and family use or for sale. Medicinal and aromatic plant collection in the ex situ status of conservation, and in situ and on farm status of conservation, represents about 10% of the Albanian flora [1].

The aim of this study was to assess the current status of the national collection of medicinal and aromatic plants in Albania.

MATERIAL AND METHODS

Data sampling: Data sampling is realized using information on the total occurrence of medicinal and aromatic plant species in Albania gathered from ex situ collection data in database of medicinal plants stored in Albanian genebank and in situ and on farm status of conservation. External data were also gathered from EURISCO database [4], from the Global Biodiversity Information Facility (GBIF) database [6], from published papers [6, 7, 11, 12], and information gathered from contact persons of Albanian genebank.

Geographic distribution: The study was conducted for all natural growing areas of medicinal and aromatic plants in 12 districts of Albania. Each population (group of individuals) represent a geo-referenced observation, where one observation supposes presence of a medicinal and aromatic plants population. All geo-referenced observations (ex situ data) chosen to carry out spatial analysis, were entered into the GIS analysis, as presence points, [8, 10] and were spatially represented as point maps using DIVA-GIS tools [9, 10].

RESULTS AND DISCUSSION

Geo-referenced data

Large amount of information was gathered and recorded for each medicinal and aromatic species. All data were checked for inconsistencies and data points without coordinates were removed from the data of the ex situ collection of medicinal and aromatic plants. Data points with incorrect coordinates on the collecting site were corrected where possible while duplicate or doubtful data were removed [17]. All medicinal and aromatic species were also screened carefully to resolve any scientific name conflicts [2]. The accessions not presented as genetic material stored in genebank were also removed.

Geographic distribution

After checking the presence or absence of accessions the data included in the medicinal and aromatic plants database with partial or complete information for a total of 450 presence points, 446 points of medicinal and aromatic plants were compiled and used to evaluate the geographic distribution of MAPs currently observed in Albania (Figure 1).

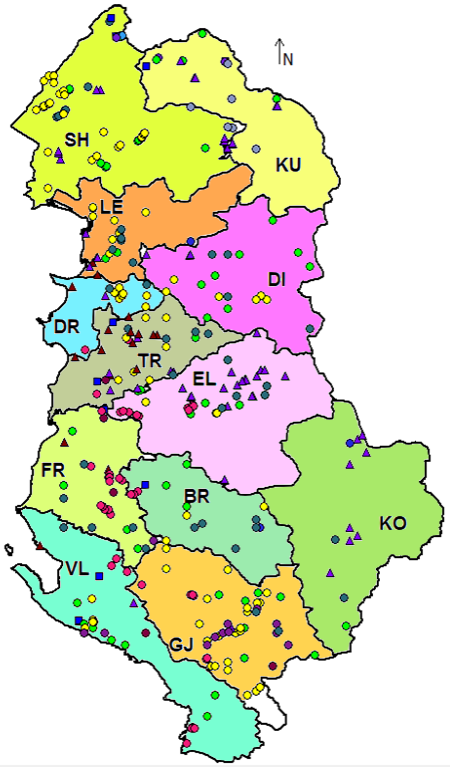

Figure 1. Geographic distribution of MAP in 12 districts in Albania

Detailed analysis of ex situ data of medicinal and aromatic plants stored in Albanian genebank shows that national inventory of medicinal and aromatic plants is compound of 450 accessions, including 17 genera . Comparisons of ex situ species data show that *Salvia officinalis* was observed and presented in 104 points - sites (observed populations) acc, *Origanum vulgare* in 74 points - sites, *Satureja montana* in 67 present points, *Thymus vulgaris* in 45 present points, *Rosmarinus officinalis* in 42 present points, *Matricaria chamomilla* in 20 present points, etc. Other species, such as *Ocimum basilicum, Achillea millefolium, Hypericum perforatum, Sideritis syrinica, Juniperus communis, Melissa officinalis, Gentiana lutea*, etc. are presented in small number of points (observed populations) ranging from 1 to 9sites, showing the endangered position or status of these species. Higher number of species was observed in Shkodra district (9 species), in Tirana and Berat districts (8 species), in Kuksi and Vlora districts (7 species) (Figure 1).

Upon analyzing composition of species diversity in all Albanian areas it was noted that in the area of Mediterranean forest and shrub habitats, the following species dominate: : laurel (*Laurus nobilis*), Dalmatian sage (*Salvia officinalis*), pomegranate (*Punica granatum*), sea onion (*Urginea maritima*), hawthorn (*Crataegus monogyna*), thorn apple (*Datura stramonium*), chamomile (*Chamomilla recutita*), greater plantain (*Plantago major*), nettle (*Urtica dioica*), elm leaf blackberry (*Rubus ulmifolius)*, etc. In the area of deciduous oak forest the most abundant were: : juniper (*Juniperus communis; Juniperus oxycedrus*), large-leaved lime (*Tilia platyphyllos*), dog rose (*Rosa canina*), wild marjoran (*Origanum vulgare*), lemon balm (*Melissa officinalis*) etc.. In the area of beech forests, the most presented were: : bilberry (*Vaccinium myrtillus*), banewort (*Atropa belladona*), common horse-chestnut (*Aesculus hippocastanum),* raspberry (*Rubus* idaeus) etc. In the area of alpine pastures were: micromeria (*Micromeria thymifolia*), cowslip (*Primula veris*) etc.

Medicinal plants are found in almost all plant families, but mainly belong: 1) Lamiaceae Family, including sage (*Salvia officinalis*), winter savory (*Satureja montana*), bushy thyme (*Thymus capitatus*), lemon balm (*Melissa officinalis*), long-stemmed thyme (*Thymus longicaulis*), mint Savory (*Calamintha grandiflora*), pennyroyal (*Mentha pulegium*), mountain tea (*Sideritis raeseri*) etc. 2) Asteraceae Family, such as mugwort (*Artemisia vulgaris*), tansy (*Tanacetum vulgaris*), chamomile (*Chamomilla recutita*), milfoil (*Achillea millefolium*); 3) and families with lower number of MAP species (e.g. Apiaceae, Pinaceae etc.).

The most frequently collected MAP species and herbs (in terms of volume) were: sage (*Salvia officinalis* juniper *(Juniperus communis),* St John's-wort (*Hypericum perforatum*), winter savory (*Satureja montana*), bearberry *(Arctostaphylos uva-ursi),* rose *(Rosa canina),* hawthorn *(Crataegus monogyna),* dandelion *(Taraxacum officinale)* and wormwood *(Artemisia absinthium).* According information of the local community, much larger quantities of these species than officially listed seem in fact to be collected and exported to international companies from Albania

Collecting missions' results

Collecting activities related to plant genetic resources including medicinal and aromatic plants were organized sporadically in Albania. During 10 collecting missions organized in 2004 – 2005 years, a total of 358 accessions (9 species) of medicinal and aromatic plants, including *Origanum vulgare* (78 accessions) , *Salvia officinalis* (115 acc.), *Satureja montana* (86 acc.), *Thymus vulgaris* (45 acc.), etc. , were collected (Table 1, Figure 2).

Table 1. Results of collecting missions organized in 2004-2005, 2009-2010, and 2013

Collecting mission	2004-2005	Collecting mission	2009-2010	Collecting mission	2013
Origanum vulgare	**78**	*Gentiana lutea*	**11**	*Origanum vulgare*	**28**
Salvia officinalis	**115**	*Artemisia absianthum*	**2**	*Thymus vulgaris*	**4**
Ocimum basilicum	**8**	*Origanum vulgare*	**2**	*Satureja montana*	**17**
Thymus vulgaris	**45**	*Hypericum perforatum*	**6**	*Hypericum perforatum*	**7**
Satureja Montana	**86**	*Thymus sp*	**1**	*Salvia officinalis*	**6**
Juniperus communis	**5**	*Syderitis raeseri*	**1**	*Micromeria juliana*	**1**
Mentha piperita	**2**	*Satureja montana*	**1**	*Achillea millefollium*	**3**
Chamomila recutita	**9**	*Teucrium polium*	**6**	*Matricharia chamomila*	**1**
Vaccinium myrtillus	**10**	*Trifolium sp*	**1**	*Sinapis arvensis*	**1**
		Melissa officinalis	**1**	*Primula vulgaris*	**1**
				Gentiana lutea	**2**
				Melissa officinalis	**1**
				Teucrium polium	**1**
				Mentha piperita	**1**
Total accessions collected	**358**		**32**		**74**

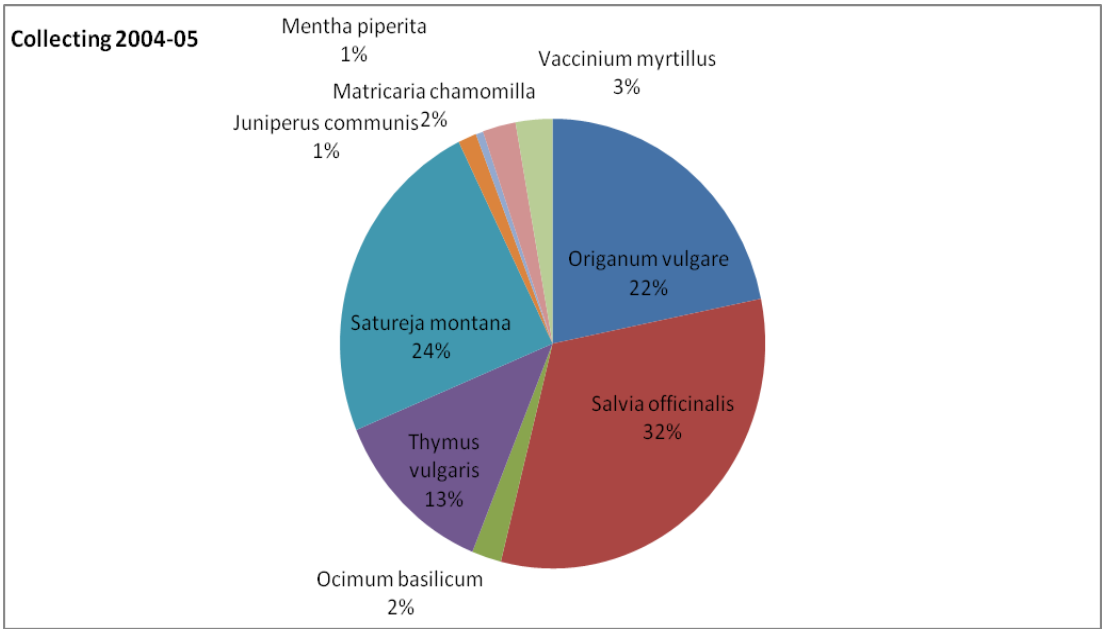

Figure 2. Collecting results of 10 collecting missions organized in 2004-2005

In 2009 and 2010 were organized 3 collecting missions, and a total of 32 accessions (9 species) of medicinal and aromatic plants, including *Gentiana lutea* (11 acc), *Hypericum perforatum* (6 acc), *Teucrium polium* (6 acc), etc., were collected (Table 1, **Figure 3**).

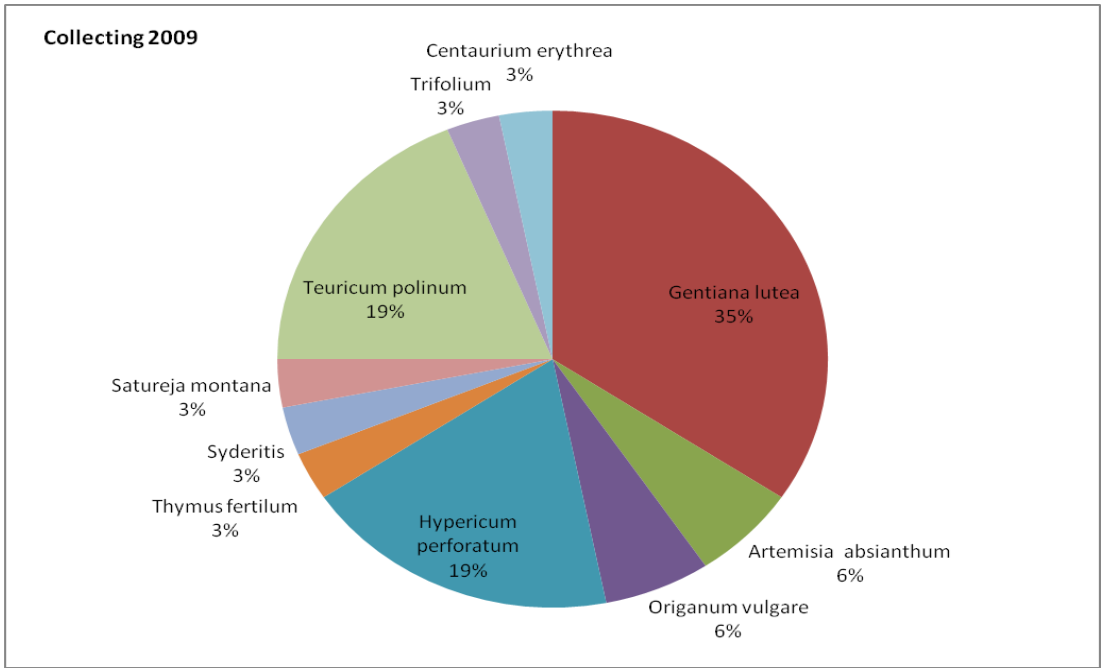

Figure 3. Collecting results of 3 collecting missions organized in 2009-2010

In year 2013. 6 collecting missions were organized and a total of 74 accessions (14 species) of medicinal and aromatic plants, including *Origanum vulgare* (28 acc.), *Thymus vulgaris* (4 acc.), *Satureja montana* (17 acc.), etc., were collected (Table 1, **Figure 4**).

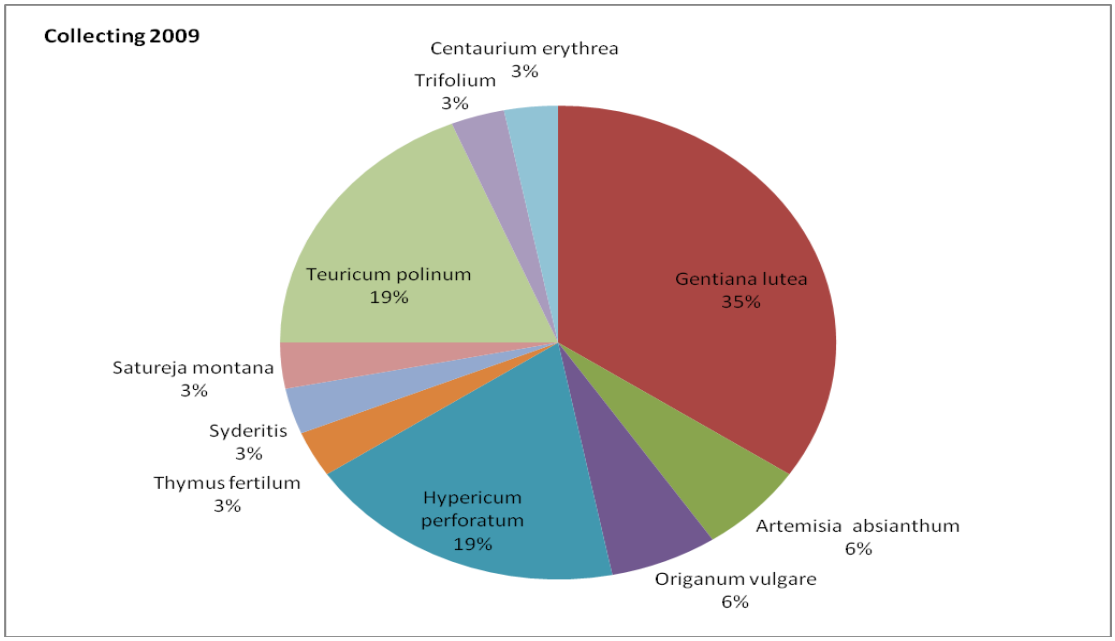

Figure 4. Results of 6 collecting missions organized in 2013

Economic Importance of Medicinal and Aromatic plants

The high costs of western pharmaceuticals put modern health care services out of reach of most of the world's population, which relies on traditional medicine and medicinal plants to meet their primary health care needs. Even where modern medical care is available and affordable, many people prefer more traditional practices. In the last decade, there has been considerable interest in resurrecting medicinal plants in western medicine, and integrating their use into modern medical systems. The reasons for this interest are various, and include:

- **low cost:** herbs are relatively inexpensive and the cost of pharmaceuticals to governments and individuals is rising
- **drug resistance:** the need for alternative treatments for drug-resistant pathogens
- **limitations of medicine:** the existence of ailments without an effective pharmaceutical treatment
- **medicinal value:** laboratory and clinical corroboration of safety and efficacy for a growing number of medicinal plants
- **cultural exchange:** expanding contact and growing respect for foreign cultures, including alternative systems of medicine
- **commercial value:** growing appreciation of trade and other commercial economic opportunities represented by medicinal plants

Trade in medicinal plants

Wild-harvesting of medicinal and aromatic plants is still widely common in the rural population, because it creates much-needed additional income. In some regions wild-

collection of MAPs is becoming increasingly important. Most collectors belong to underprivileged social groups, such as children, women, and older people who are occupied with wild-collecting MAPs during the vegetation period from early spring to the late autumn.

According to a survey, the wild-harvesting and sale of MAPs is the second-most important source of income for poorer rural households in Albania [14]. Albania reported the export of an average 7,650 tonnes of pharmaceutical plants at a value of US$ 12 million. The export was destined to 26 countries with the dominance of EU countries, which imported almost 80 % of the commodity.

The remaining 20 % were exported to other East and Southeast European countries, mainly Greece, followed by FYR of Macedonia and Turkey and to non-European countries such as the USA and, much less to Japan. Within the EU, Germany is the foremost importer of botanicals from Albania; it imported on average 2,770 tones. The second and third most important purchasers are Italy, importing on average 1,690 tones, and France, with average imports of 780 tones.

Domestic and foreign markets for medicinal plants are growing rapidly and provide important opportunities for the development and diversification of Albanian agriculture. Currently, sage (*Salvia officinalis*) dominates among the medicinal crops of Albania. Smaller surfaces are also cultivated with rosemary (*Rosmarinus officinalis*), oregano (*Origanum vulgare*), lemon balm (*Melissa officinalis*), Mountain tea (*Sideritis raeseri*), chamomile (Chamomila recutita*),* clover (*Trifolium pratense*), peppermint (*Mentha x piperita*), lavender (*Lavandula angustifolia*), basil (*Ocimum basilicum*), gentian (*Gentiana lutea),* etc.

Species Regarded Endangered in Albania

Most of the world's supply of medicinal herbs is obtained by wild collection, not by cultivation. Harvesting renewable wild resources is perfectly legitimate so long as this is conducted in a sustainable fashion that does not eliminate populations or degrade the habitat where the plants grow. There are still many medicinal plant species in Albania that are abundant in nature and can be collected in a sustainable way . When a plant is (or becomes) popular medicinally, its commercial value is likely to lead to over collection.

Many very important Albanian drug plants have been over collected to the point that the wild Albanian reserve has been designated as "threatened" (for examples, *Gentiana lutea*, *Taxus baccata*,). Some of the medicinal plants are included in the group of species of national conservation concern that are protected by National Legislation or in the National Red Data Book. They are threatened as being part of habitats where a strong human impact is observed: cuttings, intensive grazing, and deforestations in some places with aim to profit arable land for cultivation of agricultural plants or by the wrong practices of their collecting.

Sometimes cultivation is preferable even when there is a wild supply, because of the advantages of growing certain cultivars (for example, uniform maturation, or consistency of chemical composition), proximity of supply, or quality considerations (for example, being able to certify a product as organically produced).

Albania is blessed with abundant wild lands, and there is great potential for cultivation of medicinal plants.

In the last two decades several medicinal and aromatic plants are affected by the phenomenon of genetic erosion. Sixty-eight medicinal species are considered of the endangered status and 40 medicinal and aromatic plants are included in the National Red Data Book (Table 2).

Table 1. List of MAP Species Regarded as Endangered in Albania

1. *Aesculus hippocastanum L.*
2. *Allium ursinum L.*
3. *Anacamptis pyramidalis (L.) L.C. Rich.*
4. *Arctostaphylos uva- ursi (L.) Spreng.*
5. *Atropa belladonna L.*
6. *Colchicum autumnale L.*
7. *Convallaria majalis L.*
8. *Dictamnus albus L.*
9. *Dryas octopetala L.*
10. *Ephedra distachya L.*
11. *Eryngium maritimum L.*
12. *Galanthus nivalis L.*
13. *Gentiana lutea L.*
14. *Glaucium flavum Crantz.*
15. *Hypericum androsaemum L.*
16. *Ilex aquifolium L.*
17. *Juniperus oxycedrus* L.
18. *Juniperus communis L.*
19. *Menyanthes trifoliata L.*
20. *Nymphaea alba L.*
21. *Orchis coriophora L.*
22. *Orchis laxiflora Lam.*
23. *Orchis mascula L.*
24. *Orchis militaris L.*
25. *Orchis morio L.*
26. *Orchis pallens L.*
27. *Orchis papilionacea L.*
28. *Orchis provincialis Balb.*
29. *Orchis purpurea Huds.*
30. *Orchis simia L.*
31. *Orchis tridentata Scop.*
32. *Osmunda regalis L.*
33. *Paeonia peregrina Mill.*
34. *Phyllitis scolopendrium (L.) Newm.*
35. *Rhus coriaria L.*
36. *Ruta graveolens L.*
37. *Salvia officinalis L.*
38. *Sideritis raeseri L.*
39. *Taxus baccata L.*
40. *Tilia argentea L.*

Source: (Red Data Book of Albania and from official orders of Ministry of Environment)

CONCLUSIONS

- Albania has diverse climate and soil types that enable growth of several medicinal plants.
- Medicinal and aromatic plants play an important role in everyday life in Albania. These plants have enormous economic benefits.
- Despite the existing enormous potentials for production and subsequent benefits from these commodities, no strong attention has been given to improve their cultivation, production and processing technologies.
- The MAPs are highly exploited by uncontrolled harvests which people carry out for own consume or for selling (seeds, roots, leaves, flowers etc,) on the markets.
- The loss of genetic diversity in some species is at a very critical level.

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Original scientific paper

MEDICINAL AND AROMATIC PLANTS (MAP) IN VASCULAR FLORA OF THE DRENICA MOUNTAIN-REPUBLIC OF KOSOVO

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ABSTRACT

Drenica Mountain (650 to 1051 m) takes part in Kosovo's central mountains, respectively in the western mountains of the Drenica region. These terrains lie in the central part of Kosovo between Çiçavica, Plain of Kosovo (Goleshi), Llapusha, Carraleva Mountains and Anadrini. Drenica Mountain with an area of over 90 km^2 predominated by continental climate lies in the relation Carralevë-Lapushnik. It is mainly natural surface covered by forests and pastures, while there are some settlements (villages). Intensive field research was conducted in the period 2003-2006, and is complemented and completed until 2013. Of the 540 species of vascular flora present in the Drenica Mountain [24] a significant number of them are medicinal and aromatic plants (MAPs). Within the number of MAPs of these area, there are species that are nutritious, honey and toxic. Sustainable use of these species as well as reducing the negative anthropogenic impacts would result with good preservation, and advancement of this wealth of nature of the Republic of Kosovo.

Key words: *Vascular flora, MAPs, Drenica Mountain, Kosovo.*

INTRODUCTION

Drenica Mountain is a mountain area with fairly wide area, located in the territory of municipalities: Malishevë, Drenas (Gllogoc), Lipjan and Therandë (Suharekë). Explored territory covers 94.13 km^2 (9413 ha). The border of explored area begins from the Llapushnik Gorge, continues along the mountain to the right, and to the left until the Carraleva Gorge. it includes localities (villages): Arllat, Tërpezë, Lladroc, Senik, Lladroviq, Ngucat, Temeqinë, Bllacë, Carralevë, Pjetërshticë, Krojmir, Shalë, Baicë, Nekoc and Kizharekë to meet again at the starting point. Inside this boundary with a small number of residents remain localities: Berishë, Fshat i Ri, Kleçkë, Divjakë, Karaçicë, Dugë, Javor and Luzhnicë [24].

The territory of Drenica Mountain (Lapushnik-Carralevë) includes terrains of different substrates and natural habitats, the majority of which is covered by forests and pastures, and less meadows. Geomorphology is quite interesting and attractive. Geological composition is of different ages and petrology is quite heterogeneous. Elevation ranges from approximately

650 m to 1051 m. Viewed from the geological point of view most of the territory of Drenica Mountain consists of rocky sediment complexes of Mesozoic and Cenozoic periods. So the geology of the explored area is composed of limestone, serpentines, silicates and alluviums (in small areas). The Pedology is quite heterogeneous [24]. Most of the territory of Drenica Mountain soils are not conducive to the cultivation of crops except small alluvial surfaces and some small hilly areas.

Flora and vegetation of the Drenica Mountain as a whole are intensively researched in the period 2003-2006, while research on MAPs is completed until 2013 [24, 25, 26]. Although the anthropogenic factor is quite active, however MAPs represent important potential within the vascular flora of this area of the Repubic of Kosovo. Within the MAPs of the territory of Drenica Mountain are found species that are also nutritious, but the honey plants and plants with toxic effects (poisonous plants) are present as well.

MATERIAL AND METHODS

Floral material is collected by standard methods of vascular flora inventory for the purpose of final determination and collecting species in herbarium. For determining the species, we have used literature of authors: [1, 12, 17, 19, 20, 25, 34, 35, 36, 37, 38, 39, 40, 41, 43, 44, 45, 48, 50] Scientific designation is made mainly by "Flora Europaea 1-5" [48] and "Dictionary of plant names" [28]. To identify the species of MAPs, and also the nutritional, honey and poisonous plants, we are also served with literature by other authors, such as: [2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 15, 18, 21, 22, 23, 27, 29, 30, 31, 32, 33, 42, 46, 47, 49, 51, 52]. Publications by GTZ [16] and WHO [53, 54] are used also. Ranking of families, genera and species; for practical reasons is done according to the alphabet. We also used data from the Internet (www.google.com). Plant material is regulated and determined by standard methods in the period 2003-2013, and is located within the Private Herbarium (EK_Herb.Pers/I-III/1996-2013). Abbreviations used in the paper are: Natural (Nat.), Cultivated (Cult.), Phanerophyta (P), Chamaephyta (Ch), Hemicriptophyta (H), Geophyta (G), Therophyta (T), Medicinal (Med.), Nutritional (Nutr.), Honey plant (Hon.), Poisonous plant-Toxic (Tox.), Unknown floristic element (?).

RESULTS AND DISCUSSION

Research of vascular flora of Drenica Mountain is mainly realized during the period 2003-2006, and is continuously updated until 2013. Separation of MAPs by the total number of species determined, resulted in 185 MAPs within the species which has nutritional, honey but also poisonous plants.

Pteridophyta

1. Fam. Aspleniaceae. 1.Ceterach officinarum Lam. et DC. [Asplenium ceterach L.]. Nat. Euro-Asiatic. H. Med. 2.Fam. Equisetaceae. 2. Equisetum arvense L. Nat. Euro-Asiatic. G. Med. 3.Fam. Hypolepidaceae. 3. Pteridium aquilinum (L) Kuhn. in Deck. Nat. Cosmopolitan. G. Med. 4.Fam. Polypodiaceae. 4. Polypodium vulgare L. Nat. Euro-Asiatic. Ch. Med.

Spermatophyta-Gymnospermae

5.Fam. Pinaceae. 5. Pinus nigra Arnold [P. laricio Poir.] Nat. Euro-Asiatic. H. Med. 6.Fam. Cupressaceae. 6. Juniperus communis L. Nat. Cicrumpolar. P. Med. 7. Juniperus oxycedrus L. Nat. Mediterranean. P. Med.

Spermatophyta-Angiospermae

Magnoliatae [Dicotyledoneae]

7.Fam. Acanthaceae. 8. Acanthus balcanicus Heywood et Richardson. Nat. Mediterranean. H. Med. **8.Fam. Anacardiaceae**. 9. Cotinus coggygria Scop. [Rhus cotinus L.]. Nat. Euro-Asiatic. P. Med. **9.Fam. Apiaceae [Umbelliferae]**. 10. Daucus carota L. Nat. Cult. Cosmopolitan. H (T). Med. 11. Eryngium campestre L. Nat. Pontic. H. Med. 12. Petroselinum crispum (Mill.) Nyman ex A. W. Hill. [P. sativum Hoffm.] Cult. Europaean. H. Med. Nutr. **10.Fam. Araliaceae**. 13. Hedera helix L. Nat. Submediterranean. P. Med. Hon. **11.Fam. Aristolochiaceae**. 14. Aristolochia clematitis L. Nat. Submediterranean. G. Med. **12.Fam. Asclepiadaceae**. 15. Vincetoxicum hirundinaria Medicus. Nat. Europaean. H. Med. Tox. 16. Vincetoxicum huteri Vis. et Arch. Nat. Europaean. H. Med. Tox. **13.Fam. Asteraceae [Compositae].** 17. Achillea millefoliatum L. Nat. Euro-Asiatic. H. Med. 18. Bellis perennis L. Nat. Europaean. H. Med. 19. Bidens tripartita L. Nat. Euro-Asiatic. T. Med. 20. Calendula officinalis L. Cult. Europaean (Euro-Med.). T. (H.). Med. 21.Centaurea cyanus L. Nat. Europaean. H. Med. 22. Cichorium intybus L. Nat. Cosmopolitan. H. Med. Hon. 23.Conyza canadensis (L.) Cronq. [Erigeron canadensis L.] Nat. Cosmopolitan. T. Med. 24. Eupatorium cannabinum L. Nat. Euro-Asiatic. H. Med. 25. Helianthus tuberosus L. Cult. North-American plant.?. T. Med. 26. Hieracium pilosella L. Nat. Euro-Asiatic. H. Med. 27. Senecio jacobaea L. Nat. Euro-Asiatic. H. Med. 28. Tanacetum vulgare L. Nat. Euro-Asiatic. H. Med. 29. Tussilago farfara L. Nat. Euro-Asiatic. G. Med. 30. Taraxacum officinale L. Nat. Euro-Asiatic. H. Med. **14.Fam. Betulacea**. 31. Alnus glutinosa Gaertn. Nat. Euro-Asiatic. P. Med. **15.Fam. Boraginaceae**. 32. Pulmonaria officinalis L. Nat. Europaean. H. Med. **16.Fam. Brassicaceae [Cruciferae]**. 33. Capsella bursa-pastoris (L.) Med. Nat. Cosmopolitan. H. Med. 34. Lepidium campestre (L.) R. Br. in Aiton. Nat. Euro-Asiatic. T. Med. **17.Fam. Cannabaceae**. 35. Humulus lupulus L. Nat. Cicrumpolar. H. Med. **18.Fam. Caprifoliaceae**. 36. Lonicera caprifolium L. Nat. Europaean. P (NP). Med. 37. Viburnum lantana L. Nat. Europaean. P (NP). Med. Tox. 38. Sambucus ebulus L. Nat. Euro-Asiatic. G. Med. Hon. 39. Sambucus nigra L. Nat. Europaean. P. Med. **19.Fam. Caryophyllaceae**. 40. Agrostemma githago L. [Lychnis githago (L.) Scop.] Nat. Euro-Asiatic (Eurosiberian). T. Med. 41. Saponaria officinalis L. Nat. Cult. Euro-Asiatic (Eurosiberian). H. Med. Tox. **20.Fam. Celastraceae**. 42. Evonymus europaeus L. Nat. Europaean. P. Med. **21.Fam. Convolvulaceae**. 43. Calystegia sepium R. Br. [Convolvulus sepium L.] Nat. Cosmopolitan. G. Med. 44. Convolvulus althaeoides L. Nat. Mediterranean. H. Med. **22.Fam. Cornaceae**. 45.Cornus mas L. Nat. Euro-Asiatic. P (NP). Med. Nutr. **23.Fam. Corylaceae**. 46.Corylus avellana L. Nat. Europaean. P (NP). Med. Nutr. **24.Fam. Crassulaceae**. 47.Sedum acre L. Nat. Euro-Asiatic. H. Med. Hon. 48. Sedum telephium L. Nat. Euro-Asiatic. H. Med. 49. Sempervivum tectorum L. Nat. Europaean. H. Med. **25.Fam. Cucurbitaceae**. 50. Cucumis melo L. Cult. (tropical Africa and Asia) ?. T. Med. Nutr. 51. Cucumis sativus L. Cult. (India) ?. T. Med. Nutr. 52. Cucurbita pepo L. Cult. (C.& N. America)?. T. Med. Nutr. **26.Fam. Dipsaaceae**. 53. Dipsacus laciniatus L. Nat. Euro-Asiatic ?. H. Med. **27.Fam. Euphorbiaceae**. 54. Euphorbia amygdaloides L. Nat. Europaean. H. Med. 55. Euphorbia cyparissias L. Nat. Euro-Asiatic. H. Med. 56. Euphorbia helioscopia L. Nat. Cosmopolitan. T. Med. Hon. 57. Euphorbia myrsinites L. Nat. Mediterranean. G. Med. 58. Mercurialis perrenis L. Nat. Euro-Asiatic. G. Med. 59. Ricinus communis L. Cult. Paleotropical (Africa, Asia, Oceania without Australia)?. T (Ch.). Med. Tox. **28.Fam. Fabaceae [Leguminosae]**. 60. Colutea arborescens L. Nat. Mediterranean. P. Med. Hon. 61. Coronila emerus L. Nat.

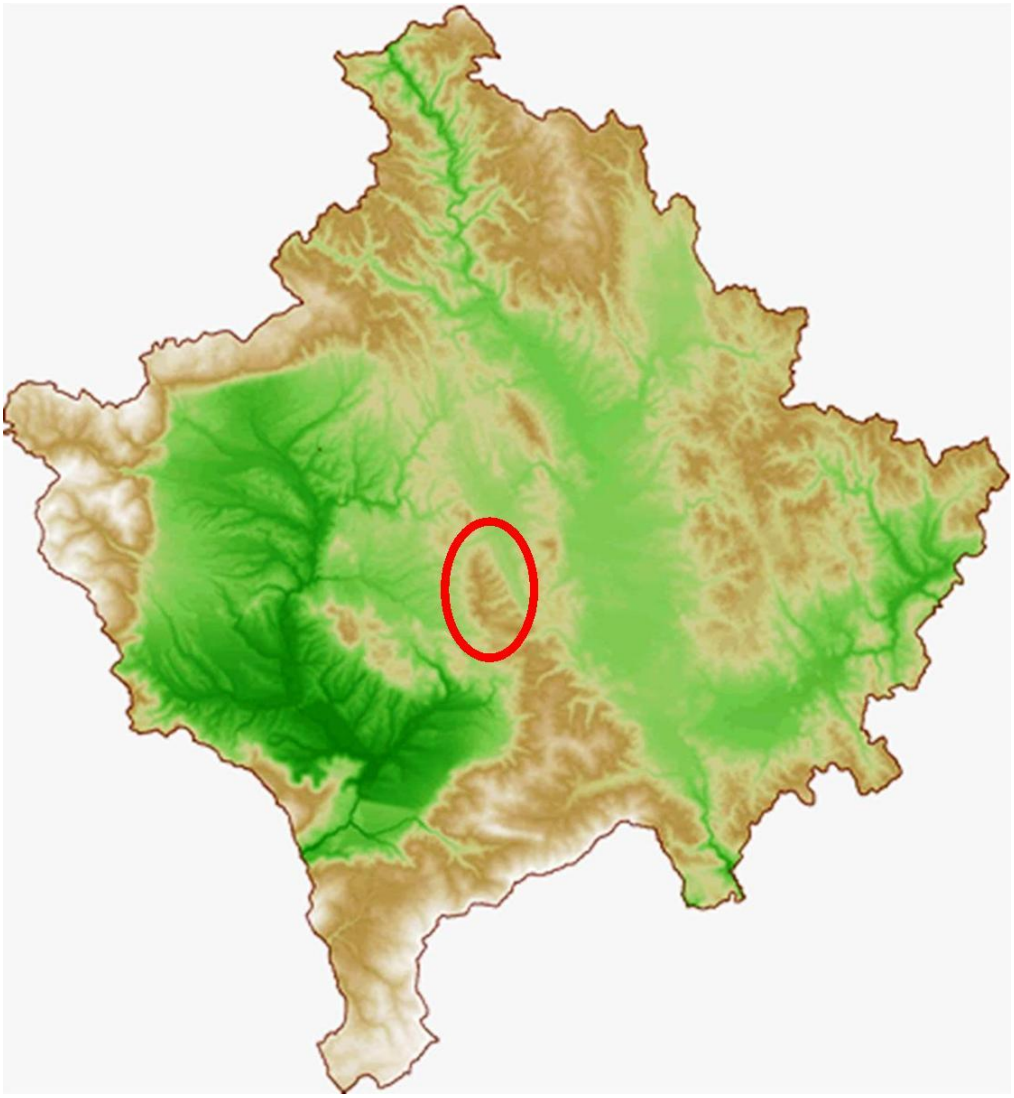
Europaean. P. Med. 62. Coronilla varia L. Nat. Euro-Asiatic. H. Med. Hon. 63. Genista ovata Waldst. et Kit. [G. tinctoria L. var. ovata (Waldst. et Kit.) Schultz.] Nat. Euro-Asiatic. Ch. Med. 64. Ononis spinosa L. Nat. Europaean. Ch. Med. Hon. 65. Medicago sativa L. Cult. Euro-Asiatic. H. Med. 66. Phaseolus vulgaris L. Cult. Plant from America (South America)?. T. Med. 67. Robinia pseudoacacia L. Cult. Plant from America (North America)?. P. Med. **29.Fam. Fagaceae**. 68. Quercus cerris L. Nat. Europaean. P. Med. **30.Fam. Fumariaceae.** 69. Corydalis bulbosa (L.) DC. [C. cava (L.) Schw. et Koerte]. Nat. Submediterranean. G. Med. 70. Corydalis solida (L.) Swartz. [C. halleri Willd.]. Nat. Fl. El. Submediterranean. G. Med. **31.Fam. Gentianaceae**. 71. Centaurium erythraea Ranfn. [C. umbellatum Gilib.] Nat. Europaean. H. Med. **32.Fam. Geraniaceae**. 72. Erodium cicutarium (L.) L' Her. Nat. Cosmopolitan. T. Med.73. Geranium sanguineum L. Nat. Europaean. H. Med. **33.Fam. Hypericaceae [Guttiferae]**. 74. Hypericum perforatum L. Nat. Cosmopolitan. H. Med. **34.Fam. Juglandaceae**. 75. Juglans regia L. Cult. Balkans (Subbalkanic). P. Med. Nutr. Native to the region stretching from the Balkans eastward to the Himalayas and southwest China. **35.Fam. Lamiaceae [Labiatae]**. 76. Calamintha officinalis Moench. [C. nepeta (L.) Savi]. Nat. Euro-Asiatic. H. Med. 77. Glechoma hederacea L. Nat. Boreal (Cirkumboreal). H. Med. 78. Marrubium peregrinum L. Nat. Mediterran. G. Med. 79. Melittis melissophyllum L. Nat. Europaean. H. Med. 80. Mentha longifolia (L.) Hudson. Nat. Europaean. H. Med. 81. Mentha pulegium L. Nat. Cosmopolitan. G. Med. 82. Origanum vulgare L. Nat. Europaean. H. Med. Hon. 83. Prunella vulgaris L. Nat. Cosmopolitan. H. Med. Hon. 84. Sideritis montana L. Nat. Euro-Asiatic. T. Med. Hon. 85. Stachys recta L. [S. czernjajevii Schost.]. Nat. Pontic. H. Med. Hon. 86. Teucrium chamaedrys L. Nat. Mediterran. Ch. Med. Hon. 87. Teucrium montanum L. Nat. Europaean. H. Med. Hon. 88. Thymus longicaulis C. Pers. Nat. Balkans. (Subbalkanic). Ch. Med. Hon. **36.Fam. Lythraceae**. 89. Lythrum salicaria L. Nat. Cosmopolitan. H. Med. **37.Fam. Malvaceae**. 90. Althaea rosea (L.) Cav. [Alcea rosea L.]. Cult. The Asian.?. H. Med. 91. Malva sylvestris L. Nat. Cosmopolitan. H. Med. **38.Fam. Moraceae**. 92. Morus alba L. Cult. The Asian (China)?. P. Med. Nutr. 93. Morus nigra L. Cult. The Asian (Cental Asia)?. P. Med. Nutr. **39.Fam. Oleaceae**. 94.Fraxinus excelsior L. Nat. Europaean. P. Med. 95. Fraxinus ornus L. Nat. Illyric. P. Med. 96. Ligustrum vulgare L. Nat. Cult. Europaean. P(NP). Med. Hon. **40.Fam. Oxalidaceae**. 97. Oxalis acetosella L. Nat. Cicrumpolar. G. Med. **41. Fam. Papaveraceae**. 98. Papaver dubium L. Nat. Europaean. T (H). Med. 99. Papaver rhoeas L. Nat. Euro-Asiatic. T (H). Med. **42.Fam. Plantaginaceae**. 100. Plantago lanceolata L. Nat. Cosmopolitan. H. Med. 101. Plantago major L. Nat. Euro-Asiatic. H. Med. 102. Plantago media L. Nat. Euro-Asiatic. H. Med. **43.Fam. Polygonaceae**. 103. Polygonum aviculare L. Nat. Cosmopolitan. T. Med. 104. Polygonum persicaria L. Nat. Euro-Asiatic. H. Med. 105. Rumex acetosa L. Nat. Euro-Asiatic. H. Med. 106. Rumex acetosella L. Nat. Cosmopolitan. H. Med. 107. Rumex crispus L. Nat. Euro-Asiatic. H. Med. 108. Rumex patientia L. Nat. Europaean. H. Med. **44.Fam. Primulaceae**. 109. Anagallis arvensis L. Nat. Cosmopolitan. T. Med. 110. Cyclamen hederifolium Aiton. [C. neapolitanum Ten.] Nat. Submediterranean. G. Med. 111. Lysimachia nummularia L. Nat. Europaean. H. Med. 112. Primula vulgaris Huds. Nat. Euro-Asiatic. H. Med. 113. Primula veris L. Nat. Europaean. H. Med. **45.Fam. Ranunculaceae**. 114. Anemone apennina L. Nat. Submediterranean. G. Med. 115. Anemone nemorosa L. Nat. Circumpolar. G. Med. 116. Clematis vitalba L. Nat. Europaean. P. Med. 117. Helloborus odorus W. et Kit. Nat. Submezic. H. Med. Tox. 118. Nigella damascena L. Nat. Mediterranean. T. Med. 119. Ranunculus ficaria L. Nat. Europaean. G. Med. 120. Ranunculus repens L. Nat. Euro-Asiatic. H. Med. **46.Fam. Rhamnaceae**. 121. Frangula alnus Mill. Nat. Euro-Asiatic. P (NP). Med. **47.Fam. Rosaceae**. 122. Agrimonia eupatoria L. Nat. Cosmopolitan. H. Med. 123. Crategus monogyna Jacq. Nat. Europaean. P (NP). Med. Hon. 124. Cydonia oblonga Mill. Cult. The Asian. ?. P. Med. Hon. Nutr. 125. Filipendula vulgaris Moench. Nat. Boreal

(Subboreal). H. Med. 126. Fragaria vesca L. Nat. Cult. Europaean. H. Med. Nutr. 127. Geum urbanum L. Nat. Cicrumpolar. H. Med. 128. Malus sylvestris Miller. Nat. Europaean. P. Med. Nutr. Hon. 129. Potentilla reptans L. Nat. Cosmopolitan. H. Med. 130. Prunus avium L. Nat. Pontic. P. Med. 131. Prunus domestica Borkh. Cult. Caucasian plant ?. P. Med. Nutr. 132. Prunus spinosa L. Nat. Euro-Asiatic. P (NP). Med. 133. Pyrus amigdaliformis Will. Nat. Mediterranean. P. Med. 134. Rosa arvensis Huds. Nat. Submediterranean. P (NP). Med. 135. Rosa canina L. Nat. Europaean. P (NP). Med. 136. Sanguisorba minor Scop. Nat. Euro-Asiatic. H. Med. 137. Sanguisorba officinalis L. Nat. Cosmopolitan. H. Med. 138. Sorbus domestica L. Nat. Cult. Europaean. P. Med. 139. Sorbus torminalis (L.) Crantz. Nat. Europaean. P (NP). Med. **48.Fam. Rubiaceae**. 140. Cruciata laevipes Opiz. Nat. Europaean. G.Med. 141. Galium aparine L. Nat. Cosmopolitan. T. Med. 142. Galium odoratum L. Nat. Europaean. G. Med. 143. Galium verum L. Nat. Euro-Asiatic. G. Med. **49.Fam. Rutaceae**. 144. Dictamnus albus L. Nat. Euro-Asiatic. Ch. Med. Hon. **50.Fam. Salicaceae**. 145. Populus tremula L. Nat. Euro-Asiatic. P. Med. 146. Salix alba L. Nat. Euro-Asiatic. P. Med. Hon. **51.Fam. Scrophulariaceae**. 147. Digitalis laevigata Waldst. et Kit. Nat. Balkans. H. Med. 148. Digitalis lanata Ehrh. Nat. Europaean. G. Med. 149. Linaria vulgaris Miller. Nat. Europaean. H. Med. 150. Veronica officinalis L. Nat. Cicrumpolar. H. Med. **52.Fam. Solanaceae**. 151. Atropa bella-donna L. Nat. Europaean. H. Hon. Med. Tox. 152. Capsicum annuum L. Cult. American plant (the tropical America)?. T. Med. Nutr. 153. Datura stramonium L. Nat. Cosmopolitan (Cosmopolitan of American origin) T. Med. Tox. 154. Hyosciamus niger L. Nat. Euro-Asiatic. T (H). Med.Tox. 155. Nicotiana tabacum L. Cult. American plant (Argentina, Bolivia)?. T. (H) Med. Tox. 156. Solanum dulcamara L. Nat. Euro-Asiatic. Ch. Med. 157. Lycopersicon esculentum Mill. [Solanum lycopersicum L.]. Cult. American plant (Central-South America, Mexico)?. T. Med. Nutr. 158. Solanum tuberosum L. Cult. American plant (South America)?. T. Med. Nutr. Tox. **53.Fam. Tiliaceae**. 159. Tilia platyphyllos Scop. [T. officinarum Crantz pro parte., T. grandifolia Ehrh.] Nat. Europaean. P. Hon. Med. 160. Tilia tomentosa Moench. [T. argentea DC.]. Nat. The Balkans. P. Hon. Med. **54.Fam. Ulmaceae**. 161.Ulmus campestris L. Nat. Euro-Asiatic. P. Med. **55.Fam. Urticaceae**. 162.Urtica dioica L. Nat. Cosmopolitan. H. Med. **56.Fam. Violaceae**. 163. Viola arvensis Murray Nat. Euro-Asiatic. T. Med. 164. Viola odorata L. Nat. Submediterranean. H. Med. **57.Fam. Vitaceae**. 165.Vitis sylvestris C. C. Gmelin [V. vinifera L. subsp. sylvestris (C. C. Gmelin) Hegi]. Nat. Cult. Its origin is not known because there are different opinions which are quite controversial, as it is known that the vine has been cultivated for thousands of years BC (before Christ) nearly Cosmopolitan. ?. P. Med. Nutr.

Liliatae [Monocotyledoneae]

58.Fam. *Alliaceae*. 166. *Allium cepa* L. Cult. Asiatic (Western-Asiatic)?. G. Med. Nutr. 167. *Allium sativum* L. Cult. Asiatic?. G. Med. Nutr. **59.Fam. *Amaryllidaceae***. 168. *Galanthus nivalis* L. Nat. Mediterranean. G. Med. **60.Fam. *Araceae***. 169. *Arum maculatum* L. Nat. Euro-Asiatic. G. Med. **61.Fam. *Asparagaceae***. 170. *Convalaria majalis* L. Nat. Submediterranean. G. Med. 171. *Asparagus tenuifolius* Lam. Nat. Mediterranean. G.Med. 172. *Polygonatum multiflorum* (L.) All. Nat. Euro-Asiatic. G. Med. 173. *Polygonatum odoratum* (Miller) Druce. Nat. Euro-Asiatic. G. Med. **62.Fam. *Iridaceae***. 174. *Iris germanica* L. Cult. Mediterranean. Cultivated and Adventive plant. G. Med. **63.Fam. *Liliaceae***. 175. *Colchicum autumnale* L. Nat. Europaean. G. Med.176. *Lilium martagon* L. Nat. Submediterranean fl. el. G. Med. **64.Fam. *Orchidaceae***. 177. *Orchis morio* L. Nat. Mediterranean. G. Med. 178. *Plantanthera bifolia* (L.) L. C. M. Richard. Nat. Euro-Asiatic. G. Med. **65.Fam. *Poaceae [Graminaceae]***. 179. *Avena sativa* L. Cult. Unknown Origin?. T. Med. Nutr. 180. *Cynodon dactylon* (L.) Pers. Nat. Cosmopolitan. G (H). Med. 181. *Dactylis*

glomerata L. Nat. Euro-Asiatic. H. Med. 182. *Phleum pratense* L. Nat. Euro-Asiatic. H.Med. 183. *Phragmites australis* (Cav.) Trin ex Stendel. [*P. communis* Trin., *Arundo phragmites* L.]. Nat. Cosmopolitan. G. Med. 184. *Poa pratensis* L. Nat. Euro-Asiatic fl. el. H. Med. 185. *Zea mays* L. Cult. American plant (Mexico), Unknown as natural, for the first time is cultivated in Mexico (was domesticated in Mexico and Central America more than 7,000 years ago from teosinthe or wild maize-*Z. mays subsp. mexicana*)?. T. Med. Nutr.



Researched area (Drenica Mountain) on the map of Kosovo (RKS)

CONCLUSIONS

Based on the results obtained for MAPs diversity within the territory of Drenica Mountain, we came up with the following conclusions:

- There are 185 recorded species of vascular flora which are also medicinal and aromatic plants (MAPs).
- These plant species belong to the 65 families of vascular flora.
- Within the MAPs identified, 160 grow in natural conditions while 31 are cultivated to grow and 6 plant species are natural but also cultivated.
- Cultivated to grow are: *Daucus carota, Petroselinum crispum, Calendula officinalis, Helianthus tuberosus, Saponaria officinalis, Cucumis melo, Cucumis sativus, Cucurbita pepo, Ricinus communis, Medicago sativa, Phaseolus vulgaris, Robinia pseudacacia, Juglans regia, Althaea rosea [Alcea rosea], Morus alba, Morus nigra, Ligustrum vulgare, Cydonia oblonga, Fragaria vesca, Prunus domestica, Sorbus domestica, Capsicum annuum, Nicotiana tabacum, Lycopersicon esculentum [Solanum lycopersicum], Solanum tuberosum, Vitis sylvestris, Allium cepa, Allium sativum, Iris germanica, Avena sativa* and *Zea mays.*
- Natural but also cultivated are 6 plant species (*Daucus carota, Saponaria officinalis, Ligustrum vulgare, Fragaria vesca, Sorbus domestica, Vitis sylvestris [V. vinifera* L. subsp. *sylvestris]).*
- Besides the medical importance, 21 species are nutritious, honey plants are 25 and 11 species are poisonous (toxic).
- Nutritious species are: *Petroselinum crispum, Cornus mas, Corylus avellana, Cucumis melo, Cucumis sativus, Cucurbita pepo, Juglans regia, Morus alba, Morus nigra, Cydonia oblonga, Fragaria vesca, Malus sylvestris, Prunus domestica, Capsicum annuum, Lycopersicon esculentum [Solanum lycopersicum], Solanum tuberosum, Vitis sylvestris [V. vinifera* subsp. *sylvestris], Allium cepa, Allium sativum, Avena sativa* and *Zea mays.*
- Honey plants are: *Hedera helix, Cichorium intybus, Sambucus ebulus, Sedum acre, Euphorbia helioscopia, Colutea arborescens, Coronilla varia, Ononis spinosa, Origanum vulgare, Prunella vulgaris, Sideritis montana, Stachys recta [S. czernjajevii.], Teucrium chamaedrys, Teucrium montanum, Thymus longicaulis, Ligustrum vulgare, Crategus monogyna, Cydonia oblonga, Malus sylvestris, Dictamnus albus, Salix alba, Atropa bella-donna, Tilia platyphyllos [T. officinarum, T. grandifolia]* and *Tilia tomentosa [T. argentea]*.
- Poisonous (toxic) plants are: *Vincetoxicum hirundinaria, V. huteri, Viburnum lantana, Saponaria officinalis, Ricinus communis, Helloborus odorus, Atropa bella-donna, Datura stramonium, Hyosciamus niger, Nicotiana tabacum* and *Solanum tuberosum.*
- Unlike other species, *Solanum tuberosum* is characterized by trait to be as nutritional medicinal and poisonous (toxic) plant while *Atropa bella-donna* is characterized by trait to be as medicinal, honey plant and poisonous (toxic) plant.
- From life forms Hemicriptophyta (H) dominates with 73 plant species, then listed as follows: Phanerophyta(P) with 40 species, Geophyta (G) with 35 species, Therophyta (T) vith 29 species and Chamaephyta (Ch) with 8 plant species.
- From Floristic elements, dominates the Euro-Asiatic floristic element with 55 plant species, then listed as follows: Europaean with 46 species, Cosmopolitan wit 25, Submediterranean with 10, Mediterranean with 12, Pontic with 3, Cicrumpolar with 6, The Balkans with 4, Boreal with 2, Illyric with 1 and Submezic with 1 plant species. Cultivated species that

originate from America are 10, Asian origin are 6 plant species, Paleotropic origine is 1 plant species, etc.

- Within the territory of Drenica Mountain, periodically is collected material of MAPs, which is done for commercial purposes by some collectors.
- Treatment with traditional use of plant species from local residents is now very small, although based on some data from local residents, plants from nature are used for treatment up to '50 years of the last century (a time when modern medicine was not developed).
- These species occupy a prominent place within the frames of phytodiversity of the Drenica Mountain in particular and in the Kosovo flora and vegetation in general.

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Original scientific paper

STATUS OF WILD-GROWING MEDICINAL PLANTS IN THE ANINEI MOUNTAINS (WESTERN ROMANIA): RECENT DATA

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ABSTRACT

The Aninei Mountains are part of the Banat Mountains, localized in the Southwest of Romania on a surface of 700 km^2. Their average height is 500-800 m. Nearly two thirds of their surface is covered by forests, the rest is occupied by pastures mostly localized in the Northern half of the area. Against this background, a rich wild-growing medicinal flora is displayed – an important asset in the context of today's spreading urbanization, industrialization and pollution. The aim of the present research was to study the composition of the medicinal flora and to localize the sites where medicinal plants occur at higher frequencies. For 49 medicinal species employed in modern phytotherapy and the local folk medicine we performed a mapping of the occurring sites. The maps were elaborated according to the TK 25 system, also employed in the transnational mapping project of the Flora of Central Europe; it provides the coordinates of the places where medicinal plants grow in larger amounts. For another 201 medicinal plants, the sites were described more generally mentioning the name of the hill, nearby villages, water courses. The rare/endangered medicinal species according to IUCN criteria were identified and localized.

***Keywords**: medicinal plant mapping, Romania*

INTRODUCTION

According to the IUCN, WWF and WHO guidelines on the conservation of medicinal and aromatic plants (MAPs), the basic needs towards a sustainable use of natural resources are the identification of MAPs, the knowledge of their distributions and abundance, and the study of the traditional knowledge on the use of plants [1]. Romania is an important country for the export of wild-growing medicinal plants. It has a remarkably high biological and ecological diversity and is rich in natural bio-resources. However, the stock of wild-growing MAPs shows a continuous decline over the last decades. Main responsible factors are overexploitation and habitat modification. Bilberry and raspberry are wild-collected in the largest amounts from Romania [2]. According to the study of Kathe et al. (2003) [2], in Romania there is no clear understanding about the current wild-stock and habitat conditions of MAPs. In this idea, we performed a research of wild-growing MPs in the South Western part of Romania, on the surface of the Aninei Mountains.

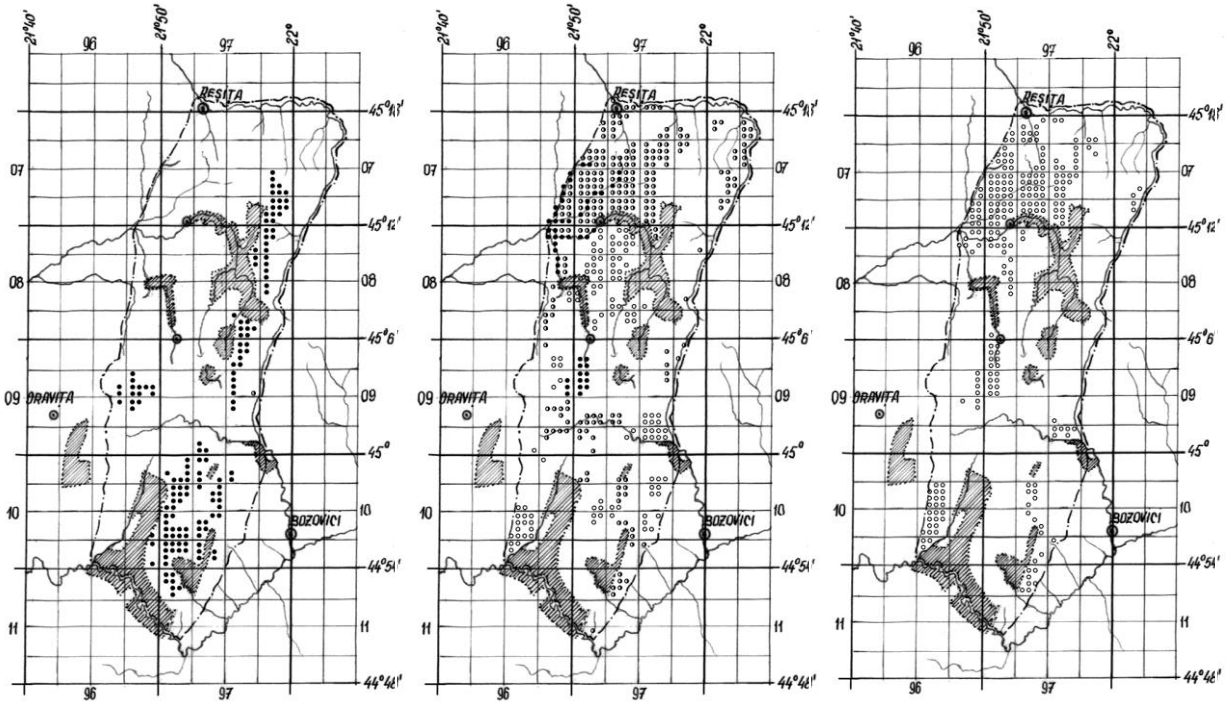
The studied territory has low altitudes (mostly 500-800 m) and has humid, temperate climate with some Mediterranean influences. There are several areas with specific vegetation structures sheltering rare and typically Banatian floristic elements. In recognition of their uniqueness, eight natural reservations were established: Gorges of Nerei-Beuşniţa, Ducin, Lisovacea, Izvorul Bigăr, Gorges of Caraş, Gorges of Gârlişte, and Buhui [3]. No form of use or exploitation of the natural stock is allowed on the territory of these protected areas. Our present research aimed at a mapping MAPs, as an important element in both, their economic valorization and efficient protection, once the vulnerabilities are recognized. It continues previous works on the medicinal flora of the Aninei Mountains, where all MAPs growing here were identified [4], their spectrum of biotypes and the areal-geographic structure were analyzed [5], and rare and vulnerable species were highlighted [6].

MATERIAL AND METHODS

The present study evaluates the medicinal flora from a surface of about 700 km^2. Mapping was performed on 49 medicinal species the total number of MAPs identified in the territory of Aninei Mountains, by marking the sites where the respective species can be more frequently found, and the relative frequency of the species. The maps are elaborated after the TK 25 system, also employed in the mapping project of the Flora of Central Europe, and give the coordinates of the places where medicinal plants grow in larger amounts. The evaluation of the relative frequency was based on the relevé method of plant sampling, which presumes the delimitation of sample-surfaces (relevé plots), followed by the counting of the medicinal plants within the surface. The plots were of 1 m^2 each, in case of the evaluation of herbaceous plants, and 625 m^2 in case of trees and shrubs. The number and positioning of the chosen sample-surfaces varied according to the frequency and constancy of the mapped species, as well as according to the geometric shape of the evaluated surfaces. In the maps, the protected areas were hachured, whereas the relative frequencies of the species are marked with circles: full circles for high density (over 4 individuals per relevé plot), half-full circles (for 1 individual per relevé plot) and empty circles for sporadic occurrence.

RESULTS AND DISCUSSION

Following the study of the wild-growing MAPs' occurence on the territory of the Aninei Mountains, it can be stated that the richest areas in medicinal plants are: the hills around the city of Steierdorf, the meadows of the rivers Caraş, Bârzava and Miniş, as well as the pastures from the South Eastern part of the investigated territory, situated at altitudes of about 600 m. For over 40 species of important MAPSs, we proceeded to the mapping of their occurrence and their frequency (figures 1-7).



Allium ursinum Achillea millefolium Cichorium intybus

Fig. 1. Occurrence of medicinal plants on the territory of the Aninei Mountains (1)

For another 201 medicinal plants, the sites were described more generally mentioning the name of the hill they grow on, nearby villages or watercourses. Due to its length, the detailed list of the places where these species vegetate cannot be included in the present paper, but is available at any time from the authors. Altogether, MAPs from the Aninei Mountains belong to 70 botanical families. A large number of plants belong to the *Asteraceae* (24 species), *Rosaceae* (22), *Lamiaceae* (20), *Fabaceae* (12), *Scrophulariaceae* (10), *Ranunculaceae* (8) and *Apiaceae* (8) families.

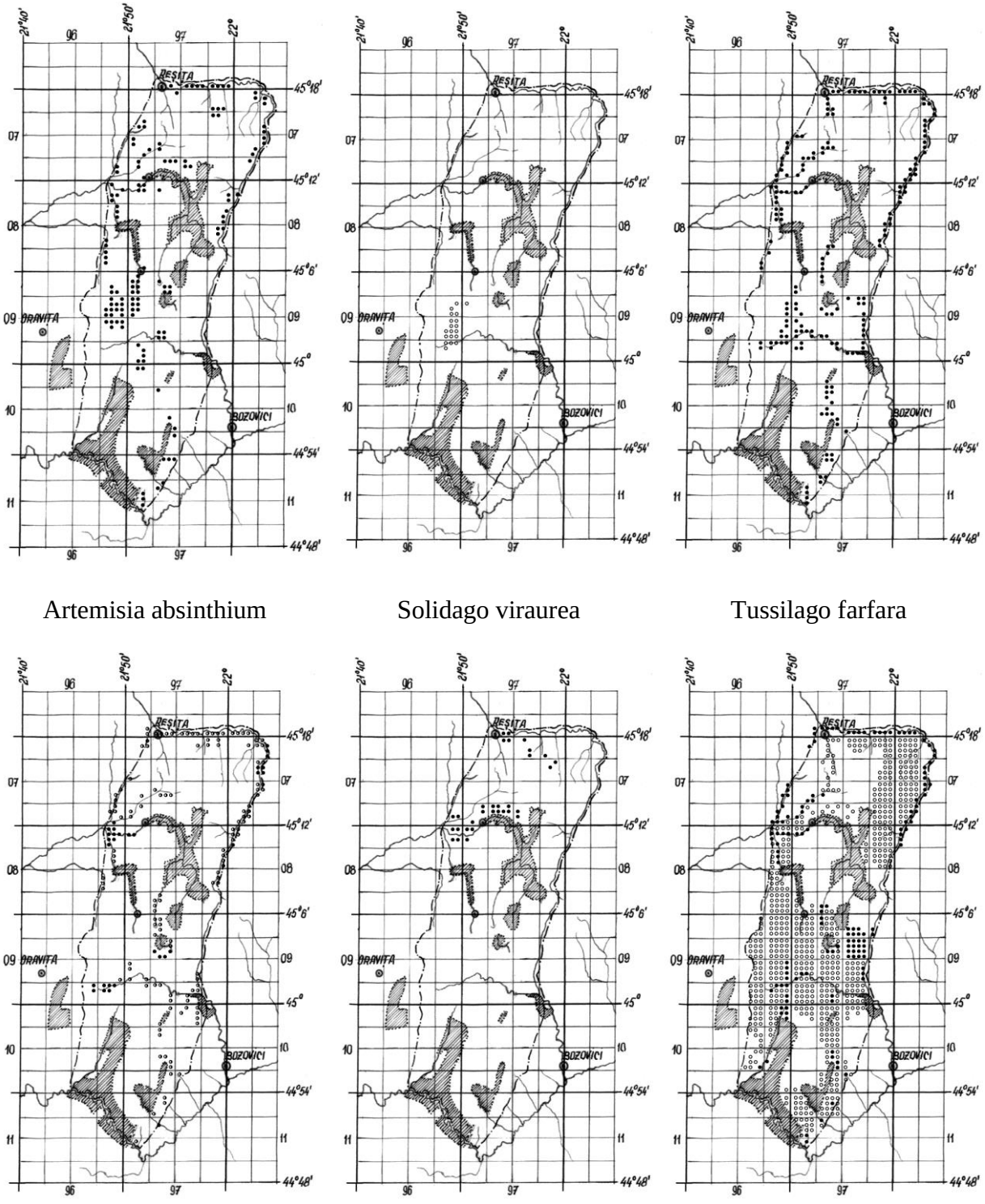
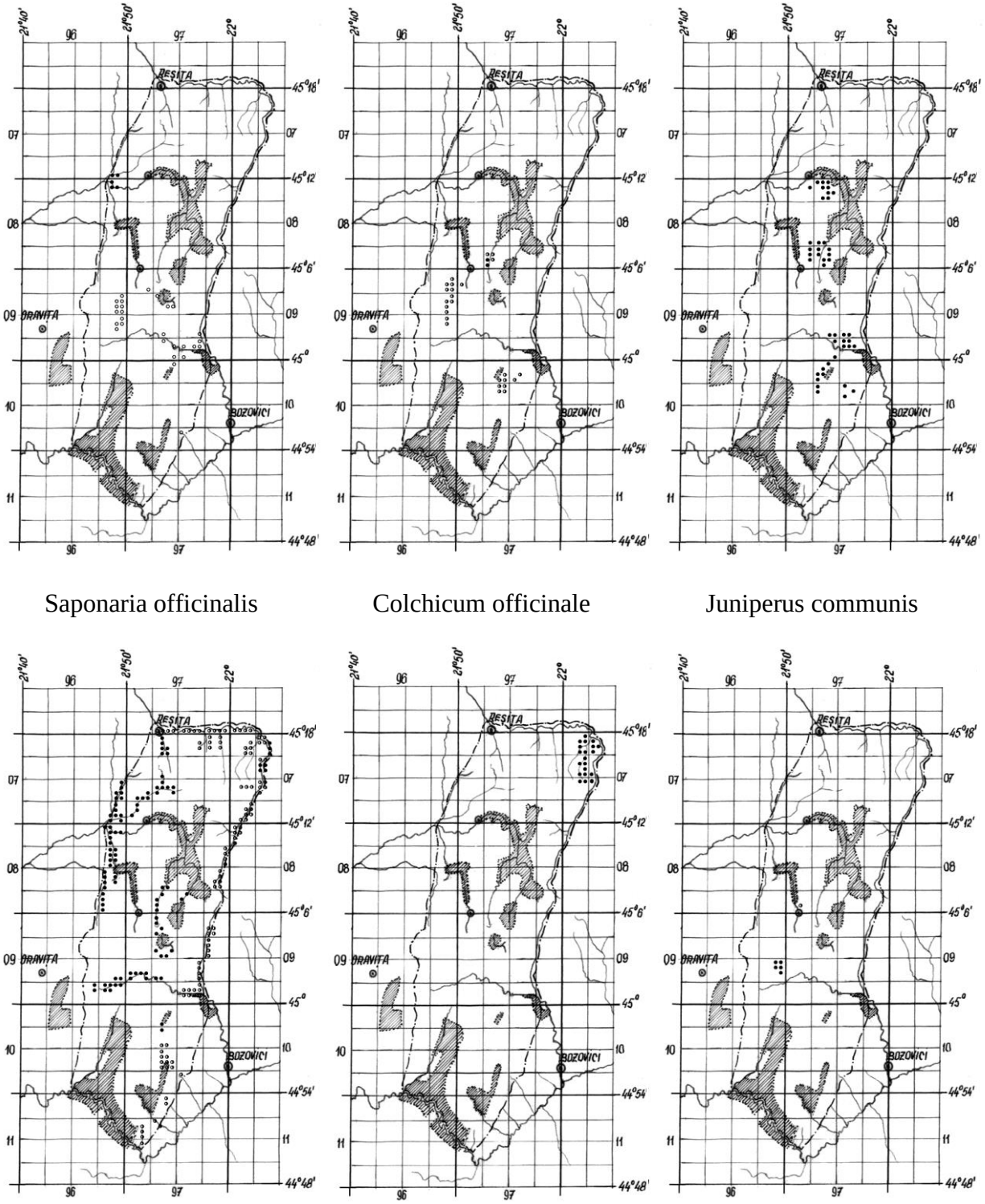


Fig. 2. Occurrence of medicinal plants on the territory of the Aninei Mountains (2)

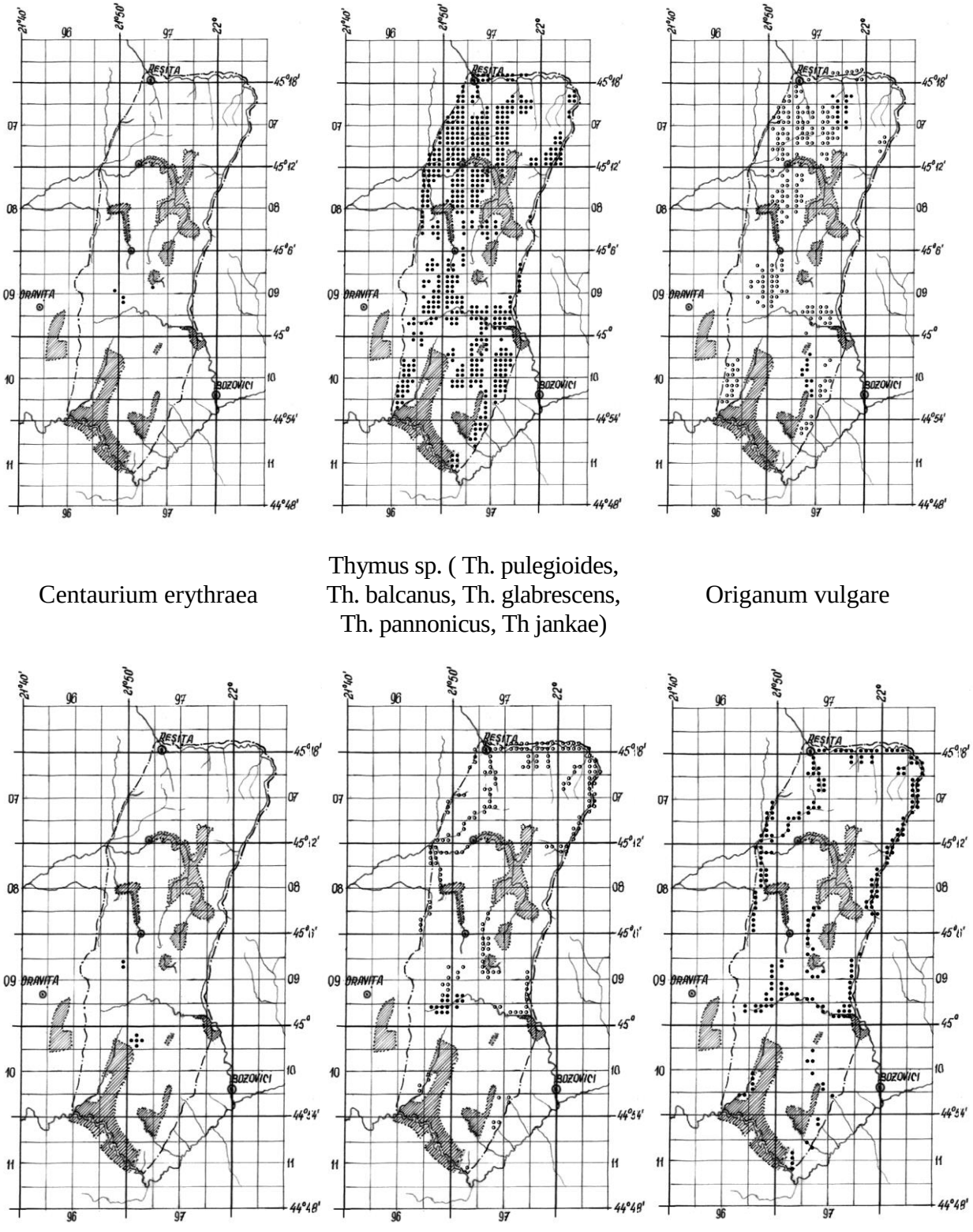


Saponaria officinalis | Colchicum officinale | Juniperus communis

Equisetum arvense | Vaccinium myrtillus | Sarothamnus scoparius

Fig. 3. Occurrence of medicinal plants on the territory of the Aninei Mountains (3)

Some valuable plants for phytotherapy (*Atropa belladonna, Angelica archangelica, Althaea officinalis, Centaurium erythraea, Solidago virgaurea*) find good conditions for vegetation and could be cultivated in the area.



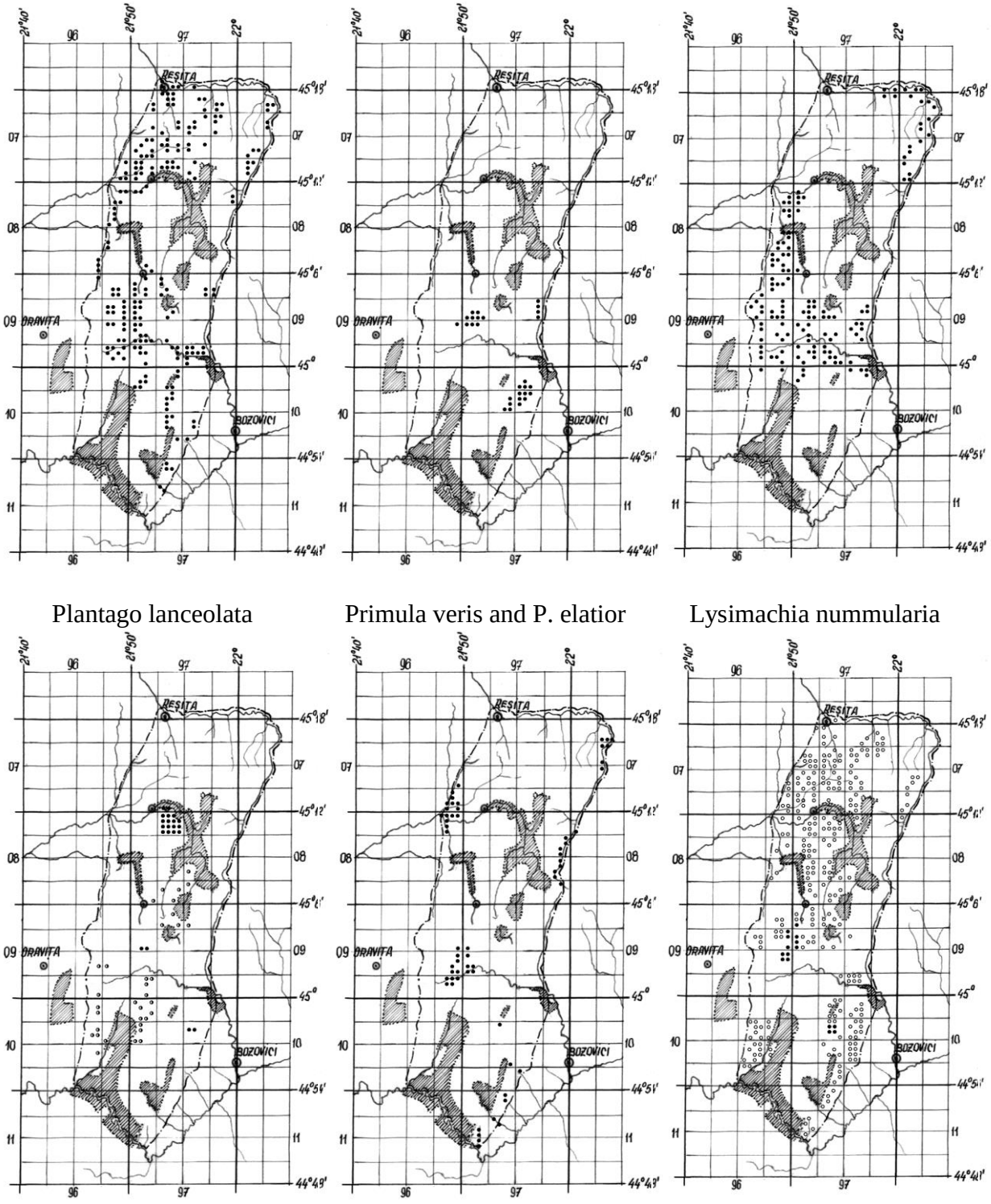
Centaurium erythraea | Thymus sp. (Th. pulegioides, Th. balcanus, Th. glabrescens, Th. pannonicus, Th jankae) | Origanum vulgare

Veratrum album | Lythrum salicaria | Epilobium sp. (E. parviflorum E. hirsutum, E. palustre)

Fig. 4. Occurrence of medicinal plants on the territory of the Aninei Mountains (4)

The rare/endangered medicinal species according to IUCN criteria were as well identified and localized: *Angelica archangelica* appears sporadically, in groups of 2-3 individuals, on the

upper course of the Miniş river, but especially in the lower course of the Bârzava river (starting from forest range Bârzăviţa, until after Văliug in the area of lake Breazova). Its status as protected species is apparently unknown in the area, and the plant is wild-collected by locals.

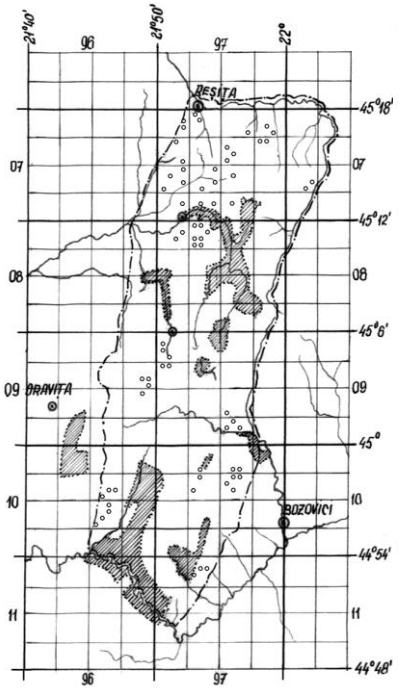


Plantago lanceolata | Primula veris and P. elatior | Lysimachia nummularia

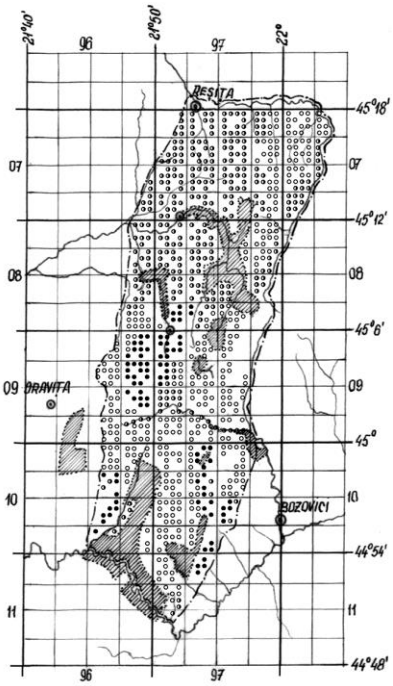
Helleborus purpurascens and H. odorus | Filipendula ulmaria | Rosa canina

Fig. 5. Occurrence of medicinal plants on the territory of the Aninei Mountains (5)

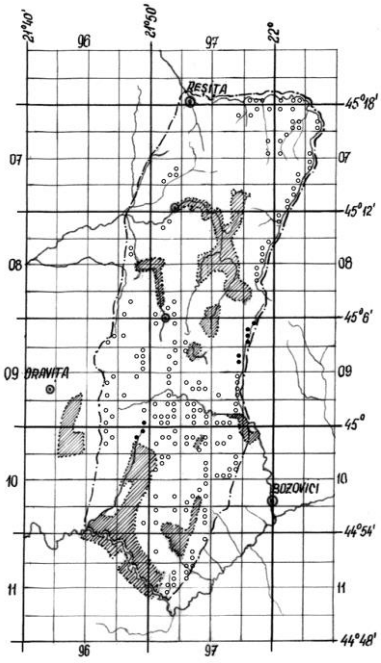
Ruscus aculeatus is only limited to natural reservations like the Gorges of Caraş and Nera, it occurs in Lisovacea, the Bigăr spring, and Ducin, where it vegetates in rare forests and brushwoods. The existence of *Taxus baccata* is getting increasingly endangered in spite of the efforts of forest rangers. *Orchis morio* grows in the Gorges of Caraş and Nera in rare forests. *Jovibarba heufelii* can be encountered on sun-exposed lime rocks from the Gorges of Caraş and Nera, Ogaşul Ursului etc. It is employed in local medicine to treat ear ailments.



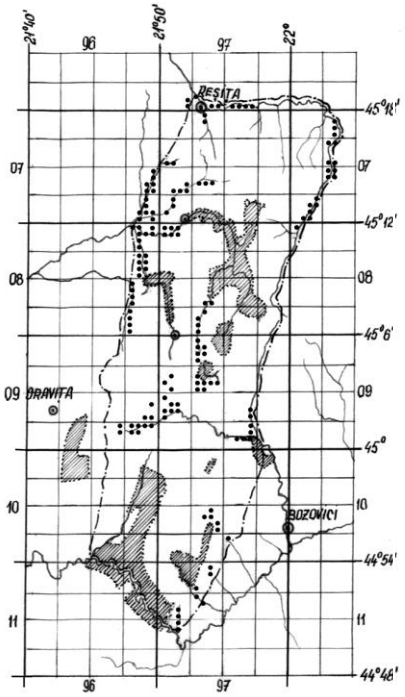
Agrimonia eupatoria



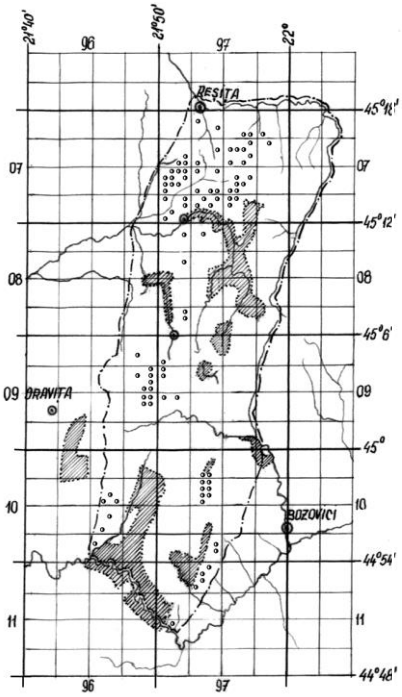
Crataegus monogyna



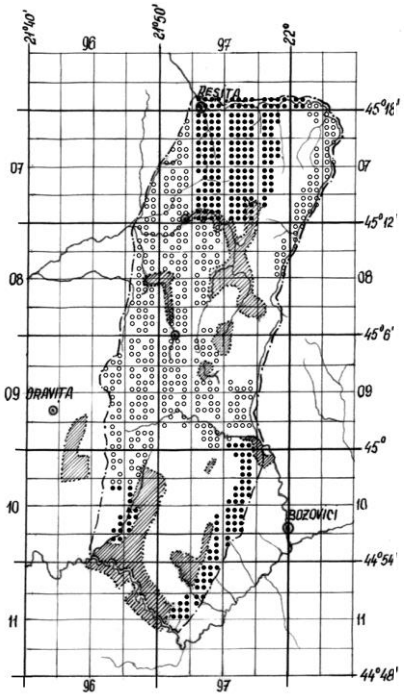
Geum urbanum



Salix alba

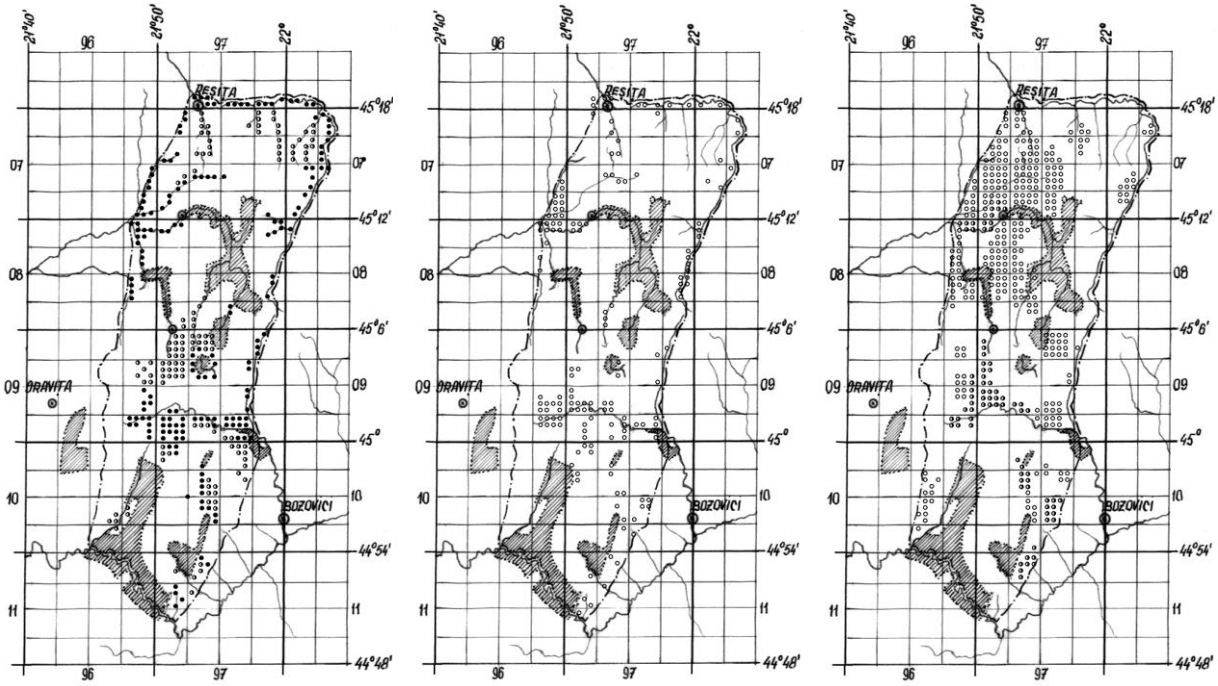


Verbascum phlomoides and V. densiflorum



Tilia tomentosa

Fig.6. Occurrence of medicinal plants on the territory of the Aninei Mountains (6)



Urtica dioica Valeriana officinalis Viola tricolor

Fig. 7. Occurrence of medicinal plants on the territory of the Aninei Mountains (7)

CONCLUSIONS

The Aninei Mountains house an impressive number of medicinal plants, employed in modern phytotherapy and in traditional medicine. Some MAP products may be wild-collected from the studied area, as the plants occur at higher frequencies: *Fraxini folium, Querci cortex, Alii ursini folium, Crataegi fructus, Abieti turione, Tiliae flos cum bracteis, Millefolii flos, Galii verii herba, Betulae folium, Urticae folium, Coryli folium, Rubi fruticosi folium et fructus, Rubi idaei folium et fructus, Equiseti herba, Pini sylvestris turiones, Plantaginis folium, Sambuci flos, Petasitidis rhizoma, Rosae pseudo-fructus, Serpylli herba, Hederae folium, Hyperici herba* and *Symphyti radix*. In case of other plants with low occurence (*Agrimonia eupatoria, Centaurium erythraea, Colchicum autumnale, Humulus lupulus, Juniperus communis, Lythrum salicaria, Primula veris, P. elatior, Saponaria officinalis, Sarothamnus scoparius, Solidago virgaurea, Veratrum album*), wild collection from the Aninei Mountains is not recommended.

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Original scientific paper

GEOGRAPHIC DISTRIBUTION AND DIVERSITY ASSESSMENT IN EX SITU COLLECTION OF ALBANIAN MEDICINAL PLANTS

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ABSTRACT

Genetic diversity and geographic distribution for 403 geo-referenced observations of ex situ medicinal plants collection data including 13 species, from twelve counties of Albania was investigated, using grids of 10 x 10 km cells to assess the number of observations per species and per district, the area of occupancy, the diversity indices and richness estimators. Geospatial analysis detects areas of high (alpha) diversity. Combination of study results for Simpson index, Dominance, Shannon-Weiner, Margalef, Brillouin, species richness and evenness, Equitability, Menhinick, Berger-Parker, and Fisher alpha diversity index show that areas of Tirana, Berat, Shkodra, Lezha, Vlora, and Kukesi counties were richer and more even than other areas. Cluster analysis on correlation matrix of diversity indices results shows presence of high similarities among Berat and Vlora counties, Tirana and Shkodra, Dibra and Lezha counties (similarity range from 64% to 80%). Presence of high species diversity in Tirana, Lezha, Berat, Shkodra, Vlora, and Kukesi counties suggests presence of a greater number species and more relative stable ecosystems, where more ecological niches are available, and environmental changes were less likely to be damaging to the ecosystem as a whole.

Key words: *Diversity indices, medicinal specie, spatial analysis, species richness.*

INTRODUCTION

Medicinal plants and especially wild species provide an invaluable source of genes for the improvement of cultivated medicinal plants. Albania is one of the Balkan countries with high level of diversity for many cultivated and wild species including currently medicinal plants. Genetic resources of medicinal plants have a major contribution to the growth of agricultural products in all regions of Albania in these two last decades. Medicinal and aromatic plants are economically, socially and culturally important crops grown over a wide range of ecological habitats in the country, in wild habitats, in forest habitats, on the hills and mountains habitats [1, 18, 19, 20, 21]. There are 300 species of medical and aromatic plants, which represent about 10% of the Albanian flora [1], and they are considered as an important source of economic revenue; 182 of them are regarded as common. Twenty-two wild species of medicinal and aromatic plants are included in the Red Book of Albania [23].

Plant genetic resources (PGR) play a key role in contributing to the sustainable development of agriculture, helping to increase agricultural food productions. Today, the conservation of genetic resources is regarded as an important need for human society. The genebanks offer the main means to explore, collect, store, and protect genetic materials, providing the raw material for the improvement of crops. The number of gene banks has increased steadily since they were first established in the 1920's. According to the second report on The State of the World's Plant Genetic Resources for Food and Agriculture, there are now some 1,750 gene banks worldwide, with about 130 of them each holding more than 10,000 accessions [5].

As the number of accessions or crops and wild species included in gene banks increases, the goals for PGR are focused on the quality of collections. In this sense, assessment of geographic distribution and genetic diversity variation present in an ex situ collection is important for conservation of PGR and especially for the quality of ex situ collections.

Geographic information systems (GIS) are useful tools for eco-geographical analysis [8, 9]. GIS studies using complex analyses visualize results of geographic distributions of biodiversity in clear maps, which are effective for management of a genebank [14, 16, 6]. GIS studies provide important information about the diversity present in specific geographic areas [16] and can be used to detect geographic distribution of a target species in ex situ collections and to identify collection priority sites [22, 9]. Since ex situ collections aim to cover the maximum amount of genetic variation and the entire range of environmental adaptation of the target species, nowadays ones of the objectives in collecting expeditions is frequently to know where to collect that means to know geographic distribution of a specific species and the amount of individuals per each species for a specific area.

Diversity indices serve as valuable tools that enable researchers to quantify diversity in a community and describe its numerical structure, and because they provide more information than simply the number of species present (i.e., they account for some species being rare and others being common) [8, 10, 15], in the analysis of diversity of an area several diversity indices and richness estimators were used [6].

Medicinal and aromatic plants are grown in all natural and suitable growing areas of medicinal plants in Albania, but genetic erosion is increasing in different areas. During the last two decades, not controlled medicinal harvesting has been rampant in many parts of Albania, especially in the poorer areas of the country. Uncontrolled medicinal harvesting occurs even inside the Protected Areas. Most of this harvesting was done to export medicinal raw products for medicinal industry. Lots of medicinal species have been most damaged by this activity because of their high quality and high selling price on the uncontrolled market. Ibraliu [13] reports harvesting techniques (uprooting) often exacerbate the threat to medicinal and aromatic plants by causing unnecessary damage, as in the cases of *Salvia officinalis*, *Sideritis raeseri*, *Origanum vulgare* (which are in endangered status), and *Gentiana lutea* which is critically endangered.

Because the Albanian territory has highly heterogeneous environmental conditions, the aim of this study was to assess the geographic distribution of medicinal plants, to optimize and make more effective results of collecting missions, and to evaluate genetic diversity of ex situ medicinal plant collection, which involved the application of GIS tools [4].

MATERIAL AND METHODS

Geographic areas and presence data: The study for assessment of geographic distribution and genetic diversity of medicinal plants is realized using ex situ data of medicinal plants collection present in Albanian Gene Bank (AGB) database. It was conducted in all natural growing areas of medicinal plants including twelve counties of Albania: Berat (BR), Dibra (DI), Durres (DR), Elbasan (EL), Fier (FR), Gjirokaster (GJ), Korçe (KO), Kukes (KU), Lezhe (LE), Shkoder (SH), Tirana (TR) and Vlora (VL). Each taxon or medicinal species and population (the group of individuals) represents a georeferenced observation point. One georeferenced observation point supposes presence of a medicinal species. All georeferenced observations, chosen to carry out spatial analysis, were entered into the GIS analysis, as presence points [10]. Each presence point is spatially represented as point map using DIVA-GIS [4, 10, 11, 12]. The geographic areas were separated into small grid square cells, and grid cells of 1 x 1 km, 5 x 5 km, 10 x 10 km were used to assess the geographic distribution, diversity indices, and richness estimators of medicinal species.

Diversity distribution: The analysis focuses only on the study of diversity at the species levels (unit of alpha diversity). Magurran [15] define species diversity as consisting of two components: the number of species (Richness) and how equally abundant the species are (Evenness). Richness in species is a straightforward measure of diversity and is commonly used for prioritizing conservation areas of either plant communities – based on number and uniqueness of observed species [7]. The measurement of diversity and geographic distribution of medicinal species was realized analyzing the number of observations per species and per district, the area of occupancy, where the total area occupied by a specific species, was selected as an indicator of abundance or rarity of a particular species.

Indices as species richness (S), dominance (D), Simpson index (1–D), Shannon-Weiner index (entropy) (H), Evenness (e^H/S), Brillouin index (B), Menhinick richness index, Margalef's richness index (D_{MG}), Equitability (J), Fisher's alpha diversity index, and Berger-Parker dominance were the indices used to assess diversity and richness taking into account the respective proportions of each species in the study area. Cluster analysis method was used to measure distance or similarity between georeferenced data using species presence/absence and diversity indices in different areas (counties). Diversity indices and richness estimators were calculated and mapped using DIVA-GIS tools [4].

RESULTS AND DISCUTION

Collecting and quality data: During collecting data a large range of information was gathered and recorded for each medicinal species. In ex situ collection of medicinal plants stored in AGB data for 436 accessions considered as presence data were gathered. For data quality [2] including the accuracy and precision of geographic coordinates firstly georeferenced or presence data were checked for inconsistencies [2, 3]. Data points without coordinates were removed from ex situ medicinal data. Data points with incorrect coordinates on the administrative unit (county) were assigned coordinates where possible while duplicate or doubtful data were removed [3, 22]. All medicinal species were also screened carefully to resolve any scientific name conflicts [3]. The accessions not present physically as genetic material stored in genebank were also removed.

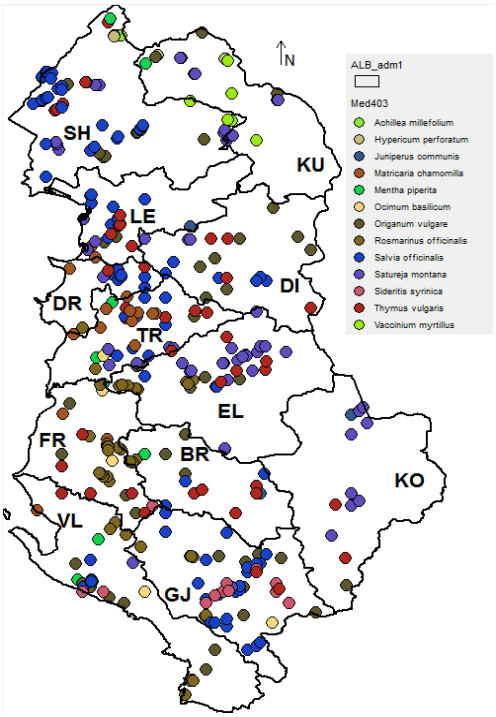

Figure 1. Geographic distribution of thirteen medicinal species

Geographic distribution: After checking the presence or absence of accessions the data included in the medicinal plants database with partial or complete information for a total of 436 presence points, in total only 403 presence points of wild populations for thirteen species of medicinal plants were compiled and used to evaluate the geographic distribution, and diversity of currently medicinal species observed in Albania (**Figure 1**).

Diversity of medicinal species: Study results for diversity species distribution based on indices and richness estimators calculated (**Table 1**) show the existence of variability between observed areas/counties related to number of individuals (presence point) and kind of medicinal species present in that geographic areas. Diversity indices values and their comparisons proved the presence of this important variability in the study areas (grid cells) analyzed. Spatial analysis detects areas of high diversity (alpha diversity). Species richness (S) clearly shows the higher number of different species occurs in SH, TR, and BR areas. In these areas the number of species (S) observed was respectively 9, 8, and 8. At the second range with highest number of species there were KU and VL counties (S = 7), and EL, DI, FR and LE counties (S = 6 species). Less species richness occurs in DR and KO areas. Simpson index (1 - D > 0.70) which calculates the probability that two individuals randomly selected from a sample will belong to different species, shows presence of higher diversity in areas of TR, LE, SH, BR and VL counties. These results were also proved by index of dominance (D) where low values show higher diversity level for the respective area (**Table 1**).

Shannon-Weiner index values (H > 1.50) show that areas of TR, BR, SH, LE, VL and KU counties were richer and more even than other areas. In these counties there were present the higher number of different species and the individuals distributed among species were more even (Table 1). In this study the Shannon index values ranges from 0.662 (DR areas) to 1.781 (TR areas) showing in general low species richness and evenness [17].

Table 1. Diversity of medicinal species based on diversity indices and richness estimators.

Indices/Counties	BR	DI	DR	EL	FR	GJ	KO	KU	LE	SH	TR	VL
Taxa/species (S)	8	6	2	6	6	5	3	7	6	9	8	7
Individuals (n)	35	27	8	39	31	42	8	28	24	76	55	30
Dominance (D)	0.218	0.339	0.531	0.312	0.303	0.265	0.406	0.296	0.212	0.212	0.195	0.271
Simpson (1-D)	0.782	0.661	0.469	0.688	0.697	0.735	0.594	0.704	0.788	0.788	0.805	0.729
Shannon (H)	1.756	1.356	0.662	1.424	1.447	1.415	0.974	1.469	1.666	1.750	1.781	1.558
Evenness (e^H/S)	0.723	0.647	0.969	0.693	0.708	0.824	0.883	0.621	0.882	0.639	0.742	0.679
Brillouin (B)	1.486	1.122	0.503	1.234	1.224	1.261	0.704	1.211	1.377	1.588	1.585	1.301
Menhinick	1.352	1.155	0.707	0.961	1.078	0.772	1.061	1.323	1.225	1.032	1.079	1.278
Margalef ($D_{MG)}$	1.969	1.517	0.481	1.365	1.456	1.070	0.962	1.801	1.573	1.847	1.747	1.764
Equitability (J)	0.844	0.757	0.954	0.795	0.808	0.880	0.887	0.755	0.930	0.796	0.857	0.801
Fisher_alpha	3.242	2.392	0.856	1.980	2.216	1.479	1.743	2.996	2.568	2.656	2.574	2.871
Berger-Parker	0.371	0.519	0.625	0.487	0.484	0.381	0.500	0.393	0.333	0.355	0.291	0.433

Margalef index values (D_{MG}) > 1.50 show presence of higher species diversity in the areas of BR, SH, KU, VL, TR and LE counties. At the DI County (D_{MG} > 1.50) there were also areas of high diversity level. The species evenness, as a measure of biodiversity that quantifies how equal the populations are numerically, show the high evenness and the less variation in populations between the species, occurs in DR, KO and LE county areas. Equitability (J), that measures the evenness, with which individuals are divided among the present species, show higher evenness result at DR, LE, KO, GJ, TR and BR county areas. Brillouin index values (B > 1.20) clearly shows that high species diversity occurs in SH, TR, BR, LE, and VL areas. Menhinick richness index, calculating the ratio of the number of taxa to the square root of sample size, shows that BR, KU, VL, and LE counties areas were with higher diversity level.

Comparisons of diversity among observed areas:

Cluster analysis method using group-average clustering of individuals and species numbers, diversity indices, and richness estimator's show clearly the presence of similarity and distances among areas where medicinal species were present, and gave a useful hierarchy of clusters shown in two dendrograms (**Figure 2**). According to distances and similarities measures of individuals and species numbers cluster analysis range each observed area (county) in different cluster group. There were high similarities among BR and VL and GJ counties (similarity range from 64% to 80 %), and among EL and TR counties (64 %), and among DI and LE (63%) (**Figure 2**, Dendrogram on the left). Cluster analysis based on diversity indices and species richness results generate a dendrogram where distinctness (distances / similarities) among counties is shown with more clear clusters, and similarity among each county is increased with 7% - 9%. Based on similarities among counties dendrogram classified these counties into three different cluster groups. There were high similarities among VL, FR, KU, DI, BR, LE, EL, and GJ counties included into the first cluster group (similarity range from 73% to 87%). There were similarity among SH and TR counties (77%), and among DR and KO counties (80%) (**Figure 2**, Dendrogram on the right).

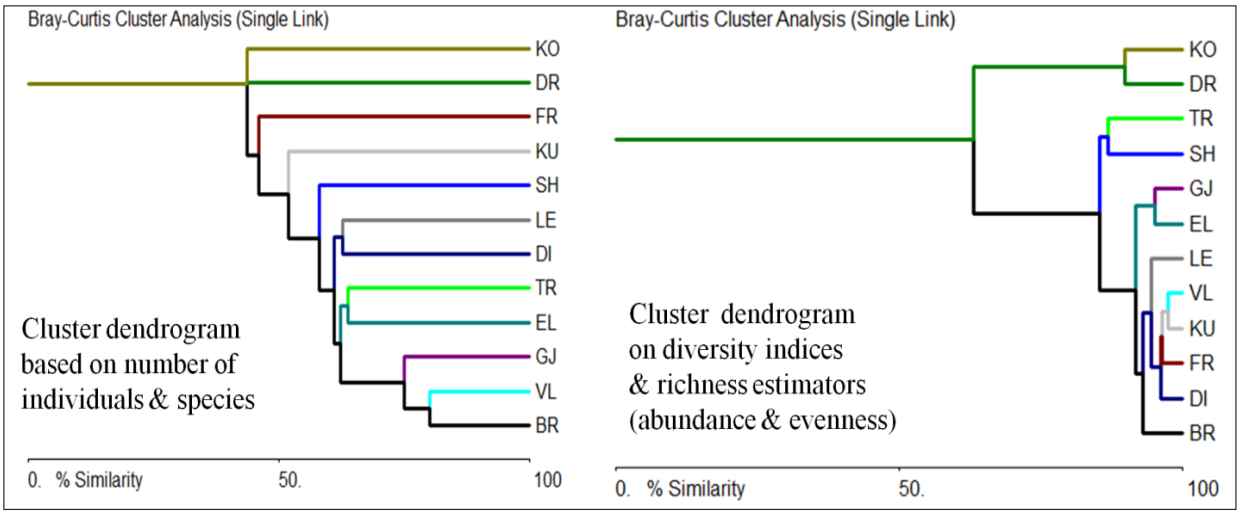

Figure 2. Cluster dendrograms based on individuals and species number (left) and diversity and richness indices (right)

Correlation analysis using similarity matrix and Spearman's Rank correlation matrix data for individuals, species, diversity indices, and richness estimators (**Table 2)** show there were very strong correlations for diversity among BR and VL and GJ counties (coefficient of correlation r range from 0.83 to 0.92), and strong correlations among DI and KO, LE, SH and TR counties (coefficient of correlation r range from 0.69 to 0.87).

Table 2. Relationships between Similarity and Spearmans Rank correlation matrix data

Comparisons		Similarity Matrix											
		BR	DI	DR	EL	FR	GJ	KO	KU	LE	SH	TR	VL
Spearmans Rank correlation matrix	BR	*	48.39	23.26	35.14	39.39	64.94	18.60	22.22	61.02	48.65	62.22	80.00
	DI	0.56	*	34.29	30.30	34.48	46.38	34.29	50.91	62.75	44.66	39.02	56.14
	DR	0.43	0.62	*	8.51	15.38	20.00	0.00	0.00	43.75	11.90	25.40	31.58
	EL	0.48	0.57	0.34	*	45.71	32.10	34.04	38.81	38.10	38.26	63.83	34.78
	FR	0.39	0.31	0.40	0.50	*	38.36	20.51	27.12	40.00	20.56	37.21	45.90
	GJ	0.88	0.55	0.57	0.52	0.39	*	8.00	25.71	39.39	44.07	45.36	75.00
	KO	0.39	0.75	0.42	0.79	0.44	0.45	*	27.78	43.75	19.05	25.40	15.79
	KU	-0.24	0.24	0.11	0.12	-0.01	-0.09	0.54	*	34.62	51.92	36.14	24.14
	LE	0.54	0.87	0.66	0.57	0.38	0.55	0.73	0.20	*	44.00	55.70	55.56
	SH	0.26	0.69	0.41	0.41	-0.11	0.32	0.66	0.53	0.74	*	58.02	41.51
	TR	0.35	0.78	0.66	0.66	0.40	0.40	0.64	0.03	0.81	0.48	*	54.12
	VL	0.83	0.55	0.60	0.55	0.56	0.92	0.37	-0.33	0.54	0.12	0.44	*

In this study both the Brillouin and Shannon Indices tend to give similar comparative measures. This information measure results to be used in favour of the Shannon index when the species differ in their capture rates. Margalef index values show high similarity with Shannon index values, and there is high similarity among species evenness and equitability estimator.

Counties as LE, BR, SH, VL and KU show high species diversity, which suggests presence of a greater number of successful species and more relative stable ecosystems. In these counties more ecological niches are available. Second group (GJ, DI, KO, FR, and EL

counties) show more less species diversity, which suggests relatively few successful species presence in these habitats, the environment is more stressful with relatively few ecological niches available. Possible hostile human activities as uncontrolled medicinal harvesting (uprooting techniques) in the future could probably have quite serious effects on species diversity, on the loss of 'biological health' level and on biodiversity indices results.

COCLUSION

- Spatial analysis detecting the areas of high (alpha) diversity were observed in SH, TR, and BR counties. Second highest numbers of species were found in KU and VL counties.
- Simpson, Shannon-Weiner, and Margalef indices show that areas of TR, BR, SH, LE, VL, and KU counties were richer and more even than other areas. Brillouin index tend to give similar comparative measures as Shannon-Weiner index.
- Cluster analysis based on individuals and species numbers shows high similarities among BR and VL, TR and EL, DI and LE (similarity range from 63% to 80 %). Cluster analysis based on diversity indices results shows less differences (or high similarities) among VL, FR, KU, DI, BR, LE, EL, and GJ counties included all into a cluster group (similarity range from 73% to 87%).
- Comparisons of diversity indices using cluster analysis method divide diversity indices in two groups. Evenness, Equitability, Dominance, Berger-Parker, and Simpson (1-D) tend to give similar comparative measures and were included into the first cluster group. Shannon, Margalef, Menhinick and Brillouin indices with similar comparative measures were included into the second cluster group.

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Original scientific paper

GEOGRAPHIC DISTRIBUTION AND SPATIAL GAPS ASSESSMENT IN EX SITU COLLECTION OF *ORIGANUM VULGARE* L. STORED IN ALBANIAN GENEBANK

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ABSTRACT

The geographic distribution and spatial gaps using circular buffer zones (1 and 10 km radius) around the ex situ and external data of 117 oregano (*Origanum vulgaris* L.) populations in ten districts of Albania was investigated. Spatial analysis and comparisons of quantitative diversity variables: richness, ex situ and external present points, no spatial gaps, mid-priority gaps, high-priority gaps, priority sites, and potential size increase of collection show the areas with higher richness of oregano populations were Kukes, Shkodra, Dibra and Gjirokastra districts areas. Study results show the areas with higher ‘‘no gaps data’’ were Lezha, Fieri and Berat districts (index range from 0.75 to 0.80), the areas with mid-priority spatial gaps were Vlora, Elbasan, Fieri and Shkodra districts (index range from 0.22 to 0.29), and the areas with high-priority spatial gaps were Tirana, Vlora, Gjirokastra, Kukes, Shkodra, and Elbasan districts (index range from 0.40 to 0.86). Higher numbers of priority sites were observed at the Shkodra, Kukesi, Gjirokastra and Elbasan district areas, which suggest these possible priority sites, can be used to increase size of oregano collection. Cluster analysis method on correlation data show clearly similarity among Shkodra and Kukes districts, among Dibra and Gjirokastra, Fieri and Vlora, and Lezha and Tirana district areas (similarity index ranges from 0.83 to 0.95).

Key words: *Diversity indices, geographic distribution, O. vulgaris L., spatial gaps.*

INTRODUCTION

Albania, a small Mediterranean country on the Balkan Peninsula in the south of Europe, is very rich in biological and landscape diversity, in cultivated crops, in wild plant species including medicinal plants. This diversity is attributable to the country's geographic position as well as geological, hydrological, climatic, and soil and relief factors. Medicinal and aromatic plants are economically, socially, and culturally important plants grown over a wide range of ecological habitats in the country, in wild habitats, in forest habitats, on the hills and mountains habitats [22, 25]. Oregano plants (*Origanum vulgare* L.), growing in natural habitats, are collected and used as raw materials in the pharmaceutical, cosmetic and food industry [22, 2].

Wild medicinal species provide an invaluable source of genes that can be used for the improvement of cultivated species, but the information on medicinal plants biodiversity in Albania is generally lacking especially in terms of species. There are still flora and taxonomic medicinal groups, which are unknown or have not been studied.

Collection of Plant Genetic Resources (PGR) provides access to the greatest possible amount of genetic variability in a particular species and helps reveal the ecological and geographic distribution of plant species [3]. A genebank is a collection of a particular crop and its wild relatives and, ideally, includes at least one example of each alternative allele for each locus [5]. Because researchers can hope to sample only a fraction of genetic variation that occurs in nature, it is important that this sample be as large as possible and contains the maximum amount of useful variation for both present and future use [1, 4]. In our days the conservation of PGR is regarded as an important need for human society, and genebanks offer the main means to store, and protect valuable raw genetic materials. As the number of accessions of different crops and wild species included in genebanks increases, improvement of quality and representativeness of ex situ collections is an important goal for PGR conservation.

Diversity is not uniformly distributed in space or among taxonomic groups, and ecological factors are a major determinant of genetic diversity [3]. Spatial distribution of a species is necessarily a product of environmental influences, including human activities [12, 10], life story traits and demographic past history of the plant species. Knowledge of spatial genetic structures provides a valuable tool for inferring these causal factors and also the underlying genetic processes such as selective pressures, gene flow, and drift [25].

Because the distribution of diversity is not known prior to data analysis, successful collecting in terms of diversity may depend on the proper identification of populations closely adapted to specific environments and land-use patterns [4]. In this case analysis of georeferenced genebank data proves useful in identifying areas of high diversity [21, 8], targeting genetic resources for breeding programs [21, 13], and selecting and designing sites for in situ conservation [13].

Geographic information systems (GIS), as useful tools for eco-geographical analysis [11], provide important information about the geographic distribution and diversity present in specific geographic areas [20] of a target species. GIS studies can be used to detect ecogeographical gaps in ex situ collections and subsequently identify where to prioritize collection efforts, which are effective for management of a genebank.

Assessment of the current conservation status of PGR, identification of gaps, formulation, and implementation of more effective collecting and conservation strategies for PGR [12], can improve genetic representativeness (GR) and the quality of ex situ collections. Since ex situ collections aim to cover the maximum amount of genetic variation and the entire range of environmental adaptation of the target species, nowadays the objective in collecting expeditions is frequently to fill gaps in the representativeness of collections [15].

The aim of this study was to assess the genetic diversity, relative spatial gaps of Oregano *(Origanum vulgare* L.), and to optimize the representativeness of medicinal plants collection stored in Albanian Gene Bank (AGB).

MATERIAL AND METHODS

Data sampling and geographic distribution: The study was conducted in the more natural growing areas of Oregano *(O. vulgare* L.) populations in Albania, and there were ten districts of Albania: Berat (BR), Dibra (DI), Elbasan (EL), Fieri (FR), Gjirokaster (GJ), Kukes (KU), Lezha (LE), Shkoder (SH), Tirana (TR), and Vlora (VL) included for geographic distribution analysis of oregano (*O. vulgaris* L.). Data sampling is realized using information on the total occurrence of oregano species in Albania gathered from ex situ collection data in database of medicinal plants stored in AGB and external data. External data, including all oregano populations surveyed (not collected) and not included in ex situ collection, were gathered from EURISCO database [7], from the Global Biodiversity Information Facility (GBIF) database [9], from publishing data [18, 19, 24], and information gathered from contact persons of Albanian genebank.

Each population (group of individuals) represent a georefernced observation, where one observation supposes presence of an oregano population [10]. All georeferenced observations (ex situ data and external data) chosen to carry out spatial analysis, were entered into the GIS analysis, as presence points [16, 10] and were spatially represented as point maps using DIVA-GIS [16, 17, 10]. The analysis focuses only on the study of geographic distribution and diversity at the population levels (unit of alpha diversity). The measurement of distribution, diversity, and relative gaps of Oregano (*O. vulgare* L.) was realized analyzing the number of observations per populations and per district, and the area of occupancy. The total area occupied, was selected as an indicator of abundance or rarity of species/ population. Maps containing geographic distribution of oregano in all Albania and per each district were created using DIVA-GIS tools [6].

Relative spatial gaps detection: Circular buffer zones (or buffer areas) that determined the distance in km under which are consider two presence or collection sites to represent in fact the same population [23], with a 1 and 10 km radius were created around the AGB ex situ data points and circular buffer zones with a 1 and 3 km radius were also created around the external data points. There were three possible situations: The first case, when the external data buffer zones intersect the AGB ex situ data buffer zones with a 1 km radius, indicates ‘‘no gaps data’’. The second case, when external data buffer zones only intersect the AGB data buffer zones with a 10 km radius, indicates mid-priority spatial gaps. The third case, when the external data buffer zones do not intersect any of the AGB data buffer zones (those with a 1 km or 10 km radius), indicates high-priority spatial gaps. Maps containing mid and high-priority spatial gaps were created for oregano (*O. vulgare* L.) using DIVA-GIS tools [6].

Assessment of geographic distribution: Assessment of diversity distribution and relative spatial gaps of Oregano (*O. vulgare* L.) populations is carried out using several quantitative diversity indices or variables. Richness of oregano population (S), ex situ present points (EXS-PP), external present points (EXT-PP), ‘‘no spatial gaps data’’ (NGD), mid-priority spatial gaps (MPG), high-priority spatial gaps (HPG), number of priority sites (NPS), and potential size increase (PSI %) of oregano collection (calculated by the ratio NPS/S) were the quantitative diversity variables calculated and used in this study. A value from 0 to 1 was given to each variable showing respectively 0 no variability and 1 high variability.

Analysis of distances: A cluster analysis method on correlation was used to identify groups of relatively similar material [14], and to measure distances/similarities between georeferenced data using presence/absence population of oregano and quantitative diversity indices/variables in different observed areas of ten districts in Albania. Quantitative indices or variables were calculated using the SAS JMP Statistical Discovery [26].

RESULTS AND DISCUTION

Collected and quality data: In ex situ collection of medicinal plants stored in AGB data for 125 populations of oregano were gathered. Data quality including the accuracy and precision of geographic coordinates firstly presence or georeferenced data were checked for inconsistencies. Data points without coordinates were removed from ex situ medicinal data. Data points with incorrect coordinates on the administrative unit (county) were assigned coordinates where possible while duplicate or doubtful data were removed [27]. After checking the presence or absence of accessions the data included in the medicinal plants database with partial or complete information for a total of 125 presence points, in total only 117 presence points (72 ex situ presence data and 45 external verified presence data) of oregano populations were compiled and used to evaluate the geographic distribution, diversity of currently oregano populations observed per each district and in all Albania.

Geographic diversity distribution: Geographic distribution results of observed oregano populations given on the map as present points in all Albanian territory (left map, **Figure 1**) show the areas with higher richness of oregano populations were KU, SH, DI and GJ districts areas. At the second range of richness were BR, FR, and VL district areas (Table 1). A more detailed analysis related to ex situ present points (points in red colour) and external present points (points in blue colour) presented on the right map (**Figure 1**) show higher number of ex situ present points were at the KU, SH, DI and GJ districts areas, and higher number of external present points were in KU and SH districts areas. In these areas possible oregano populations can be collected and stored in genebank.

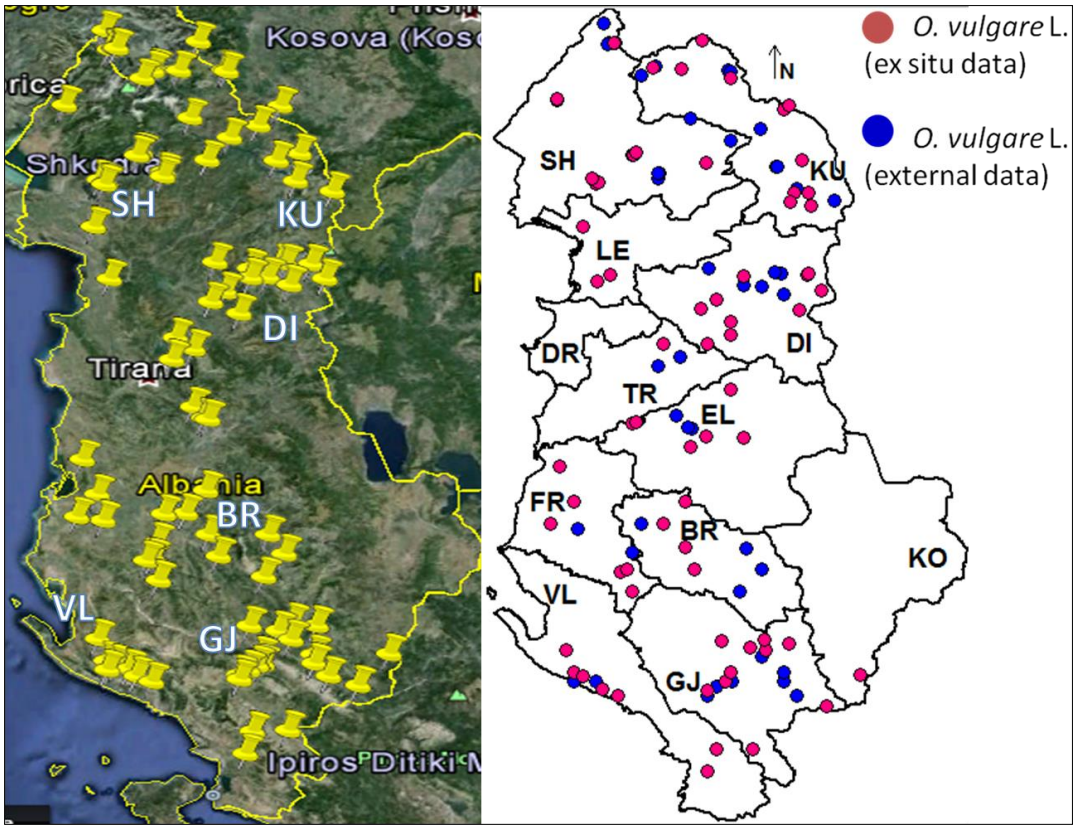


Figure 1. Geographic distribution of oregano (*O. vulgare* L.) populations in Albania (left map). Distribution of ex situ data (points in red colour) and external data (points in blue colour) per each district areas (right map).

Relative spatial gaps: Quantitative analysis of diversity and spatial gaps indices (Richness-S, EXS-PP, EXT-PP, NGD, MPG, HPG, NPS, and PSI) (Table 1) show presence of significant differences among districts areas analyzed. Quantitative variables results and their variances (Table 1) show the higher significant differences related to oregano (*O. vulgare* L.) distribution and spatial gaps were expected at the KU, SH, DI and GJ districts areas (where variance σ^2 range from 34.29 at DI district to 68.19 at KU district). To identify the relative spatial gaps of oregano, geographic distribution maps of ex situ data and external data containing mid spatial gaps and high-priority spatial gaps were superimposed and possible relative spatial gaps per each district were evaluated (**Figure 2**).

Study results show the areas with higher ‘‘no gaps data’’ were LE, FR and BR districts (NGD index range from 0.75 to 0.80) (**Table 1**). In these areas most of the external data buffer zones intersect the AGB ex situ data buffer zones with a 1 km radius, showing ‘‘no gaps’’ as it would imply that collecting of oregano populations located less than 2 km from the ex situ present point is represented in the AGB database (**Figure 2**).

Table 1. Quantitative variables used to assess the diversity and spatial gaps of oregano

Variables	BR	DI	EL	FR	GJ	KU	LE	SH	TR	VL	Average
Richness (S)	8	16	7	8	17	21	5	20	5	9	11.6±6.22
INS-PP	0.50	0.63	0.57	0.75	0.59	0.52	1.00	0.55	0.60	0.78	0.65±0.15
EXT-PP	0.50	0.38	0.43	0.25	0.41	0.48	0.00	0.45	0.40	0.22	0.35±0.15
NGD	0.75	0.69	0.14	0.75	0.53	0.43	0.80	0.40	0.60	0.56	0.56±0.20
MPG	0.00	0.13	0.29	0.25	0.12	0.19	0.00	0.25	0.00	0.22	0.14±0.11
HGP	0.25	0.31	0.86	0.25	0.47	0.57	0.20	0.60	0.40	0.44	0.44±0.15
NPS	2	5	6	2	8	12	1	12	2	4	5.40±4.09
PSI (%)	25%	31%	86%	25%	47%	57%	20%	60%	40%	44%	44% ± 20
Variance (σ^2)	8.09	34.3	8.83	7.98	41.5	68.2	3.09	62.92	3.08	10.85	22.9±22.4
St. Dev.	2.84	5.86	2.97	2.82	6.44	8.26	1.76	7.93	1.75	3.29	4.25±2.34

The areas with mid-priority spatial gaps were VL, EL, FR and SH districts (MPG index range from 0.22 to 0.29) (**Table 1**). In these areas the external data buffer zones intersect the AGB ex situ data buffer zones with a 3 to 10 km radius, showing ‘mid-priority spatial gaps’ as it would imply collecting of oregano populations located between 3 to 10 km from the ex situ present point represented in the AGB database (**Figure 2**).

The areas with high-priority spatial gaps were VL, GJ, KU, SH, and EL districts (HPG index range from 0.44 to 0.86) (**Table 1**)**.** In these areas the external data buffer zones do not intersect the AGB ex situ data buffer zones with a 10 km radius, showing ‘high-priority spatial gaps’ as it would imply collecting of surveyed (not collected) of oregano populations located out of buffer zones with a 10 km radius from the ex situ present point represented in the AGB database (**Figure 2**) Presence of oregano populations (external data) out of zones with a 10 km radius (ex situ data) increased the difficulties of trying and collecting of those oregano populations.

Results of the study show the total number of priority sites (54 sites) includes external and ex situ data for all Albanian territory. Higher number of priority sites (38 sites) were observed at the SH, KU, GJ and EL district areas. The concentration of priority sites (70%) only in four districts (SH, KU, GJ and EL) suggests in these areas the possible priority sites can be used to increase size of oregano collection, and the priority of genebank is to identify the real

geographic position of these priority sites. Identification of priority sites increases the effectiveness of collecting missions in these four district areas having the possibility to increase (ideally) the potential size of oregano (*O. vulgare* L.) collection with 32 – 33% (ratio between priority sites and total present points).

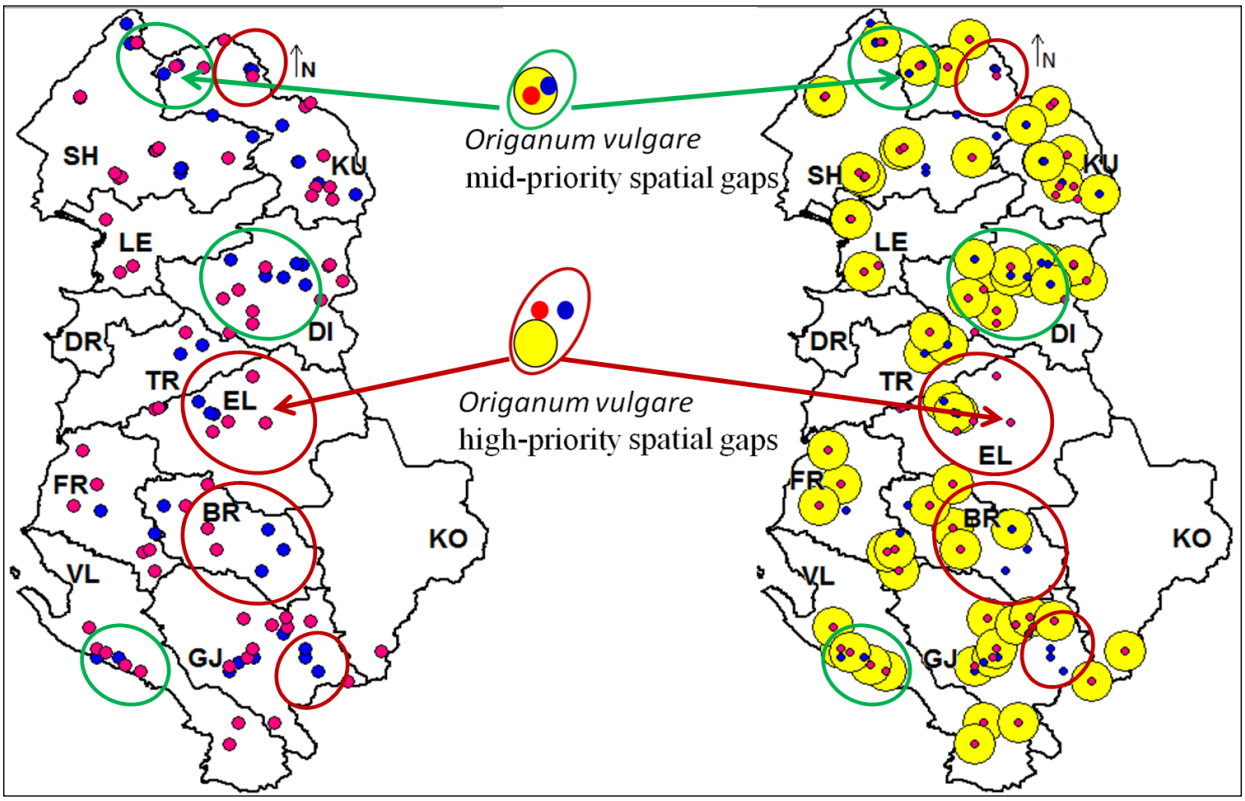
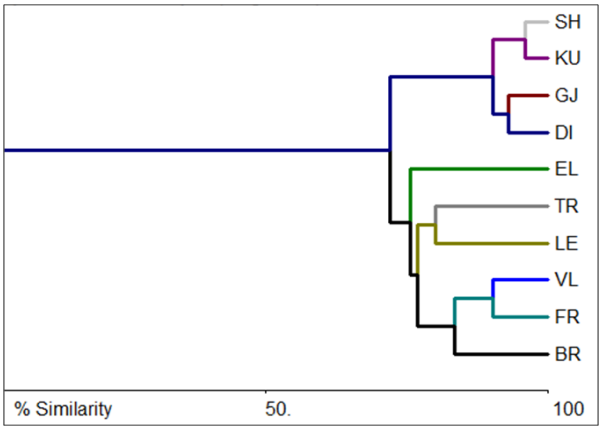


Figure 2. Geographic distribution map, mid-priority and high-priority spatial gaps of oregano (*Origanum vulgare* L.)

Cluster analysis on correlation: Cluster analysis method using similarity and correlation matrix data proved the presence of similarity among areas where oregano populations, mid-priority, and high-priority gaps were observed.



Clusters	Distance	Similarity	Leader	Joiner	Leader	Joiner	Coeff. r
9	4.065	95.935	6	8	KU	SH	0.96
8	7.0707	92.929	2	5	DI	GJ	0.95
7	9.8039	90.196	4	10	FR	VL	0.92
6	9.9099	90.09	2	6	DI	KU	0.88
5	16.667	83.333	1	4	BR	FR	0.82
4	20.001	80.009	7	9	LE	TR	0.83
3	23.077	76.923	1	7	BR	LE	0.66
2	24.444	75.556	1	3	BR	EL	0.48
1	28.002	72.008	1	2	BR	DI	0.76

Figure 3. Cluster dendrogram and similarity indices among oregano district areas observation

Cluster dendrogram show clearly similarity among KU and SH districts, DI and GJ, FR and VL, LE and TR district areas (**Figure 3**). For all observed areas the similarity matrix indices range from 72 to 95.9 and coefficient of correlation (r) range from 0.48 in cluster 2 to 0.96 in cluster 9. Low similarity and higher distances was among BR and DI counties (distance index 28.00, similarity index 72.00)

CONCLUSION

- Ex situ collection of oregano (*Origanum vulgare* L.) stored in Albanian genebank represent 61.5% of potential size of oregano collection.

- The study identified the areas with higher richness of oregano populations were KU, SH, DI and GJ districts. The areas with mid-priority spatial gaps were VL, EL, FR and SH districts, the areas with high-priority spatial gaps were TR, VL, GJ, KU, SH, and EL districts.

- Higher number of priority sites were observed at the SH, KU, GJ and EL district areas. The concentration of priority sites (70%) only in four districts suggests in these areas the possible priority sites can be used to increase size of ex situ oregano collection.

- Application of GIS tools and gap detection methodology can improve representativeness of gene-bank collections through identifying prioritized collecting sites and potential priority areas for in situ conservation.

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Review paper

MEDICINAL MUSHROOMS AND THERAPY: TRANSLATING A TRADITIONAL PRACTICE INTO THE WESTERN MEDICINE

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ABSTRACT

Medicinal mushrooms are used in traditional medicine for many years. Searching for new medicinal substances that come from mushrooms and their studying is significant for medicine and pharmacy. However, most of these substances have not been clearly characterized.

Modern medical practice relies heavily on the use of highly purified pharmaceutical compounds whose purity can be easily assessed and whose pharmaceutical activity and toxicity show clear structure - function relationships. In contrast, many herbal medicines contain mixtures of natural compounds that have not undergone detailed chemical analyses and whose mechanism of action is not known. Traditional folk medicine and ethno - pharmacology coupled to bioprospecting have been an important source of many medicines. With the current decline in number of new molecular entities from the pharmaceutical industry, novel agents are being sought from traditional medicine. As an example of medicinal mushrooms demonstrates, translating traditional Eastern practices into acceptable evidence - based Western therapies is difficult. Different manufacturing standards, criteria of purity and under - powered clinical trials make assessment of efficacy and toxicity by Western standards of clinical evidence difficult. Purified bioactive compounds derived from medicinal mushrooms are a potentially important new source of agents; their assimilation into Western drug discovery programs and clinical trials also provides a framework for the study and use of other traditional medicines.

This review includes some of the isolated and identified substances from mushrooms which express promising antitumor, immune modulating, antiviral, antibacterial and antiparasitic effects. The studied species belong to the different genera of Basidiomycetes such as: *Boletus, Amanita, Calvatia, Tricholoma, Russula, Lactarius*…. Species from almost all mentioned genera are found in the Republic of Macedonia, with different distribution and frequency. In suitable conditions some species develop rapidly.

***Key words**: medicinal mushrooms, medicine, therapy, effect, etnomycology*

INTRODUCTION

Mushrooms are heterotrophic eukaryotic organisms. So far, approximately 100,000 species of mushrooms are known. They are able to grow in all climatic regions and develop in all possible substrates. Some species are cosmopolitan, and some are specialized in certain substrates and develop under certain conditions [1]. From about 2250 species of mushrooms that are considered to occur in the Republic of Macedonia, a large part is medicinal, and about 500 species are edible. Mushrooms despite nutritional value [2-9] have a medicinal value as well. Far East has more than 1,000 years of tradition in the use of mushrooms for healing. Western civilization in the last decades of the twentieth century began to discover this wonderful world.

Through the synthesis of secondary metabolites e.g. polysaccharides, steroids, triterpens, nucleotides etc., most visibly are displayed out the tremendous opportunities of mushrooms as Biosystems that man can manipulate for advantageous purposes. Although most of these metabolites are isolated from representatives of subtypes Zygomycota and Ascomycota, recent research has increasingly turned towards Basidiomycota. The advantage of the higher mushrooms is that in most cases laboratory cultivation is not needed, but their macroscopic fruiting bodies can easily been identified in the nature and to be collected.

The purpose of this research is to show that mushrooms are easily available and inexpensive, are found everywhere and have greater applications in medicine and pharmacy. Considering the fact that mushrooms are a weakest investigated group of organisms, however, it is the reason for their low utilization for therapeutic purposes. Therefore the results of scientific research and implementation of scientific facts is of great importance, and will allow faster implementation of preparations based on medicinal mushrooms from diet supplements in drugs.

This paper is based on data derived from primary and secondary literature. The primary literature provides insight into today's medical knowledge about medicinal mushrooms in a highly professional journal e.g. International Journal of Medicinal Mushrooms, Journal of Food and Agriculture, Edible Fungi of China etc. Secondary literature covers scientific studies published on: www.pubmed.gov, www.sciencedirect.com, www.wipo.int, www.lib.bioinfo.pl, www.cefe.cnrs.fr, www.begellhouse.com/journals/, www.cancerletters.info. Data from research papers published in publications of congresses and data from books are also used.

GLOBAL DIVERSITY OF MUSHROOMS IN R. OF MACEDONIA

In the Republic of Macedonia according to the current research around 2250 species of mushrooms are known and systematized into 6 types e.g.: Basidiomycota, Ascomycota, Myxomycota, Chytridiomycota, Oomycota and Zigomycota, out of classes Ascomycetes and Basidiomycetes belong 250 and 2000 species, respectively. Within the class Basidiomycetes, most species are registered among the Aphyllophorales and Agaricales. So far, in the

Republic of Macedonia there is registered the total of 117 species of poisonous mushrooms, of which 31 species are hallucinogenic [10].

In this paper emphasis is placed on a distribution of the most investigated medicinal mushrooms in the world at the level of the Republic of Macedonia. There are 11 species explored, including: *Agaricus bisporus, Clitocybe nebularis, Coprinus comatus, Ganoderma lucidum, Griphola frondosa, Hericium erinaceus, Neolentinus lepideus, Phalus impudicus, Pholiota adiposa, Piptoporus betulinus* and *Pleurotus ostreatus*. According to the action of their metabolites in mushrooms are explored their antibacterial, antifungal, antiviral and antitumor (cytostatic) effect.

Agaricus bisporus (J.E. Lange) Imbach - Family *Agaricaceae*

Well known "Shampignon" is commonly cultured species worldwide which is used in the diet. It is an excellent source of vitamins of the B group, especially riboflavin, as well as minerals like K, Se and Cu. This mushroom possesses antioxidant activity, antihyperlipidemic and antitumor effects. In addition goes and many studies that has investigated and confirmed the antimicrobial activities of the components such as: 1-octen-3-ol and 10-oxo-trans-8-dodecanoic acid [11]. The research which included studies of the extracts from fruiting bodies of nine common mushrooms, with gel electrophoresis demonstrated the strongest activity of the A. bisporus extracts extracted with cold water [12]. Applied powder of A. bisporus in lipidemic, normal and control rats caused a significant reduction in total cholesterol, LDL levels and increase HDL levels, without significant changes in the values of triglycerides in the lipidemic samples. This reduces the risk of coronary artery disease. Reduced atherogenic indexes, cardiac risk factor, reduced liver mass are recorded [13]. Research in 2001 demonstrated that extracts of A. bisporus caused significant dose-dependent suppression of the activity of the enzyme aromatase (estrogen synthetase) obtained in situ, which has a dominant role in tumor proliferation in human breast cancer in women. The active components of the extracts of A. bisporus soluble in water were stable at high temperature [14]. Secondary metabolite agaritin of fruiting body of the mushroom A. bisporus is phenyl hydrazine derivate of glutamic acid. Though is fined in high concentrations in the mushroom its biological effects on humans are not sufficiently explored [15]. Lectins of A. bisporus tested in vitro showed anti proliferative activity. With that they inhibit the development of proliferative disease vitreoretinophy [16].

Clitocybe nebularis (Batsch) P. Kumm. - Family *Trocholomataceae*

This species belongs to the group of mushrooms that possess the combined biological characteristics. The spectrum of antimicrobial activity is broad and includes: antibacterial, antiparasitic and antiviral action. Possess and anticancer properties. The mushroom produces klitocibin, cysteine protease inhibitor that is of potential interest for the treatment of cancer since it is known that cysteine protease is involved in the development of malignant diseases [17]. Serine protease inhibitor CnSPI was isolated from *C. nebularis* and its role in intra / extra cellular proteolysis was established [18].

Coprinus comatus (O.F. Müll.) Pers - Family *Agaricaceae*

In this mushroom, most of the biological activity is accounted to β-glucan. It is established that the mushroom *C. comatus* possesses an enormous amount of β-glucan in comparison with 18 other medically important mushrooms [19]. With the aid of the NMR spectroscopy

fucogalactan is characterized in the aqueous extract of the mushroom [20]. Aqueous mushroom extract showed potential in vitro activity against breast cancer [21]. Alkaline protein was isolated from the fruiting bodies of *C. comatus,* called y3 which inhibits the growth of the cell line of gastric cancer with IC_{50} of 12μg/mL [22]. Extract from micellar polysaccharides administered intra peritoneal in white mice at doses of 300 mg/kg inhibits the growth of Sarcoma 180 and Ehrlich tumors up to100 % and 90 % respectively [23]. Research in China, conducted on mice demonstrate the ability of polysaccharide extracts of *C. comatus* to increase the activity of lysozyme, which is a general indicator of the state of the immune system [24]. Recently is published an in vivo study in which proinflammatory and immunomodulatory effects of the extract from *C. comatus* are demonstrated [25]. There are many studies in the past decades that research the hypoglycaemic capacity of the aqueous extracts of *C. comatus* [26-28]. *Coprinus comatus* contains components that kill nematodes, particularly soil nematodes, *Panagrellus redivivus* and *Meloidogyne arenaria* [29]. Researchers in the mushroom *Coprinus comatus* discovered the existence of ergotionen (thiol), a substance with antioxidant properties [30,31].

Ganoderma lucidum (W.Curt.:Fr.) Karst. - Family Ganodermataceae

Scientists from China found that Reishi mushroom improves the blood pressure and reduces the need of oxygen of the heart muscle [32]. Similar findings come from Japanese researchers [33,34]. Reishi contains ganodermic acid (triterpens) that reduces high blood pressure, reducing high levels of cholesterol and inhibits platelet aggregation. Its power as an antioxidant and antimicrobial agent in the prophylaxis and treatment against gram positive bacteria has been demonstrated [35]. Studies on Reishi antitumor effect are widely distributed in Japan, Korea, China as well in Europe and America. A *G. lucidum* mycelia soy extract (GCP) is a product rich in polysaccharides and isoflavones, genistein and diadzein, with a significant potential for the treatment of an advanced prostate cancer [36]. Active antitumor components from Reishi are called hetero-β-D-glucans e.g. β-D- glucan, glukurono-β-D-glucan, arabinoksilo-β-D-glucan, ksilo-β-D-glucan, mano-β-D-glucan, ksilomano-β-D-glucan. β-D-glucan modulates the immune system by activating the immune cells such as macrophages, T helper-cells, increases values of immunoglobulins which produces enhanced response to foreign cells (bacteria, viruses or tumors) [37]. Because of the role of the immune system scientists in 1990 from the Health science center from San Antonio found that Reishi can be effective in the treatment of arthritis, conjunctivitis and rheumatism without side effects [38]. On the Far East Reishi is prescribed for the treatment of chronic hepatitis with total efficiency in the interval from 70.7 to 98.0 % [39]. In Japan is recorded efficacy of Reishi in the treatment of hepatic insufficiency [40]. Researches on the role of *Ganoderma lucidum* in the treatment of HIV / AIDS are conducted in Africa. Improvement of the immune response has been documented with the use of the products of *Ganoderma lucidum* in the treatment of patients with HIV / AIDS [41]. From Reishi are isolated some low and high molecular inhibitors of asparagine protease that can be used as a potential pharmacological substances in the treatment of Alzheimer's disease [42].

Griphola frondosa (Dicks.:Fr.) S.F. Gray - Family Meripilaceae

Maitake mushroom in China and Japan is used in the treatment of cancer, diabetes, HIV infection, osteoporosis, hepatitis, liver protection, cardiac arrhythmia, atherosclerosis, thrombosis, infection, coronal artery disease, prophylaxis of heart attack, blood pressure regulation [43]. In the mushroom polysaccharide structure named as D-fraction was discovered, which has biological activity. It is believed that this mushroom achieves its

effects by stimulating macrophages, HK cells, T lymphocytes, IL - 1 and superoxide anions [44]. Diet with large amounts of *G. frondosa* in hypertensive rats reduces blood pressure. Isolated was ACE - inhibitory peptides in the mushrooms that showed competitive inhibition with ACE. The most potent ACE - inhibitory activity was detected in the extracts with cold water with IC_{50} of 0.95 mg [45]. Antiviral effects against HIV (AIDS) have been confirmed by the U.S. National Cancer Institute. Studies in Japan have shown that the with the use of D-fraction from Maitake mushroom killing of the T helper-cells by the HIV can be prevent up to 97 % in vitro [43] .

Hericium erinaceus (Bull.:Fr.) Pers. - Family Hericiaceae

The mushroom called "hou tou gu" in Kina or "yamabusitake" in Japan has been used for centuries in the Far East countries. Studies on the pharmacological features recently started. Documented is that *H. erinaceus* has effect on gastric and duodenal ulcers by strengthening the immune system [46,47]. The pharmacologically active ingredients include: hericenon, lecithin, chitin and β-glucan. Erinacin has the strongest activity in the regeneration of nerve and enhancing the dementia of the Alzheimer type [48]. Neurotropic activity is demonstrated also and improved myelinization in mature myelin fibers, intact cell growth in vitro and the absence of toxic effects or damage to nerve cells [49]. In Japan it is found that yamabusitake is effective for improving dementia in patients diagnosed with mild cognitive impairmant [50]. In the study of Wang et al. in the methanol extract of *H. erinaceus* were identified D-treitol, D-arabinol and palmitic acid. It showed a dose-dependent reduction in blood glucose level in hyperglycaemic rats [51].

Neolentinus lepideus (Fr.) Redhead & Ginns - Family Polyporaceae

Extracts of the mushroom *L. lepideus* cause reinforcement of immunity and immunmodulator activity. The mushroom contains heteroglucan, free amino acids, ergosterol, anise and eburoic acid, lentinamicin A, B, β-1-3-glucan that promote granulocyte system to proliferation and cell differentiation in experimental mice [52].

Phalus impudicus L.: Pers. – Family Phallaceae

This mushroom was used by Baltic and Slavic peoples for abdominal pain, renal diseases, for washing and treating wounds, as a remedy for rheumatism, and bones pain. In European traditional medicine is called "stinkhorn", and usually is used as a juice from fresh fruiting bodies. In China *P. impudicus* is used as a cure for cancer in oral cavity. According to the physician Caro this mushroom has been used as a medicine in carcinoma cutaneum and internal tumors. Recent experiments and clinical studies show that *P. impudicus* has antithrombotic activity in the treatment of thromboembolic disease [53]

Pholiota adiposa (Batsch) P. Kumm. - Family Strophariaceae

Polysaccharides extracted from the culture of mycelium of *P. adiposa* administered intra peritoneal in white mice showed inhibition of the growth of Sarcoma 180 and Ehrlich solid tumors, up to 70 % and 80 % respectively [54]. Antitumor potential was later confirmed by a study in Korea, which consisted of a screening test of Sarcoma 180 cells transplanted into mice [55]. Later, these findings have been complemented with a new study where the tumor size was reduced, clearance of carbon particles and phagocytosis were increased [56]. 60 %

methanol extract of *P. adiposa* showed antimicrobial activity against *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Streptococcus pyogenes and Mycobacterium smegmatis* [57]. In addition to the potential of *P. adiposa* antihypertensive effect hence the results of many studies that confirm the presence of components that have ACE - inhibitory activity. New pentapeptide with ACE - inhibitory activity was isolated from the fruiting body of *P. adiposa* [58]. Methanol extracts from fruiting bodies of *P. adiposa* inhibit the enzyme 3-hydroxy- 3metil glutaryl CoA reductase, a key enzyme in the regulation of cholesterol synthesis [59]. Studies of the effect of the *P. adiposa* extract on fatty tissue in hyperlipidemic mice showed a marked reduction of retroperitoneal fats [60].

Piptoporus betulinus (Bull.) P. Karst. - Family Fomitopsidaceae

This mushroom has a long tradition in folk medicine for the treatment of cancer and stomach diseases. Experimentally is confirmed that the fruiting bodies have antibacterial, antiviral, anti-inflammatory, antiparasitic, antiproliferative and immunemodulator effects [61]. (E)-2-(4-hydroxy-3metil-2-butenyl)-hydroquinone and C polyporenic acid were isolated as matrix metalo-proteinase (MSEs) inhibitors from *P. betulinus* [62]. Experimentally was confirmed that compounds derived from *P. betulinus*, have anti-inflammatory effects and are useful for application in ear [63].

Pleurotus ostreatus (Jacq.:Fr.) Kummer - Family Pleurotaceae

This mushroom has anti-inflammatory, antioxidant, antitumor, hypoglycemic effect, causing antithrombotic effects, reduces blood pressure and lowers plasma lipid concentrations [64]. *P. ostreatus* is safe and effective alternative to statins in the treatment of hyperlipidemia in patients with HIV [65]. *P. ostreatus* produces health benefits with effect on the atherogenic lipid profile in hyperholesteremic condition [66].

CONCLUSION

A choice of small part of mushrooms was elaborated in this paper. Almost all mentioned species are registered in the Republic of Macedonia and have different distribution and frequency. In favorable conditions a massive growth of some species occurred. This paper should be a guide for that in which direction the scientific research should be develop, which can only be stimulated by the evidence presented in this and similar works.

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Review paper

USE OF ST JOHN'S WORT THROUGH THE AGES

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ABSTRACT

Hypericum perforatum L. is a plant which is well known for ages because of its medicinal properties. St. John Wort was even prescribed by Hippocrates, the father of medicine, Dioscorides the father of pharmacognosy and Pliny. This plant was used as a treatment for puncture wounds, headaches, burns and infections.

It is believed that the black - red dots and the translucent perforations on the plant contain the most medicinal properties. The name *Hypericum* refers on the use of the plant to wart off evil, by hanging plants over a picture in the house during St. John's day. The name *perforatum* refers to the presence of small oil glands in the leaves. *Hypericum perforatum* L. has long been used as an ancient folk cure for maladies including wounds kidney, lung ailments and depression.

The European physicians used the plant for vertigo, burns, paranoia and spontaneous abortion. In ancient time this plant has a history in magic. Also it, was used to protect the live from ailments, protections from death and discover the length of life of family members.

The black - red dots on the petals of the plant symbolize the blood shed by St. John at his beheading. In addition the translucent spots on the leaves represent the tears shed because of the event. It has wide range of medical uses because of its chemical composition. St. John's wort contains procyanidins, antrachinons, carbohydrates, alcohols, triterpenes, sterols, hetrosides of phenolcarbonic acids and flavonoids, naftodiantronic compounds, phenolic acids, phloroglucinols and essential oils. In the 21st century, science has offered scientific explanation of St. John's wort's features and explained its application, as well as its important role in pharmacy and medicine with its antibacterial, antiviral and antidepressive effects.

Presentation of the development of the ideas associated with St. John's wort, and the evolution of the notions, increased the ability of the pharmacists and physicians to respond to the challenges of their professional services in facilating the human life.

***Key words**: Hypericum perforatum, St. John Wort, depression, usage, history*

INTRODUCTION

The use of medicinal plants as a means of healing is old, as much as mankind. The search for the source of health launched in nature, which has an answer to every question and for every disease, was a need [1]. Paracelsus enrolled "Health is everywhere around us, we are part of nature and the need for the drug for ourselves we have to demand in it and not out of it" [2].

St John's Wort (*Hypericum perforatum* L. fam Hypericaceae) has a long historical use, as one of the most used and the most curative plants on the planet, which is widely distributed in the territory of the Republic of Macedonia. Times have changed, but preference towards the plant *Hypericum perforatum* L. is still actual, and its use is more disseminated in all parts of the world. Hence importance to research the history of this plant is enormous.

CHRONOLOGY FROM THE PAST TO THE PRESENT

LEGENDS

Christians have their own legend about St. John's wort derived from the color of its petals and this is associated with the wounds of Jesus Christ. According to the legend, when the beloved devotee of Jesus Christ, in his sadness he stood under the cross on which the Saviour was crucified, began carefully to collect plants bedewed from the holy blood to give to the honest believers as a precious memory of the death of the Saviour. Mysteriously red juice from the aromatic plant caused an impression as one drop of the blood of Jesus Christ lives hidden in the red color of the golden-yellow flower [3].

In England in the pre-Christian period St. John's wort was used in religious rituals and many legends has been written about it. Because of its bright yellow color St. John's wort was often associated with the sun and was used to predict marriage. To predict the chances of a marriage blessing, young girls have collected this plant and left to remain till morning. If the next morning flowers remain fresh they will have a happy marriage, and if faded bad luck would followed them.

Kim Fletcher [4] in his book "The Penguin Modern Australasian Herbal" explained how St. John's wort was denominated. During the medieval period to *Hypericum perforatum* has been prescribed magical powers for protection against evil spirits because of its affiliation with St. John the Baptist. According to this legend the plant was called St. John. The name may derive from the red spots on the petals which symbolize the blood of Saint John, and occur in late August and early September when is the day denoted to the Holy martyr's death i.e. when he was beheaded. Transparent spots on the leaves represent the shed tears because of the occurrences. Lastly, perhaps the plant was named basically on the folk belief that if you put a branch bellow the pillow before the feast of the birth of St. John, the saint will appear in a dream and the dreamer will be blessed and protected from death the following year.

In Macedonia there are many legends and tradition of St. John's wort usage. According to the folk tradition of Prespa's and Bitola's region St. John's wort is most curative if it is gathered on July 7th the day of St. John (Ivanden) in Orthodox calendar. In this region St. John's wort could be found under the name "navaliche" - (navala = influx) because the craftsmen and traders gathered St. John's wort on this day and weave it in cross that put in a prominent

place, usually above the door, believing that it will bring a influx of customers. In their homes the also hang Cross from St. John's wort for an influx of health, gladness and happiness in the family. Domestic Oleum Hyperici in these regions made from St. John's wort gathered on this day in the most mature stage is the best and the most curative.

According to the work of Mary Treben [3] a Serbian people collect St. John's wort on the day of St. John (Ivanden) too which is a symbol of the power of light and warmth. St. John's wort then glares in its most glorious floral splendor. In ancient times Serbian girls weave it in wreaths and each of the girls that played around St John's fire had to wear this crown. In that mysterious night in the fire were thrown branches of St. John's wort and girls that were already for marrying on the faded flowers could seen what kind of fortune would have with the boys next year.

In Austria villagers according to the old folk customs were putting this plant between two slices of bread and gave it to the domestic animals for they could be spared from the diseases. This custom was kept up to date in some rural families [3].

HISTORY OF ST. JOHN'S WORT NAME

Hypericum is an ancient name and can have many meanings. "Ypericon" (a Greek word) was first mentioned by Europhon, Greek physician from 288 BC. A simpler explanation of the name "Ypericon" is that originated from the words "ereike" meaning high and "hyper" meaning upward. The remaining authors originated from "hypo" - under and "erikn" or "ereikn" - desert, which explains its finding places in nature. Later Linnaeus (1707-1778) believed that the etymology of the name *Hypericum* originated from "hyper" - translucent and "eikon" - an image [6]. This refers to the traditional usage of the St. John's wort to protect against evil with hanging the plants above the picture in the house, on the day when they celebrate St. John. Linnaeus explained this picture as transparency of petals [9]. In antiquity St. John's wort was used to exorcise evil spirits, so the plant was holding on the icons as protection.

Name *perforatum* refers to the presence of small oil glands in the leaves that look like windows, and can be seen when the plant is held on sun light.

Aristotle (384-322 BC) this plant mentioned by name "egg on the ground". *Hypericum perforatum* in antiquity was known also as "Fuga Daemonium" because it was used to chasing demons.

HISTORY OF ST. JOHN'S WORT USAGE

St. John's wort has a long historical usage. Historical data indicate that the ancient nations in their medicine used plants. Very interesting is the fact that, according to their records, they accurately knew the properties of plants, how and where to apply them. The first recorded, written usage of St. John's Wort for medicinal purposes dates back to the ancient period. This old medicinal plant was found in the writings of the ancient Greeks and Romans, and later in

other nations [5, 6]. St. John's wort was used in antic period to treat burns, fever, snake bites and some mental disorders [7, 8].

Theophrast (371– 287 BC) recommended St. John's wort as a remedy for external usage. Pliny the Elder (23-79 AD) thought that St. John's wort should be taken with wine against snakebite [5].

Dioscorides (40-90 AD) the most famous physician and pharmacognosist in ancient period mentioned 4 species of *Hypericum: Upericum, Askuron, Androsaimon* and *Koris,* which he recommended for the treating of sciatica by drinking them mixed with hidromel (mixture of water and honey). He believed that St. John's wort could cure cholera but if was continually drunk. Also, he thought that St. John's wort can heal burns [6].

Medical properties of St. John's wort were described by Galen (131-200 AD). He recommended this plant as a medicament for treatment of wounds and for the purification of blood [10].

One of the treatises of the abbes Hildegard of Bingen (1098-1179 AD) was a textbook of *Materia medica* which, although influenced writings, also contains a great deal of information about folk medicine. Among the indigenous medicinal herbs she mentioned St. John's wort [11].

Paracelsus (1493-1541 AD) was one of the proponents of chemically prepared drugs from raw plants and mineral substances; nonetheless, he was a firm believer that the collection of those substances ought to be astrologically determined. He continuously emphasized his belief in observation, and simultaneously supported the "Signatura doctrinae" –the signature doctrine. According to this belief, God designated his own sign on the healing substances, which indicated their application for certain diseases e.g. St John's wort't would be beneficial for treatment of wounds and stings given that the plant leaves appear as if they had been stung.

Matioli (1568 AD) in his work Discorsi wrote about St. John's wort as a diuretic, emenagog and antimalaric, especially recommending St John's Wort usage, for treatment of burns [6].

Culpper (1650 AD) described the relationship of plants with zodiac signs. He considered *Hypericum* under the heavenly sign Leo in the dominance of the Sun. According to him St John's Wort is the unique plant for medical treating of wounds primarily burns, for reducing inflating and healing the wounds, for healing bites of poisonous animals. It is now confirmed by the modern science.

In 1696 Englishwoman Aubrey told the story of St. John's wort usage to exorcise evil spirits.

In the New World, Indians used St. John's wort as a means for abortion, dermatological medicament and for raising the immunity [12].

St. John's wort is mentioned in the Macedonian medicine book by E. Sprostranov who collected from the Ohrid's region the etnophytotherapy during the whole Middle Ages. Macedonian Medicine books are manuscripts created on the territory of Macedonia that contains recipes for treating diseases in people and animals are written in original Macedonian folk language in Macedonian Cyrilic alphabet. The data presented in Ohrid's

medicine book dating from the time of St Clement (ninth century). According to E. Sprostranov St. John's wort has been used against burns, cuts, hemorrhoids. Oleum Hyperici was used as an antiseptic and for internally against diseases of the liver, stomach and intestinal parasites [13].

By the 1850s, St. John's wort has been used to determine the living life of family members. Each family member had placed a bunch of St. John's wort on the roof of the house. Next morning was investigating which of bunch is the most withered. It meant that this family member will die soon.

In 1876 King in his work King's American Dispensatory mentioned the use of *Hypericum perforatum* against diarrhea, intestinal worms, menorrhagia, hysteria, jaundice, nervous disorders, depression and as *Hyperici* tincture for external usage against bruising. Felter - Lioyd performed revision on the work King's American Dispensatory and add the recipes of a St. John's wort preparations for curing the spine pain, shock, hysteria and contusions.

THE HEALING POWER OF ST. JOHN'S WORT

From a phytochemical point of view, St John's wort is one of the best investigated medicinal plants. A series of bioactive compounds has been detected in *Hypericum perforatum*, namely flavonol derivatives, biflavones, proanthocyanidines, xanthones, phloroglucinols and naphthodianthrones.

Hyperforin and other phloroglucinol derivatives act antibacterial. Hyperforin is well - known for its anti - inflammatory, anti - tumor, anti - bacterial, and antioxidant properties. The application of a hyperforin - rich verum cream could strengthen the skin barrier function by reducing radical formation and stabilizing stratum corneum lipids [14]. A similar effect has naphtodianthrone component e.g. hypericin, but for him it hasn’t been confirmed any activity against viruses [15]. The antiviral and antineoplastic activities of hypericin and its derivatives and its mode of action have been widely studied, in the last three decades [16]. However, clinical research in this field is still scarce. Due to the significant amount of tannins concentrated infusion of St. John's wort has been used as antidiarrhoic. Recently there has been a scientific research on defining the antidepressant effect of St. John's wort. Carriers of this action are xanthone ingredients (MAO) inhibitors. Since, couple of years ago the effect of hypericin against HIV virus is confirmed. Maybe this old drug would gain new therapeutic indications.

Commonly St. John's wort oily extract has been used internally and externally against gastric ulcer and ulcers.

There is a large numbers of different other phyto preparations of St John's wort in almost all pharmaceutical forms. From St. John's wort is produced the antibacterial preparation Imanin® which could be applied externally in fresh and infected wounds, burns, ulcers etc. The preparation stimulates tissue regeneration [9]. In European countries several different phyto preparations of St John's wort are in use: Hyperforat® (coated tablets, drops, and ampoules), Jarsin® (coated tablets), Psychotonin® (tincture) and Neurapas® (film - coated tablets).

At present, almost all pharmacopoeias in the world e.g. Ph. Eur. 6 [15], British Herbal Pharmacopoeia [16] etc. proscribe *Hypericum perforatum* preparations of a real medicinal value. German Commission E also prescribes the impact of *Hypericum perforatum* and its preparations [17]. German Commission E is a therapeutic guide in herbal medicine, complied by a special expert commission of German Federal Institute of Medicines and Medical Inventions. Recently this medicinal plant is less used in traditional purposes. More recently is considered as an antidepressant and antiviral mean for human application.

SIDE EFFECTS

Hypericin has photo sensible properties and as such increases the sensitivity of the skin to the sunlight [18]. In animals with light skin that graze St. John's wort with sun exposure occur toxic changes that appear on the skin called Hypericismus [9]. For the photo toxicity of this plant naftodiantronic compounds are responsible [8].

Plant medicines used by patients in self-treatment contain powerfully acting active substances which can be a source of adverse events including interactions with synthetic medicines. Usage of St. John's wort causes high risk of various complications. St. John's wort preparations shouldn't be combined with antidepressants without physician's consultation [20]. Different extracts of *Hypericum perforatum* have shown a considerable influence on pentobarbital and diazepam pharmacodynamics and paracetamol pharmacokinetics, in correlation with their naphtodianthrone concentrations [19].

Patients using St John's wort concomitantly with cyclosporine or other medications with similar absorption and / or metabolism to cyclosporine need close monitoring, because St John's wort interacts with cyclosporine, causing a decrease of cyclosporine blood levels [22, 23].

CONCLUSION

St. John's wort was for ages appreciated by the various civilizations and was used externally against cuts, burns, hemorrhoids, for healing of wounds and as an antiseptic. Internally against liver aches, kidney aches, asthma, lungs aches, stomachaches, diarrhea, hysteria, etc.

Today, it should be encouraged the isolation and preparation of preparations from St. John's wort with verified active components in dosage amounts.

Effective and safe phytotherapy requires a lot of knowledge about the properties and toxicity of preparations used and accurate monitoring of the consequences of their actions.

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Original scientific paper

MICROMORPHOLOGICAL RESEARCH REGARDING THE GLANDULAR HAIRS OF *THYMUS PRAECOX* OPIZ SSP. *POLYTRICHUS* (A. KERN. EX BORBAS) JALAS

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ABSTRACT

Thymus praecox ssp. *polytrichus* grows in alpine and subalpine regions especially on crystalline rocks. In Romania, it is frequently found in all mountain areas. The *purpose* of the paper is to identify and characterize the morphological types of glandular hairs of *Thymus praecox* ssp. *polytrichus* according to vegetation stage. *Thymus praecox* ssp *polytrichus* was collected from Parang Mountains, during 2013, in different vegetation phases. For the micro-morphological research, the vegetal material has been fixed and preserved in 70% ethylic alcohol. The micrographs were performed by means of a Novex (Holland) microscope, using a Canon A95camera. The glandular hairs of the analyzed samples present a unicellular base implanted between epidermal cells, a unicellular pedicel and a secretory gland consisting of one or more secreting cells covered by a very thin cuticle. The hairs with unicellular and pluricellular gland are present in all aerial vegetative organs, in all stage of plant. The hairs with bicellular gland are rarely observed, being frequent mostly in the vegetative stage of plant. In a surface section of the leaf blade is observed that glandular hairs are numerous per unit area, being bounded by elongated epidermal cells, with straight sidewalls.

Keywords: *Thymus, micro-morphology, glandular hair, frequency, vegetation stage*

INTRODUCTION

Most Lamiaceae species have a strong aromatic character due to the presence of glandular structures that produce volatile oil [1]. Studies on secretory structures of plants of this family show the presence of two types of secretory trichomes: peltate and capitate [2, 3, 4]. Several morphological, structural, ultrastructural aspects and secretory types have been studied [5, 6,

7, 8, 9, 10, 11, 12, 13], due to the economical importance of volatile oils produced by the secretory structures previously mentioned.

Part of the Lamiaceae plant family, *Thymus* Genus can be considered one of the most important genera of the family due to the great number of species it comprises [14]. *Thymus* Genus is frequently found in the Mediterranean region, where it does not overcome 50 cm in height, being very well adapted to drought and heat [14]. In România 17 species of *Thymus* can be found, out of which 16 are spontaneous, and one (*Thymus vulgaris* L.) is cultivated [15]. A common feature of the species of this genus is the presence of different forms of secretory hairs that produce volatile oils with inviting aromas, this probably being one of the reasons that humans have been attracted by these plans, using their oils in numerous ways.

The authors of this article aim to identify and characterize the main morphological types of secretory hairs, bearing in mind the ontogenetic stage of the plant.

MATERIAL AND METHODS

During the year 2013, the plant material consisting in Thymus praecox ssp. polytrichus plants has been collected in the Parang Mountains (Romania) on different altitude levels (1600 m; 2069 m (Dengheru Peak); 2145 m (Udele Gorge); 950 m (Ranca)) and during different vegetation stages (Vegetative, Anthesis and Fructification) of the plants.

The collected plants were verified by PhD prof. Nicolae Ștefan, taxonomist at the Faculty of de Biology of the „Al. I. Cuza" University of Iași. For the micro-morphological research, the vegetal material has been fixed and preserved in 70% ethylic alcohol. The micrographs were performed by means of a Novex (Holland) microscope, using a Canon A95camera.

RESULTS AND DISCUSSION

Previous studies regarding volatile oils secretory structures in Lamiaceae showed that in this plant family two kind of secretory hairs can be found (peltate and capitate), located on the aerial plant organs, especially on the leaf. Usually a secretory hair (either peltate or capitate) has a basal area comprising one or many cells, a stalk made of one or many cells and a gland consisting of one or many secretory cells [16]. Above these structures, some authors consider that the epidermis cells radial disposed around the basal part of the secretory trichome are part of the secretory trichome structure. Such cells have been observed in the *Thymus* species we studied (Plate I, c). It is believed that these basal cells do not function as typical epidermis cells but as an accessory for the secretory trichome that has a role in volatile oil production. The size, shape, display of these cells and the vacuole frequency in the cells probably concurs to photosynthesis product collecting and also their carriage to the basal cells of the secretory trichomes. Subsequently, these products will end up to the secretory gland throughout the cells that form the stalk of the secretory trichome where they will serve to volatile oil forming through enzyme action of secretory cell cytoplasm [17].

Secretory trichoms are considered being exclusive sites of biosynthesis of volatile oils, so, their number is directly proportional to the quantity of volatile oil that is being produced.

Consulting the existing bibliography we have ascertained that a relative small number of papers on the study of secretory trichomes in the Genus *Thymus* exist, from a structural point of view and on the frequency and their distribution on the aerial plant organs also.

To identify the types of secretory trichomes, cross sections and superficial sections of the aerial organs of *Thymus* plants taken into study have been carried out. Alongside the morphological description of the secretory structures of volatile oils, highlighting the possible differences that could occur based on phenophase and environment influences is one of our goals.

Thus, our studies have highlighted the fact that secretory hairs in the analyzed plants have (fig. 2):

- a *base* consisting of one cell, located between the epidermis cells;
- a *stalk*, usually consisting of one cell, sometimes two or three, especially in the one cell gland trichomes;
- a *secretory gland* consisting of one or many secretory cells, covered on the exterior by an extremely fine cuticle.

Based on the number of cells that construct the gland, there are three categories of observed secretory trichomes (Plate I):

- *trichoms with unicellular gland* – found on all the vegetative aerial organs; usually these trichoms have a unicellular, rarely bi- or tricellular stalk; the glandular (secretory) cell produces volatile oil that crosses the thin cellulose wall and cambers the cuticle covering the gland;

- *trichoms with bicellular gland* – not so often observed, especially in the species in their vegetative stage, the cuticle covering the gland is detached from the cell wall;

- *trichoms with a multicellular gland* – present on all the aerial vegetative organs and in all ontogenetic plant stages; the gland is composed by 4, 8, or 12 secretory cells. In young leaves and on the epidermis that covers the tip of the stem; different stages of development of the trichomes can be observed. Multicellular trichomes in different development stages differentiate themselves from the uni- or bicellular ones by shape of the basal cell and stalk, and also by the fact that glandular cells have a very obvious cuticle.

Concerning the layout and frequency of secretory hairs, they are found on the stem and most of all on the leaf.

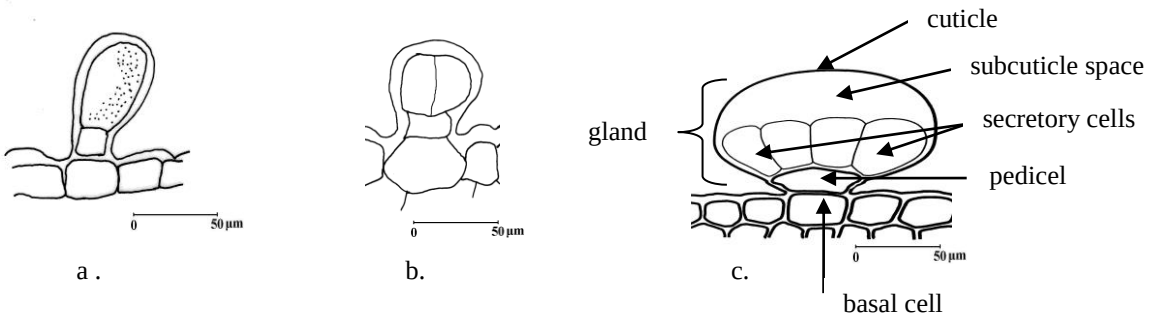


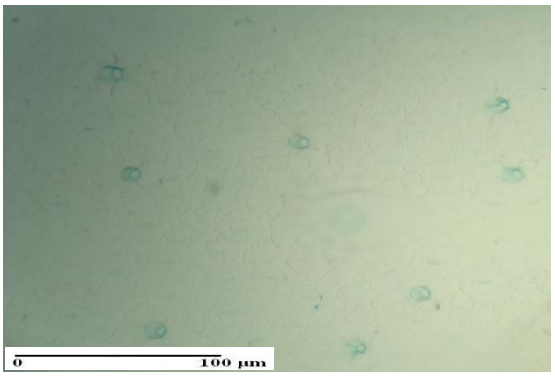
Figure 2. Morphological types of secretory trichomes in *Thymus* sp.: a. Trichome with unicellular gland, b. Trichome with bicellular gland păr, c. Trichome with tetracell gland (a, b – original images, c. processed image after Fahn, 1979).

In all analyzed samples, secretory trichomes are present on all the length of the stem, their frequency growing from bottom to top. They consist of a unicellular base placed between the epidermis cells, a stalk and a gland consisting of 1, 3 or many secretory cells. The most frequent are the unicellular ones.
Trichome frequency is the same on both sides of the leaf, margins and along the main vein, being even more frequent on the base of the leaf.
In cross section, glandular trichomes are present on both sides of the leaf, in very deep crevasses, sometimes opposed to each other, where the mesophyllum is extremely thin.

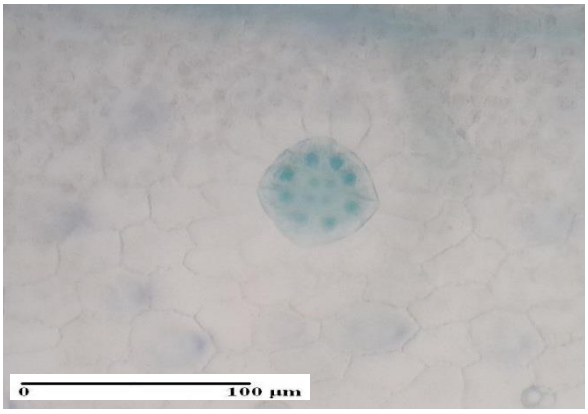
Generally, during secretion stage, the cuticle covering the secretory cells is two or three times thicker on top of them, compared to the cuticle covering the lateral gland walls. When the secretory trichome reaches maturity, gland cells begin to produce volatile oil that diffuses trough the exterior wall and cambers the cuticle. These trichomes are frequent on relatively young leaves and remain this way on mature leaves also.
Data obtained using photonic microscopy represent preliminary results in investigating secretory trichomes in the studied samples. These data will be integrated along the ones obtained using electron microscopy (SEM and transmission), date that will provide additional information on their structure and information regarding their ultra-structure and different stages of their development, aspects that cannot be highlighted using photonic microscopy.

PLATE I. Types of secretory trichomes in *Thymus praecox* ssp. *polytrichus,* collected from different altitude levels in Parâng Mountain.

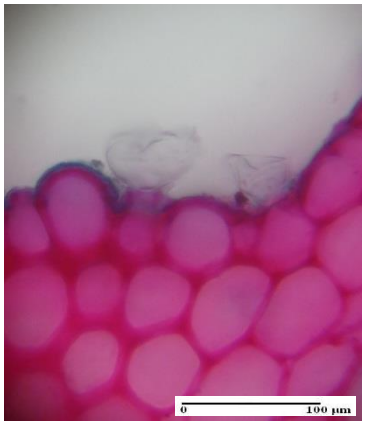
a. Secretory trichome with multicellular gland in leaf cross section (950 m altitude – vegetative stage).



b. Secretory trichomes with unicellular gland in leaf superficial section (950 m altitude- vegetative stage).

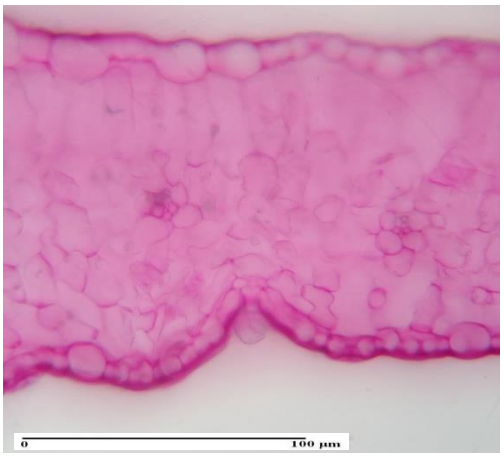


c. Secretory trichome with multicellular gland in leaf superficial section (1600 m altitude – anthesis stage).



d. Secretory trichomes with unicellular and bicellular gland in stem cross section (950 m altitude – vegetative stage).

e. Secretory trichome with multicellular gland in leaf cross section (2069 m altitude – anthesis stage).



f. Secretory trichome with unicellular gland in leaf cross section (2145 m altitude – fructification stage).

In a superficial section through the leaf, numerous secretory trichomes can be observed on the surface, being bordered by radially elongated epidermis cells with straight lateral walls.

CONCLUSION

In *Thymus praecox* ssp. *polytrichus* the authors observed three categories of secretory trichomes: *trichomes with unicellular gland* – found on all the vegetative aerial organs; usually these trichomes have a unicellular, rarely bi- or tricellular stalk; the glandular (secretory) cell produces volatile oil that crosses the thin cellulose wall and cambers the cuticle covering the gland; *trichomes with bicellular gland* – not so often observed, especially in the species in their vegetative stage, the cuticle covering the gland is detached from the cell wall and *trichomes with a multicellular gland* – present on all the aerial vegetative organs and in all onthogenetic plant stages; the gland is composed by 4, 8, or 12 secretory cells.

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Original scientific paper

DISTRIBUTION OF *SIDERITIS RAESERI* BOISS. ET HELDR. IN ALBANIA – STATE OF ITS POPULATIONS AND RECOMMENDATIONS FOR CONSERVATION

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ABSTRACT

Sideritis raeseri Boiss.et Heldr. belongs to the section *Empedoclia* of genus *Sideritis* L. (Lamiaceae). The extensive use of this species in Mediterranean traditional medicine is one of the reasons for the current threatened and vulnerable status of its populations. The species is endemic for the Balkan Peninsula and is naturally distributed in Albania, Greece and Macedonia. This paper reports the current state of populations of *Sideritis raeseri* in Albania. It is included in the Red Data Book of Albania with the category of endangered species. The current study provides information about its biological characteristics, ecological requirements, vegetation dynamics, place of the species in habitats and population state and structure. The field study was carried out in July and August 2013. Three localities were investigated: Gramozi Mt, Ostrovica Mt and National Park of Llogora. List of vascular plant diversity was prepared for each locality. The main purpose of the study was to focus the attention to conservation of this valuable species and to provide recommendations for its conservation and sustainable use and management.

Key words: *Sideritis raeseri Boiss.et Heldr., populations, conservation*

INTRODUCTION

The genus *Sideritis* L. (fam. Lamiaceae, subfam. Lamioideae) represents a complex taxonomical group, requiring extensive experimental investigation. This trend extends also to the Balkan representatives of the genus. For example, according to Flora Europaea [1] *Sideritis raeseri* Boiss.et Heldr. is a synonym of *Sideritis syriaca* L., together with *Sideritis taurica* Stephan ex Willd., *S. cretica* Boiss., and *S. sicula* Ucria. Taken in this broad sense, *S. syriaca* has a wide distribution and it is spread all over Southern Europe – from Sicily to

Crimea (Albania, Bulgaria, Crimea, Greece, Italy, Macedonia, Russia and Sicily). According to Flora of Albania [2], *Sideritis raeseri* is a distinct species indigenous to Albania, Macedonia, and Greece, and is therefore a Balkan endemic. Its populations are considered threatened and vulnerable and therefore, it is included in Red Data Book of Albania [3] with the category of endangered species. The studies on the variation of its natural populations to date are limited to Gramozi Mt (Albania) and were done by evaluating morphometric characters [3].

Sideritis raeseri is a perennial herb with slightly woody base and has a stem 10-50 cm in length. Basal leaves are spatulate with 5-20 mm petiole. Cauline leaves are narrowly elliptic to oblong, acute. Verticillasters have number between 3 and 15 and are always spaced apart each other.

Sideritis raeseri, known in Albania as "Caj Mali" has been an object of interest for a long time due to its valuable therapeutic properties. Being a native plant from the Mediterranean region, it is used as a herbal tea in the treatment of inflammations (against anti-human immunodeficiency virus replication), gastrointestinal disorders (anti-ulcerogenic, stomachic, carminative), coughs (analgesic), and as a tonic, while the extract is used as a component of dietary supplements for anemia. Phytochemical investigations of *Sideritis raeseri* were focused to the phenolic fraction and the antioxidant activity of flavonoids from its methanolic extract was studied [4]. Two apigenin derivatives were isolated and structural elucidated - apigenin 7-glucoside and apigenin 7-(4-O-ß-glucosyl-trans-p-coumarate) [5]. The content of potential bioactive phenolics of Macedonian *Sideritis* species used for medicinal "Mountain Tea" was studied [6]. Like many species of the Lamiaceae Family, *Sideritis raeseri* possess essential oils secreted by glandular hairs. The antibacterial activity of essential oils and their derivatives has been recognized for a long time. The chemical composition of the essential oils and its antimicrobial activities has been studied [7]. Phytochemical analyses of the *Sideritis raeseri* cultivated in an experimental field in Serbia have been performed. The essential oil composition, phenolic compounds and mineral content were analyzed in four different stages of flower development [8]. Investigations of the effect of the ethanol extract of *Sideritis raeseri* on the contractile responses of isolated rat ileum were done and the extract caused inhibition of spontaneous contraction, similar to the spasmolytic agent papaverine [9]. The aim of the present study was to describe the natural plant communities of *Sideritis raeseri*. This could help its better conservation and sustainable use.

MATERIAL AND METHODS

The field observations were carried out during July and August, 2013. Transect method was used for inventory of the flora. Transects were selected in order to cover maximum area. During the field observations GPS-coordinates, altitude, some biotic and abiotic factors were described; digital photographs of the species and their localities were taken. Distribution areas, populations' size and status of the habitats were investigated. The taxa were determined according to Flora Europaea [1] and Flora of Albania [2]. The floristic catalogue is alphabetically arranged by families, genera and species. The geographical floristic elements are described according to Walter [10]. Life form definitions were based on Raunkiaer's classification [11]. The specimens are presented in the Appendix.

Study area

Three localities of the species are investigated – at the base of Gramozi Mt, Ostrovica Mt and Llogora.

Gramozi Mt

Gramozi is a mountain in southeastern part of Albania, on the eastern edge of the Kolonja district with the highest peak of Peç-i (2523m), and it sits on the border of Albania with Greece. It is composed mainly by paleogenic flysch less from the lower flysch and limestone. It has a complicated tectonic structure. There are multiple forms of glacial relief at heights above 1500-1600 m. From the lower part of Gramozi Mt arise some branches of Osumi River. Northern and central part is rich in alpine pastures, while in the southern part of the mountain there are beech and conifer forests [12].

Ostrovica Mt

Ostrovica is a mountain located between Korça district and Skrapar, with the highest peak of Faqekuqi Peaks (2383m). It is one of the highest mountains of the southeastern part of the country. Consist of Mesozoic limestone and flysch. Numerous springs emerge from the foot of Ostrovica that feed some branches of Devolli and Osumi River. There are steep slopes, on ridge observed glacial forms of karstic landscape. Vegetation consists mainly of alpine pastures [13].

Llogora

The National Park of Llogora lies in the Northwest of Cike-Lungare mountain range. Cika Mountain (2045 m) and the Peak of Qore (2018 m) are the highest peaks of the zone that gradually get down to the Gorge of Llogora (1027 m) and the valley of Dukat to Northwest. From the geological viewpoint the zone of the National Park of Llogora consists of carbonate deposits of Mesozoic and Paleocene [13].

RESULTS AND DISCUSSION

Habitats where *Sideritis raeseri* grows are typical rock formations. It is distributed at open and sunny high mountain slopes with poor and eroded soils. In many places there is an outlet on basic rock.

The population of *Sideritis raeseri*, located in Ostrovica Mt can be classified in the most volatile state (N 40.19305, E 19.60326). The altitude is 1037 m a. s. l. and the slope varies between 25-40°. The exposition of the investigated area is northwest. In many places sites are more or less devoid of soil cover and the marble basic rocks are revealed. The population of *Sideritis raeseri* is almost destroyed. There are only leaves at the base of 5 - 7 plants per hectare - flowering stalks were harvested before the period of full bloom. The main threat to the deposit is its proximity to trunk road. The projective cover of vegetation is about 30 - 40%. The communities are species-poor and formed by strongly dominating of *Quercus coccifera* L., *Phlomis fruticosa* L., *Thymus capitatus* Hoff. et Link.

The localities of *Sideritis raeseri* in Llogora are in relatively good state due to limited access, the rules and regulations at the National Park of Llogora (N 40.2145, E 19.5809). The plant communities where *Sideritis raeseri* is found are dominated by *Pinus nigra* Arnold, *Pinus heldreichii* Christ., *Juniperus communis* L. or *Daphne oleoides* Schreb. The altitude varies between 1300 and 1700 m a.s.l.

In Gramozi Mt there are several localities of *Sideritis raeseri* and the one that we investigated is located in close proximity to the border with Greece (N 40.43903, E 20.79541). The study area lies at the altitude above 1700 m, with an inclination of 40° and southwest exposure. The basic rock is limestone with poor and dry soil layer. Because of the remoteness of the locality, the lack of good roads and the restricted access, the studied population is in a stable state. The species population consists of more than 200 well developed generative individuals; each of them with 2 - 12 flowering stems. The plant community had two vertical layers of vascular plants: shrub (with layer coverage of about 60 %) and herb (with layer coverage of about 50%). In many places *Sideritis raeseri* reaches 10 percent ratio herb layer. The dominant species in shrub layer is *Juniperus communis* L.

As a result of floristic investigations 116 plant taxa belonging to 83 genera and 31 families were recorded for the three localities of *Sideritis raeseri*. The analysis of the taxonomic structure of the flora in the three fields established that the families with the greatest diversity of species are: *Asteraceae* (17), *Poaceae* (13), *Lamiaceae* (13), *Fabaceae* (10), *Apiaceae* (7), *Caryophyllaceae* (7), *Rubiaceae* (6) and *Scrophulariaceae* (5). The locality with the largest number of species is that of Gramozi Mt – 86, followed by Ostrovice with 44 species, and Llogora with 41 species. The floristic list is presented in the Appendix.

Perennial herbs (70 species) prevail among the biological types but the number of annual herbs is also great (21 species).

Correlation of life forms determined the biological spectrum, which could serve as indicator of the climate and of the specific conditions in the area under study (Raunkiaer, 1905). In the terms of their life form, hemicryptophytes prevailed and accounted for 56.64%, followed by therophytes – 20.35 %, phanerophytes – 8.85%, chamephytes – 6.12%, the intermediate group of therophytes to hemicryptophytes – 6.12% and geophytes with 1.77%. The prevalence of hemicryptophytes is consistent with that in temperate geographical zone. On the other hand – the high percentage of therophytes showed association with Mediterranean Region.

The main geographical elements are those with Mediterranean origin or close to it - 70 species are divided between the following groups: Med (16 species), subMed (25 species), Eur-Med (14 species), Eur-subMed (3 species) and Med-As (2 species). The number of species with European origin (20 species) also takes important role in the floristic composition. Relatively low contribution of Boreal and subBoreal elements (8 species) is observed. The dominance of floristic elements with Mediterranean origin indicates ecological conditions in many cases and it is not only a result of competition. This is due to the absence of well-defined subalpine belt as well as increased Mediterranean influence that enters the southeast part of Albania. Balkan endemics and subendemics have comparatively great participation – 9 species.

Relation to humidity is another important parameter reflecting the biological and ecological peculiarities of plants. In terms of this parameter, the identified plant species are classified as follows: 51.33% are typically xerophytic species and 20.35% are typically mesophytic species. Along with this there are plants of greater or poor drought resistance: mesoxerophytes – 21.24% and xeromesophytes – 7.08%. The distribution of species in these groups corresponds to the observed dominance of the group of heliophytes (105 species) than that of sciophytes (8). This is due to the ecological conditions and the geographical location respectively.

Taxon	Locality			Phyto-geographic element	Requirements to the light	Requirements to the humidity	Biological Type	Life Form
	Gra	**Ostr**	**Llo**					
LYCOPODIOPHYTA								
Lycopodiaceae								
Huperzia sellago (L.) Bernh. Ex Schrank & Mart		+		Kos	H	M	P	Ch
PINOPHYTA								
Cupressaceae								
Juniperus communis L.	+	+	+	subBoreal	H	M	B	Ph
Pinaceae								
Pinus nigra Arnold		+	+	subMed	H	M/X	T	Ph
Pinus heldreichii Christ.		+	+	Ap-Bal	H	M/X	T	Ph
MAGNOLIOPHYTA								
MAGNOLIOPSIDA								
Apiaceae								
Bupleurum fontanesii Guss. ex Caruel	+			Med	H	X	A	Th
Bupleurum praealtum L.	+			subMed	H	X	A	Th
Eryngium amethystinum L.	+			Med	H	X	P	H
Eryngium campestre L.	+	+	+	Pont-Med	H	M/X	P	H
Pimpinella tragium Vill.	+			Pont-subMed	H	X	P	H
Pimpinella saxifraga L.	+		+	Eur-As	H/Sc	X	P	H
Trinia glauca (L.) Dum.	+	+		subMed	H	X	A-P	H/Th
Asteraceae								
Achillea clypeolata Sm.	+		+	Bal	H	X	P	H
Achillea collina J. Becker ex Rchb.	+	+		Eur-subMed	H	M/X	P	H
Achillea millefolium L.	+		+	Eur-Sib	H	M	P	H
Anthemis cretica L.	+			Med	H	X	P	H
Artemisia santonicum L.	+	+		Eur-Med	H	M/X	P	H
Carlina acanthifolia All.	+		+	Eur	H	X	P	H
Carlina corymbosa L.	+			Med	H	X	P	H
Carlina vulgaris L.	+	+	+	Eur-Med	H	M/X	A	Th

Centaurea calcitrapa L.	+			Med	H	X	A	Th
Centaurea Rhaponticoides group	+							
Cichorium intybus L.	+			Eur-Sib	H	M	P	H
Cirsium ligulare Boiss.	+	+	+	Med	H	M/X	P	H
Hieracium pilosella L.	+	+	+	Eur-Med	H	M	P	H
Hieracium pannosum Boiss.	+	+		Bal-Anat	H	X	P	H
Inula hirta L.	+			Eur-Sib	H	M	P	H
Onopordum acanthium L.	+	+	+	Eur-Med	H	X	A	Th
Xeranthemum annuum L.	+	+	+	subMed	H	X	A	Th
Boraginaceae								
Anchusa stylosa Bieb.	+			subMed	H	X	A	Th
Echium italicum L.		+		subMed	H	M/X	A	HTh
Echium vulgare L.	+			Eur-As	H	M/X	A-P	HTh
Onosma sp.	+	+	+					
Brassicaceae								
Aethionema saxatile (L.) R. Br.	+			subMed	H	M/X	P	H
Thlaspi praecox Wulfen	+			subMed	H	M/X	P	H
Caryophyllaceae								
Dianthus deltoides L.	+			Eur-Sib	H	M	P	H
Gypsophila muralis L.	+	+		Eur-As	H	X	A	Th
Minuartia caespitosa L.		+		Eur-Med	H	M/X	P	H
Paronychia kapela (Hacq.) A. Kern.		+		subMed	H	X	P	H
Silene coronaria (L.) Clairv.	+			Med-OT	H	M/X	P	H
Silene bupleuroides Chater & Walters			+	Pont-subMed	H	M/X	P	H
Spergularia rubra (L.) J. & C. Presl	+			subBoreal	H	M/X	A	Th
Chenopodiaceae								
Polycnemum arvense L.	+	+		Eur-Sib	H	X	A	Th
Cistaceae								
Helianthemum nummularium (L.) Mill.	+	+		Alp-Med	H/Sc	X	P	Ch
Convolvulaceae								
Convolvulus cantabrica L.		+		Pont	H/Sc	X	P	H
Crassulaceae								
Sedum acre L.		+		Eur-Med	H	X	P	Ch
Sedum hispanicum L.		+	+	Eur-	H	X/	A-	HT

				Med		M	P	h
Dipsacaceae								
Dipsacus laciniatus L.	+			Eur-Med	H	M/X	A	HTh
Scabiosa ochroleuca L.	+			Eur-Sib	H	X/M	A-P	HTh
Scabiosa triniifolia Friv.	+			Bal	H	X	A	HTh
Euphorbiaceae								
Euphorbia cyparissias L.	+			Eur	H	X	P	H
Euphorbia myrsinithes L.	+	+	+	subMed	H	X	P	H
Fabaceae								
Chamaecytisus absinthioides (Janka) Kuzmanov	+	+	+	Bal	H/Sc	M	B	Ph
Coronilla scorpioides (L.) C. Koch		+		subMed	H	X	A	Th
Dorycnium herbaceum Vill.	+		+	Eur-Med	H	X	P	H
Lotus corniculatus L.	+			Eur-Med	H	M	P	H
Onobrychis montana DC.			+	Carp-Bal	H	X	P	H
Ononis arvensis L.	+			Eur-As	H	M	P	Ch
Ornithopus compressus L.	+	+	+	subMed	H	M/X	A	Th
Trifolium alpestre L.			+	Eur-Sib	H	X/M	P	H
Trifolium angustifolium L.		+		Med	H	X/M	A	Th
Trifolium medium L.	+			Eur-As	H/Sc	M	P	H
Fagaceae								
Quercus coccifera L.		+		Med	H	X	T	Ph
Geraniaceae								
Geranium cinereum Cav. ssp. *subcaulescens* (L'Her. ex DC.) Hayek	+			Med	H	X	P	H
Hypericaceae								
Hypericum perforatum L.	+		+	Kos	H	M	P	H
Lamiaceae								
Acinos alpinus (L.) Moench	+		+	Alp-Carp	H	X/M	P	H
Micromeria cristata (Hampe) Griseb.		+		Bal-Anat	H	X/M	P	H
Phlomis fruticosa L.		+		Med	H	X	B	Ph
Prunella laciniata (L.) L.	+			Eur	H/Sc	X/M	P	H
Salvia argentea L.	+			Med	H	X	P	H
Salvia triloba L.		+		Med	H	X	P	H
Sideritis montana L.	+	+	+	subMed	H	X	A	Th

Sideritis raeseri Boiss. & Heldr.	+	+	+	Bal	H	X	P	H
Teucrium chamaedrys L.	+	+	+	subMed	H/ Sc	X/ M	P	H
Teucrium montanum L.	+			subMed	H	X	P	Ch
Teucrium polium L. ssp. *capitatum* L. (Arcangeli)	+	+	+	Pont-Med	H	X	P	H
Thymus capitatus Hoff. et Link.		+		Med - OT	H	X	P	Ch
Thymus sp.	+		+		H	X	P	Ch
Morinaceae								
Morina persica L.	+			Med-OT	H	X	P	H
Oleaceae								
Fraxinus ornus L.		+	+	subMed	H	X	T	Ph
Phillyrea latifolia L.		+		Med	H	X	B	Ph
Plantaginaceae								
Plantago lanceolata L.	+		+	Kos	H	M	P	H
Plantago major L.	+			Boreal	H	M	P	H
Plumbaginaceae								
Plumbago europaea L.			+	subMed	H	X	P	H
Primulaceae								
Primula veris L.	+		+	Eur-Med	H	M	P	H
Ranunculaceae								
Nigella arvensis L.	+		+	subMed	H	X	A	Th
Rosaceae								
Agrimonia eupatoria L.	+		+	Eur-Med	H	M	P	H
Prunus spinosa L.	+		+	SPont	H	X	B	Ph
Sanguisorba minor Scop.	+			subBoreal	H	M/ X	P	H
Rubiaceae								
Asperula aristata L.f.	+			subMed	H	M/ X	P	H
Asperula purpurea (L.) Ehrend.	+			subMed	H	X	P	H
Cruciata laevipes Opiz.	+		+	subMed-Cas	H	M/ X	P	H
Galium divaricatum Poirret ex Lam.	+			Med	H	M/ X	A	Th
Galium verticillatum Danth. ex Lam.	+			Med-As	H	X	A	Th
Galium verum L.			+	Eur-As	H	M	P	H
Scrophulariaceae								
Digitalis ferruginea L.	+		+	subMed	H	M	P	H
Parentucellia latifolia (L.) Caruel	+			Med	H	X	A	Th
Scrophularia canina L.	+			Eur-Med	H	X	P	H
Verbascum phlomoides L.	+	+	+	Eur	H	M	A-P	H
Veronica austriaca L.	+	+		Eur-	H/	X	P	H

				Med	Sc			
Thymeleaceae								
Daphne oleoides Schreb.	+			subMed	H	X	B	Ph
LILIOPSIDA								
Liliaceae								
Scilla autumnalis L.	+			Pont-subMed	H	X	P	G
Veratrum lobelianum Bernh.	+			Eur-As	H	M/X	P	G
Poaceae								
Aegilops geniculata Roth.	+	+		Med	H	X	A	Th
Bromus intermedius Guss.		+		Med-subMed	H	X	A	Th
Bromus mollis L.	+			Boreal	H	M	A	Th
Bromus squarrosus L.			+	subMed	H	X	A	Th
Cynosurus cristatus L.	+			Eur	H	M/X	P	H
Cynosurus echinatus L.	+			subMed	H	X	A	Th
Dactylis glomerata L.			+	Eur-As	H	M	P	H
Festuca nigrescens Lam.	+			Eur	H	M	P	H
Festuca rubra L.	+			Boreal	H	M/X	P	H
Festuca valida (Uechtr.) Penzes	+			Bal	H	X	P	H
Melica ciliata L.	+	+	+	Eur-subMed	H	X	P	H
Phleum pratense L.	+			Eur-subMed	H	M	P	H
Stipa capillata L.	+			Pont-Med	H	X	P	H

Legend: Biological types: A (annual herb), P (perennial herb), B (bush), T (tree).
Life forms: Ph (phanerophyt), Ch (chamaephyt), H (hemicryptophyt), G (geoptophyt), T (therophyt).
Phytogeographic elements: Ap-Bal (Apenino-Balkan), Bal (Balkan endemic), Bal-Anat (Balkan-Anatolian), Boreal (Boreal), Eur (European), Euro-As (Euro-Asian), Euro-Med (Euro-Mediterranean), Euro-SubMed (Euro-SubMediterranean), Euro-Sib (Euro-Siberian), Kos (Cosmopolitian), Med (Mediterranean), Med-CAs (Mediterrano-Central Asian), Pont-Med (Pontic-Mediterranean), Pont-SubMed (Pontic-Submediterranean), SubBoreal (Sub-Boreal), SubMed (Sub Mediterranean), sPont (South Pontic)

Picture 1 *Sideritis raeseri* in Gramozi Mt.

Picture 2 Gramozi Mt.

Picture 3 *Geranium cinereum* Cav. ssp. *subcaulescens* (L'Her. ex DC.) Hayek

CONCLUSION

The floristic inventory in the localities of *Sideritis raeseri* proved that the species has relatively poor plant communities. Altogether 116 taxa were established in its natural localities and most of them belong to the typical species for the very specific habitats, which is an indicator of relatively conserved natural physiognomy of the habitats. Based on the investigations it is not clear whether the habitats of the species are endangered or not. However, the conservation of species in the wild requires a complex effort, involving a wide range of disciplines and institutions. An urgent task will be to assess the natural localities for their conservation status and if necessary, to undertake measures for their conservation. This will help the conservation of the species as a whole. The other important step need to be taken is increasing the awareness of people who live close to the localities with emphasis on threats of the species and the risk of its extinction.

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Original scientific paper

FLORA AND VEGETATION OF BERATI CASTLE IN ALBANIA

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ABSTRACT

The flora of Berati castle was studied for the first time based on field research conducted in 2011-2013. Castles represent a specific environment, which is partly similar to rocks and rock fissures.

The vascular flora consist of 153 genera 54 families and 200 taxa, growing in the castle walls, rocks within the castle area and directly adjacent to the castle. In the life – spectrum, therophytes are the prevailing (41%). Seventeen chorological groups are distinguished, with the Euri- Mediterranean element (30%) as dominant. Of all plant species recorded, a considerable part belongs to medicinal plants, comprising about 48% of the total number. Besides the local abiotic conditions, macroclimate and availability of diaspores from the surrounding ruderal or semi natural vegetation types, a human influence play a significant role in shaping the species composition on walls. The urban and rural landscape and technology of wall building influence a range of plant species, which are able to colonize this habitat.

Key words: *castle flora, life forms, chorology.*

INTRODUCTION

Castles are usually situated on craggy rocks and together with their impressive fortification walls create a unique environment for plant life in many respects, e.g. low availability of room for settlement, hardness and alkalinity of the substratum, scarcity of soil and humus, high inclination, extreme temperatures and low humidity [1]. The study of the wall flora is of special importance for the maintenance and preservation of archaeological monuments. Moreover, castles and their surroundings are ideal places to study plant invasions and link them to historical and current human activities. Considering vertical division, walls usually consist of three different zones: the base, the vertical wall surface with joints (fissures) and the wall top. Species composition of basal zone consists of plants growing on vertical surface and species of nearby vegetation. This is caused by favorable environmental conditions of the basal zone (more moisture and nutrients). This are the first results of the study now in

progress focused on the vascular flora of the Berat castle. Berat is a town located in south-central Albania and the capital of both the District of Berat and the larger County of Berat. Berat Castle (40.7067° N, 19.9522° E) is a fortress overlooking the town of Berat. It dates mainly from the 13th century and contains many Byzantine churches in the area and Ottoman mosques. It is built on a rocky hill on the left bank of the river Osum and is accessible only from the south. After being burned down by the Romans in 200 B.C., the walls were strengthened in the fifth century under Byzantine Emperor Theodosius II, and were rebuilt during the 6th century under the Emperor Justinian I and again in the 13th century under the Despot of Epirus, Michael I Komnenos Doukas, cousin of the Byzantine Emperor. The main entrance, on the north side, is defended by a fortified courtyard and there are three smaller entrances. The fortress of Berat in its present state, even though considerably damaged, remains a magnificent sight. The surface that it encompasses made it possible to house a considerable portion of the cities inhabitants. The buildings inside the fortress were built during the 13th century and because of their characteristic architecture are preserved as cultural monuments. The population of the fortress was Christian, and it had about 20 churches (most built during the 13th century) and only one mosque, for the use of the Turkish garrison (of which there survives only a few ruins and the base of the minaret). The churches of the fortress were damaged through years and only some have remained. The castle hill rises 187 m above the sea level with an area of 9.6 hectares. There are 24 towers of different forms and sizes along the impressive Hellenistic and medieval walls. The interior of the castle is still inhabited today.

Berat district is part of the Mediterranean, which is characterized by mild winters and hot dry summers [2]. Total annual amount of solar radiation is 1673 kWh/m^2, with minimum values recorded in December (51.9 kWh/m^2) and maximum in July (230.4 kWh/m^2). The average maximum temperature in July for the city of Berati reaches almost 33.8^{0}C while the average minimum in January is found to be around 2.6^{0}C. Regarding the higher temperatures observed, the absolute maximum temperatures in Berat was recorded with a value of 47^0 C, while the lowest value, (as absolute minimum) was recorded to be -10^0 C, in January. Average annual rainfall vary from 1169 mm [2].

STUDY AREAS AND METHODS

Flora of Berati castle consist of a mixture of native, wide distribution and alien species. An extensive field study was conducted from 2011 to 2013 to record the vascular flora growing on the walls of the Berati castle. One visit was made after every two months. Thus a total of six visits were made for the field observations in a year.

During our investigations, voucher herbarium specimens were collected. We also photographed native and introduced species (some of them rare or endangered). Only plants spontaneously growing in the study area, both native and alien plants are included in the floristic list. A compiled floristic list of plants, and an analysis of this list, is shown in Tables 1, 2.

Fig. 1. Berati Castle

Families, genera and species are arranged alphabetically within the major units of classification, Dicotyledoneae and Monocotyledoneae. The scientific names of species and families are presented according to the latest nomenclatural checklist of vascular plants of the Euro+Med Plant Base and Flora Europaea [3,4]. Information concerning the distribution and life form of the taxa is taken from the above literature and additionally from [5]. Specimens are deposited in the Herbarium of the Museum of Natural Sciences of the University of Tirana (TIA).

RESULTS AND DISCUSSION

Flora of castles often shows greater diversity of species than that found in more natural areas. The increase in biodiversity can minimize the impact (or destruction) by a specific insect or disease on a particular species or genus, but it can also involve a risk to native plants if any of the introduced species behaves as an invading species, as these can come to compete with or displace the autochthonous species. Since the Berati Castle is situated within the urban and rural landscape, the composition of the wall flora is strongly influenced by the surrounding ornamental, ruderal, and semi natural vegetation types

In the Berati castle 200 taxa (species and sub-species) were recorded, representing 54 families and 153 genera. The prevailing representation of Mediterranean plants in the flora of the Berati Castle demonstrates that this flora is, despite the exposure to durable anthropogenic influence, still developing under the prevailing influence of the Mediterranean climate conditions. 22 taxa of the Berati castle flora is represented by the alien species or c.10% of the total flora. Most aliens are of American (5.4% of the total flora) and Asiatic origin (4.5%). In the chorological spectrum (Table 1), elements of Mediterranean origin 90 in total or 44.7% of the total flora prevail, closely followed by those of wide distribution 81 taxa or 40.2%, while Balkans and European comprise 8 taxa (3.9%).

As for the alien species found in Berati Castle, these include some naturalized trees such as *Acer negundo*, *Agave americana*, *Arundo donax*, *Sophora japonica*. There are also other species acting as invaders, such as *Acacia dealbata*, *Elaeagnus angustifolia*, *Eriobotrya japonica*, *Eucalyptus camaldulensis*, *Eucalyptus globulus*, *Gleditsia triacanthos,* as well as *Ailanthus altissima*, *Opuntia ficus-indica* and *Robinia pseudoacacia.*

The higher number of species were found in Poaceae family 13,5 % of total species, followed by Asteraceae 13% and Fabaceae with 9%. The highest proportion of species of Asteraceae

family found on studied walls is related to its high species number in Central Europe and the remarkable success of this family in terms of dispersal and establishment [6].

The walls under investigation host vegetation of different growth forms, ranging from mosses and lichens, to ferns and herbs, and to shrubs and trees. The flora on the walls consisted predominately of heliophilous to shade-tolerant plants. In the life – spectrum, therophytes are prevailing (41%), followed by Hemichryptophytes with 31%. Therophytes are the prevailing life form in the interior castle area, the walls and the surrounding area. Their percentage is higher in the interior area and on the walls than in the surrounding area, reflecting the more microclimatic conditions of the limestone rocks and walls. Other noteworthy characteristics of the area include the good representation of Hemicryptophytes and Geophytes, both in the interior area and on the walls.

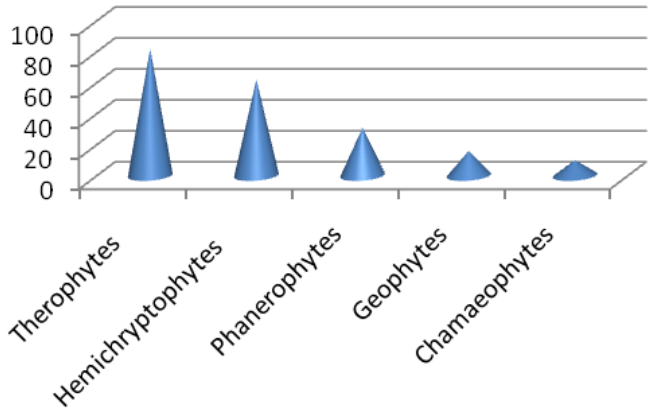

Fig. 1. Life – form spectrum of the flora of Berati castle

Life forms (%)	Interior	Walls	Surrounding area	Total area
Therophytes	59.8	54.6	47.5	53.4
Hemichryptophytes	23.0	20.3	19.3	21.4
Phanerophytes	6.1	12.7	12.6	11.6
Geophytes	4.9	6.0	11.6	7.8
Chamaeophytes	6.2	6.4	9.0	5.8

Table 1. Life – form spectrum of the flora of Berati castle and its surrounding area

Seventeen chorological groups are distinguished, with the Euri- Mediterranean element (30%) as dominant, followed by Paleotemporale 14 %, and Cosmopolite 10 %.

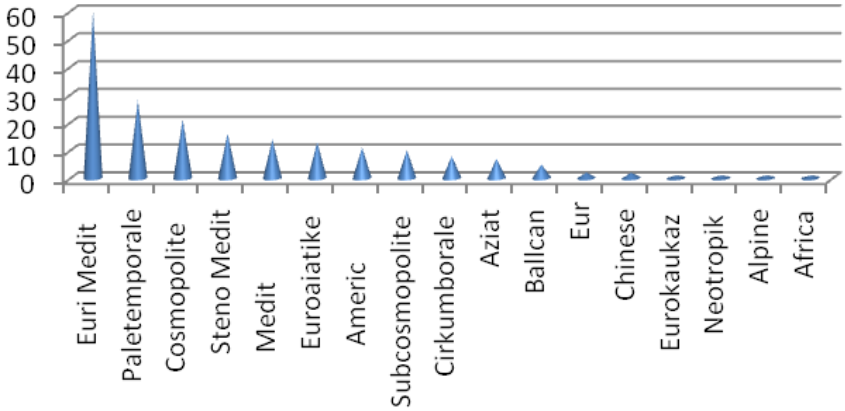

Fig. 2. Chorological spectrum of the flora of Berati castle

Chorological group	Origin	Taxa	Interior	Walls	Surrounding area	Total area
Balkan and European (8)	Balkan	5	1.1	1.3	1.7	1.0
	European	2	1.1	1.0	0.0	1.2
	Alpine	1	1.0	0.0	0.0	1.1
Mediterranean (90)	Mediterranean	14	12.5	4.0	12.2	8.0
	Steno-Mediterranean	16	7.7	16.5	12.1	15.2
	Euri-Mediterranean	60	24.2	14.5	10.3	11.0
Wide distribution (81)	Eurasiatic	13	5.2	9.5	4.4	6.2
	Euro-Caucastian	1	1.0	0.0	0.0	1.0
	Cirkumboreal	7	1.3	4.3	0.9	1.6
	Paleotemporal	28	0.9	1.7	2.2	2.3
	Cosmopolitan	21	30.6	30.0	40.1	38.1
	Subcosmopolitan	10	7.7	15.0	13.0	7.9
Alien (22)	American	11	1.7	0.0	0.9	1.2
	Asian	7	1.0	1.2	2.2	1.0
	China	2	1.0	1.0	0.0	1.1
	Neotropical	1	1.0	0.0	0.0	1.1
	African	1	1.0	0.0	0.0	1.0

Table 2. Chorological spectrum of the flora of Berati castle and its surrounding area

The interior castle area is the species-richest area, containing 133 species. Thirty species were recorded on the walls and 37 species were found in the surrounding area. The great plant species richness of the castles is already known.

The differences between castle floras and surrounding flora and vegetation were studied by botanists in the 19th and 20th centuries [7]. Around castles, the number of species of wild flowering plants can be double that in similar areas in the vicinity [7].The whole area of Berati castle belongs to the Mediterranean vegetation zone and phrygana communities with *Phlomis fructicosa* dominate it. Phrygana is perhaps the best example of the appearance and maintenance of a specific degradation stage of vegetation under a long-term and permanent impact by human activities (tree cutting, purposeful forest fires, grazing by livestock). Their soil (where these plant communities are distributed) is usually poor and scattered stones and rocks are abundant on the surface. *Phlomis fruticosa* play an important role in the

composition of the phryganas on dry shallow soil over limestone in the Berati castle area. *Pinus halepensis* clumps are limited to the southwest side of the study area, while cultivated fields, mainly of olive trees, are concentrated in the flat and low slope areas. The natural vegetation cover in the interior castle area has been strongly affected by intense human activities that have lasted for centuries. The most wide-spread species in that area are: *Phlomis fruticosa, Euphorbia characias, Micromeria juliana, Micromeria greacca, Hedera helix, Spartium junceum.* Moreover, a significant number of urbanophilous species were recorded in the interior castle area and on the walls, *Capsella bursa-pastoris, Chenopodium album, Chenopodium ambrosioides, Galium aparine, Galium verum*.

Urtica pilulifera, Lactuca serriola, Hordeum murinum etc., reflecting the long time changes in inhabitable habitats and the effects of tourist development. High numbers of accidental species like *Salix babilonica , Populus alba, Ficus carica* that reach only low covers on walls indicate the influence of surrounding vegetation on species composition on these castle, as well as strong limitation by wall environment and/or competitive exclusion in a small-scale habitat. *Parietaria judaica* is the most common species like in other countries of southern Europe [8]. Others were spices or food plants and others had a technical use – e.g. the Yellow Chamomilla (*Anthemis tinctoria*) for dyeing or the Pellitory-of- the-wall (*Parietaria diffusa*) for cleaning.

The walls represent a specific environment, which is partly similar to rocks and rock fissures [8]. Occurrence of the wall species varies consider ably with respect to their position on the walls. Usually wall species occur in three wall positions: 1. horizontal top – the flat end of the wall, 2. vertical surface – the vertical wall surface with joints (fissures) and 3. the base – 30 to 50 cm from the wall base towards the top. These three zones represent a characteristic feature of the wall flora and vegetation. In Berati castle, the horizontal top of the walls has a usually dense plant cover supported by disintegration of the building material. This is the species-richest wall zone and it is characterized by the presence of many annual species. The widest-spread secies of the horizontal top are: *Bromus sterilis, Hordeum murinum, Micromeria graeca, Micromeria juliana, Thymus sibthorpii, Trifolium repens, etc*. The vertical surface of the walls is a harsh environment for most plant species and, as a result, a poor, mainly chasmo-phytic flora similar to that of the adjacent rocky slopes inhabits them. Some characteristic plants of this zone are: *Capparis spinosa subsp. spinosa, Ephedra distachya, Micromeria graeca, Micromeria juliana*. The species composition of the basal zone consists of some plants growing on vertical surface and mainly species of nearby vegetation. The favourable environmental conditions of this zone (more moisture and nutrients) make possible the establishment of a relatively rich flora that include, among others, the following species: *Euphorbia exigua, Malva sylvestris, Malva parviflora, Parietaria diffusa, Urtica dioica, Urtica pilulifera,* etc. There are several wall-dwelling plants that seem to be common inhabitants of the Mediterranean walls, such as *Ephedra distachya, Calendula arvensis, Avena barbata, Bromus hordeaceus, Urtica pilulifera, Capparis spinosa, Hordeum murinum, Lactuca serriola, Senecio vulgaris,* etc.

Berati is the most known city in Albania for cultivation of Medicinal Aromatic Plants, planted in 1256 ha area with a production 522 tons, while some of the species which are cultivated in large areas *Satureja Montana L., Origanum vulgare L. var. viridis Boiss., Salvia officinalis L., Mentha piperita etc.*

Below is the list of plants with Life-Form, Chorological Spectra and the Medicinal Aromatic plant (MAPs) founded in the Berati Castle identification by A. Mullaj, J.Shehu.

Pteridophyta

Aspleniaceae

Asplenium trichomanes H. Cosm tem. MAPs

Asplenium ruta muraria H.Circum-bor

Ceterach offichinarum H. Euroaziatike. MAPs

GYMNOSPERMAE

Cupressaceae

Cupressus sempervirens P. E-Medit

Juniperus phoenicea P. Euri-Medit. MAPs

Ephedraceae

Ephedra distachya NP. NW-Medit. MAPs

Ephedra fragilis Desf. P. *Medit. MAPs*

Pinaceae

Pinus halepensis P. Steno-Medit

ANGYOSPERMAE

DICOTYLEDONEAE

Amaranthaceae

Amaranthus albus T. Nordamer

Amaranthus blitoides T. Nordamer

Amaranthus retroflexus T. Nordamer

Apocynaceae

Nerium oleander P. S-Medit

Araceae

Arum italicum G. Steno – Medit. *MAPs*

Araliaceae

Hedera helix *P. Submedit-Subatl. MAPs*

Asteraceae

Anthemis arvensis T. Steno-Medit.

Artemisia campestris Ch . Circumbor. MAPs

Aster squamatus T. Neotropike

Bellis perennis H. Euro-Kaukaz. MAPs

Calendula arvensis T.Euri-Medit. MAPs

Carduus pycnocephalus H. Euri-Medit

Carlina corymbosa H. Steno-Medit. MAPs

Chamomilla recutita T. Subcosm. *MAPs*

Chondrilla juncea H. Euri-Medit.

Chrysanthemum leucanthemum H. Euri-Medit.

Cichorium intybus H.Cosmop. *MAPs*

Conyza albida T. America Tropic

C. bonariensis T. America Tropic

Dittrichia viscosa H. Euri-Medit

Erigeron canadensis H. North America

Lactuca serriola H. Euri-Medit

L. viminea H. Euri-Medit

Scolymus hispanicus H. Euri-Medit.

Senecio vulgaris T. Euri-Medit.

Silybum marianum H. Medit. MAPs

Sonchus asper T. Euroasiat.

S. oleraceus T. Euroasiat.

Taraxacum officinale H.Circumbor. MAPs

Tragopogon porrifolius H. Euri-**Medit.**

Tussilago farfara H Euroasiat. MAPs

Urospermum picroides T. Euri-Medit.

Boraginaceae

Buglossoides purpurocaerulaea H. S-Europ.-pontico. MAPs

Echium italicum H. Euri-Medit

Heliotropium europaeum T. Euri-Medit

Myosotis ramosissima T. Europeo-W-Aziat

Brassicaceae

Brassica oleracea Ch. Medit.

Capsella bursa-pastoris H. Cosmop. MAPs

Cardaria draba G. Medit.

Clypeola jonthlaspi T.Steno-Medit.

Erophila verna T. Cirsumbor.

Cactaceae

Opuntia ficus-indica Ph.Neotropik (invaziv)

Capparaceae

Capparis spinosa NP. Euroaziat. MAPs

Caprifoliaceae

Lonicera etrusca P.Euri-medit

Lonicera caerulea L. P. Alpine

Caryophyllaceae

Cerastium glomeratum T. Euri-Medit

Herniaria hirsuta T.Paleotemp. MAPs

Stellaria media T.Cosm. MAPs

S. pallida T.Cosm

Chenopodiaceae

Atriplex rosea T. Euri-Medit

Chenopodium album T. Subcosmop

Ch. ambrosioides T.Cosm

Convolvulaceae

*Convolvulus althaeoide*s H.Steno-Medit.

C. arvensis G.Paleotemp.

Cuscuta sp. T. Cosmop. *MAPs*

Crassulaceae

Sedum album Ch. Euri-Medit.

S. sediforme Ch. Steno-Medit.

Sedum acre Ch. Steno-Medit. MAPs

Cucurbitaceae

Ecbalium elaterium G. Euro-Medit. MAPs

Euphorbiaceae

Euphorbia exigua T. Eur-Medit.

E. helioscopia T. Cosmop.

Mercurialis annua T. Paleotemp.

Ricinus communis P. Paleotrop. MAPs

Geraniaceae

Erodium ciconium T. Euro-Medit.

E. cicutarium T. Euro-Medit.

Geranium molle T. Euro-Asiat.

G. rotundifolium T.Paleotemp

Guttiferae

Hypericum perforatum H. Paleotemp. MAPs

Lamiaceae

Ballota nigra H.Euro-Medit.

Lamium amplexicaule T. Paleotemp.

Marrubium peregrinum H.Ballcan. MAPs

Mentha piperita H.Paleotemp. MAPs

Mentha pulegium H.Paleotemp. MAPs

Micromeria juliana Ch. Steno-Medit. MAPs

Micromeria graeca Ch. Steno-Medit. MAPs

Phlomis fructicosa Ch. Steno-Medit

Salvia verbenaca H. Medit.-Atl

Teucrium polium H. Medit. MAPs

Thymus sibthorpii Ch. Ballcan.

Fabaceae

Spartium junceum Ph.Euro-Medit

Hippocrepis unisiliquosa T. Euro-Medit.

Lotus corniculatus H. Cosmop.

Medicago lupulina T.Paleotemp.

M. minima T. Euri-Medit.

M. orbicularis T. Euri-Medit.

M. sativa H. Cosmop.

Melilotus alba T. Euras. MAPs

M. officinalis H. Euras. MAPs

Ononis spinosa Ch. Eur-Medit. MAPs

Spartium junceum P. Euro-Medit . MAPs

Robinia pseudacacia P. Nordamer. MAPs

Trifolium fragiferum H. Paleotemp.

T. repens H. Paleotemp.

T. resupinatum T.Paleotemp

Trigonella monspeliaca T. Euri-Medit. MAPs

Vicia sativa T. Medit.

V. villosa T.Euro-Medit.

Malvaceae

Althaea officinalis L. H. Eurosib. *MAPs*

Malva moschata H. Euri-Medit. MAPs

M. parviflora T. Euri-Medit. MAPs

M. sylvestris H. Eurosib. MAPs

Moraceae

Broussonetia papyrifera Ph. Aziatic.

Ficus carica Ph. Medit. MAPs

Morus alba Ph. Aziatic. MAPs

Oleaceae

Fraxinus ornus L. P. Eur-Medit. MAPs

Olea europaea P. Medit.

Oxalidaceae

Oxalis acetosella G. Circumbor.

O. corniculata H.Euri-Medit.

Papaveraceae

Fumaria officinalis T. Paleotemp. MAPs

Papaver rhoeas T. E-Medit. MAPs

Plantaginaceae

Plantago psyllium T. Steno-Medit.

P. lanceolata H. Euroasiat.

P. major H. Euroasiat.

Polygonaceae

Fallopia aubertii P. Centroasiat.

Polygonum aviculare T. Cosmop.

Rumex conglomeratus H. Euroasiat.

R. cristatus H. NE-Medit.

R. pulcher H. Euro Medit

Portulacaceae

Portulaca oleracea T. Cosmop. MAPs

Primulaceae

Anagallis arvensis T. Euro-Medit. MAPs

Cyclamen hederifolium Ait. G. Eur. MAPs

Primula veris L. H. Medit. MAPs

Punicaceae

Punica granatum P. Asiat. MAPs

Ranunculaceae

Clematis vitalba P. Eur. MAPs

Consolida ajacis T. Euri-Medit. MAPs

C. regalis T. Euri-Medit. MAPs

Helleborus odorus. G. Eur. MAPs

Ranunculus arvensis T. Paleotemp.

Resedaceae

Reseda lutea H. Euro.

Rhamnaceae

Paliurus spina-christi P. Ballcan. MAPs

Rosaceae

Crataegus monogyna P. Paleotemp. *MAPs*

Cydonia oblonga P. Asiat. MAPs

Potentilla reptans H. Paleotemp. MAPs

Prunus dulcis P. S-Medit. MAPs

Sanguisorba minor H. Paleotemp. MAPs

Rubiaceae

Galium aparine T. Euroasiat. MAPs

Galium verum H. Euro-Asiat. MAPs

Rubia tinctorum H. Asiat. MAPs

G. spurium T. Euroasiat.

Salicaceae

Salix alba P. Euro-Asiat. MAPs

Salix purpurea P. Euro-Asiat. MAPs

Salix babilonica P. Chinese

Populus alba P. Paleotemp

P. nigra P. Paleotemp

Saxifragaceae

Saxifraga tridactylites T. Euri-Medit.

Saxifraga rotundifolia T. Euri-Medit.

Scrophulariaceae

Antirrhinum majus Ch. Medit.

Cymbalaria muralis H. Ballcan.

Verbascum sinuatum H. Euri-Medit.

Veronica anagallis-aquatica H. Cosmop.

V. persica T. W- Asiat. MAPs

Simaroubaceae

Ailanthus altissima Ph. China.

Solanaceae

Datura stramonium T. Americ. MAPs

Hyoscyamus niger T. Euro-Medit. MAPs

Physalis alkengi H. Euro-Asiat. MAPs

Solanum nigrum T. Cosmop. MAPs

Solanum dulcamara N.P. Paleotemp. MAPs

Ulmaceae

Celtis australis Ph. Euro-Medit.

Ulmus minor P. Europeu-Caucas.

Umbelliferae

Apium graveolens H. Paleotemporale. MAPs

Daucus carota H. Paleotemporale. MAPs

Eryngium campestre H.Euri-Medit.

Foeniculum vulgare H. S-Medit. MAPs

Petroselinum sativum H. E-Medit. MAPs

Torilis arvensis T. Subcosmop. MAPs

T. nodosa T. Euri-Medit. MAPs

Urticaceae

Parietaria diffusa H. Euri-Medit

Urtica pilulifera T. S-Medit. MAPs

U. urens T. Subcosm. MAPs

U. dioica H. Subcosm. MAPs

Verbenaceae

Verbena officinalis H. Paleotemp. MAPs

Vitaceae

Parthenocissus quinquefolia P. Nordameric.

Vitis vinifera P. Cosmop. MAPs

Zygophyllaceae

Tribulus terrestris T. Cosmop. MAPs

MONOCOTYLEDONEAE

Cyperaceae

Cyperus longus G.Paleotemp.

Scirpus holoschoenus G. Steno-Medit.

Commelinaceae

Tradescantia virginiana G. Nordameric.

Poaceae

Agropyrum repens *H. Eur. MAPs*

Agrostis stoloniferia H. Circumbor.

Arundo donax G. Subcosm.

Avena barbata T. Euri-Medit.

A. sterilis T. Euri-Medit.

Bromus hordeaceus T. Subcosm.

B. sterilis T. Euri-Medit.

B. tectorum T. Paleotemp.

Cynodon dactylon G. Cosmop. MAPs

Dactylis glomerata H. Paleotemp.

Dasypyrum villosum T. Euri-Medit.

Digitaria sanguinalis T. Cosm.

Desmazeria rigida T. Euro-Medit.

Eleusine indica T. Cosm.

Hordeum murinum T. Circumbor.

H. vulgare T. Africa.

Koeleria cristata H.North-Central Europ.

Lolium perenne H. Circumbor.

Lophochloa cristata T. Paleotemp.

Melica ciliata H. Euri-Medit.

Paspalum paspaloides G. Subcosm.

Phalaris canariensis T. Macaronesia.

Poa annua T. Cosm.

Poa trivialis H. euroazia

P. bulbosa H. Paleotemp.

Setaria viridis T. Subcosm.

Sorghum halepense G. Cosm.

Liliaceae

Allium sativum G. Aziat. MAPs

Asparagus officinalis G. Euri- Medit. MAPs

Asphodelus fistulosus H. Paleosubtropikale.

Colchicum automnale G. Eur. MAPs

Muscari comosum G. Euri-Medit.

Ornithogalum umbellatum G. Euro-Medit.

Scilla autumnalis G. Euri-Medit.

CONCLUSION

The interior castle is the richest - species area and the natural vegetation covering this area has been strongly affected by intense human activities that have lasted for centuries.

Flora of the walls of Berati castle is also an indicator for medicinal herbs species growing in the area around. A considerable part plant species recorded belonged to medicinal plants, which covered about 48% (96 species) of the total number.

The investigation of the survival rate of plants growing on walls would be a very interesting phenomenon to examine on permanent plots.

The highest proportion of species of *Asteraceae f*amily found on studied walls is related to its high species number in Central Europe and the remarkable success of this family in terms of dispersal and establishment.

A combined effort in the conservation of both biodiversity and historical monuments is necessary to keep alive the memory of medieval history represented not only in the walls and towers of the castles but in the plant species growing in their vicinity as well.

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Original scientific paper

ON THE REPRODUCTIVE BIOLOGY OF *SIDERITIS SYRIACA* L. (LAMIACEAE)

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ABSTRACT

The paper presents a study on the features of the embryological structures and processes in the male and female generative sphere and two mean parameters of the reproductive biology – pollen and seed viability of *Sideritis syriaca.* The anthers are tetrasporangiate. The anther wall develops according Dicotyledonous-type and consists of epidermis, fibrous endothecium, one ephemeral middle layer and glandular tapetum. The sporogenous tissue is one-layered. After regular meiosis in microspore mother cells and simultaneous microsporogenesis predominantly tetrahedral microspore tetrads form. The mature pollen grains are two-celled. The ovule is anatropous, tenuinucellate and unitegmic in which a unicellular archesporium forms. The embryo sac develops after *Polygonum* (monosporic)-type. In result of porogamous double fertilization an Onagrad-type embryo forms. These features reveal *Sideritis syriaca* as sexually reproducing species. The established during the study high pollen and seed viability provide to the species a high reproductive capacity.

Key words: *Sideritis syriaca, embryological structures, pollen and seed viability*

INTRODUCTION

Sideritis syriaca L. (Lamiaceae Lindl.) belonging to the section *Empedoclia* (Rafin.) Benth. is one of the variable species growing on mountain rocks in South Europe – from Sicily to Crimea [1]. There are many difficulties for accurate taxonomy determination of the species due to the interspecific hybridization occurring in the genus. Hayek (1931) accepted *S. raeseri* for the flora of Albania, Greece and Macedonia and *S. syriaca* as endemic for Crete [2]. According to Flora Europaea [1] the species includes all of these synonyms: *S. raeseri* Boiss. and Heldr., *S. sicula* Ucria, *S. taurica* Stephan ex Willd., *S. cretica* Boiss. In Mountain flora of Greece [3] *S. raeseri* and *S. syriaca* are Balkan endemics and are different plant species respectively. Initial information in Bulgaria was that *S. taurica* occurs [4], while later Asenov [5], influenced by Heywood [1] reported instead *S. syriaca.*

In Bulgaria *S. syriaca* has a very restricted distribution – in only three localities in Strandzha Mt [6] and it is included under the category "Critically Endangered" in the Red list of Bulgarian vascular plants [7] and Red Data Book of the Republic of Bulgaria, Vol. 1. Plants and fungi [8]. It is under the Biodiversity Law [9].

S. syriaca is a valuable medicinal plant known as "Cretan Mountain Tea", "Greek Mountain Tea", "Malotiras", "Shepherd's tea" or "Mountain Tea". According to the Greek tradition, it is used as a panacea for any illness – the plant has figured in local herbal remedies for more than 2400 years. Rimstidt (2010) announces that Hippocrates was the first that wrote about the healing benefits of *S. syriaca* around 400 B.C [10]. Recent studies have proved the special attributes of the plant: antimicrobial activity; immune stimulator [11, 12].

Up to now, *S. syriaca* was mainly an object of karyological [13] and anatomical studies focused to its taxonomy [14] and phytochemical analyses revealing its antioxidant and anti-inflammatory activities [11, 12 and 15].

In Bulgaria, there are few reports on karyological [16], chorological [6] and phytochemical investigations [17] on this species.

The aim of the present study is to reveal the mode of reproduction and embryological features of *S. syriaca* as well as to estimate two main parameters of its reproductive biology, namely pollen and seed viability that define the reproductive capacity of this species. The embryological study on *S. syriaca* is conducted for the first time.

MATERIAL AND METHODS

One natural population of *S. syriaca* from Strandzha Mt (Southeast Bulgaria) was studied.

The material for the embryological study (flower buds and flowers at different developmental stages) was collected and fixed in a mixture FAA (formalin:glacial acetic acid: 70 % ethanol in ratio 5:5:90 parts), embedded in paraffin, cut into 8-12 μm sections with a rotary microtome and treated according to classical paraffin method [18]. The sections were stained with Heidenhain's haematoxylin and included in Enthelan.

To estimate the pollen viability a common method by staining with acetocarmine and direct count was applied [19]. Therefore, anthers from the open flowers were collected and suspended in a solution containing acetocarmine – 1% acetocarmine was used for staining [20]. The pollen viability was estimated on temporary slides after the stained in red (viable, fertile) and unstained (nonviable, sterile) mature pollen grains in 30 anthers were counted (visual field – at an augmentation 100x) using light microscope. The total number of pollen grains examined for viability was 735 (Table 1).

To estimate the seed (embryo) viability, a quick tetrazolium test that conducted in a short time period with minimal equipment [21] was applied. Initially, the tetrazolium solution is colorless, but changes to red when put in contact with the hydrogen (a reduction) derived from the enzymes in the respiratory process of the embryos. Embryos showing active respiration turn red and are considered viable (the darker the color, the higher is the respiratory activity in the seed). For the tetrazolium testing approximately 200 mature seeds of the population of *S. syriaca* were preliminary incubated in water for 24 h at 30-35^{o}C. Then the seeds were cut deeply in its chalazal part and incubated in a diluted (1 %) solution of 2,3,5-triphenyltetrazolium chloride for 24 h. The quality of mature seeds (embryos isolated from the seeds) and their viability potential were determined after the application of tetrazolium test by observations of the staining patterns of embryos isolated from the seeds.

The embryo viability was estimated depending on the intensity of staining: viable embryos display entire embryo staining or staining of his basal part – the root (normal staining); nonviable embryos display abnormal or no staining. The manner of staining reveals the live and dead areas of the embryos and gives a possibility to determine whether the seeds have a capacity to produce normal seedlings [22].

Data obtained during the study are presented in a table and figures. The observations carried out were done using light microscope "Olympus" CX21 and micrographs – with digital camera (1,4MP).

RESULTS AND DISCUSSION

Embryological features

Anther and development of the male gametophyte

The anthers are tetrasporangiate. The formation of placentoids between the anther locules shown in *S. scardica* [18], are also observed in *S. syriaca* and may be considered as a characteristic features for *Lamiaceae* because of their presence in other species of the genera of this family: *Lavandula* L., *Salvia* L., *Stachis* L, *Hyssopus* L., *Agastahe* Clayt. [19, 20, 21, 22]. The wall formation follows the Dicotyledonous-type [23, 24]. The anther wall consists of four layers: an epidermis, an endothecium, one middle layer and a tapetum that at the beginning of the anther ontogenesis are almost similar in shape but later on they begin to distinguish – the tapetum even at stage of sporogenous tissue (Fig. 1) while the other layers – after the formation of microspore tetrads (Fig. 4). The epidermis comprises one row of almost rectangular uninucleate cells that vastly enlarge during the anther ontogenesis. The middle layer is ephemeral that completely degenerates up to end of the meiosis in microspore mother cells (MMCs) like *Sideritis scardica* [23] and the most representatives of *Lamiaceae* [24, 25, 26]. At the beginning of anther ontogenesis, the endothecium comprises cells that are much similar in size and shape to the epidermal ones. Subsequently, they radially elongated and after the formation of one-nucleated pollen develop fibrous thickenings and divide in a radial direction to form two-rowed endothecium layer (Fig.5). Similar multiplication of the rows of endothecium we reported too in other species from different families: *Solanaceae – Atropa belladonna* L. [31] and *Oleaceae – Olea europaea* L. [32]. The one-layered tapetum is glandular during the whole anther ontogenesis. Initially, its consisting cells are one-nucleate but even at the stage of MMCs they become two-nucleate (Fig. 3) as result of mitotic division. At the stage of mature pollen, the anther wall consists of one-rowed endothecium, partially conserved epidermis and traces of degenerating tapetum cells (Fig.6).

The sporogenous tissue is one-rowed (Fig. 1). Initially, its cells are polygonal and fit close each other. Later on, they elongate, round up and differentiate into MMCs (Fig.2). The meiosis in MMCs passes with insignificant deviations. After simultaneous type of microsporogenesis predominantly tetrahedral microspore tetrads form (Fig.4). At the time of shedding the pollen grains are two-celled, usually morphologically uniform, tricolporate.

Ovule and development of the female gametophyte

The gynoecium is syncarpous, inferior – typical for the family *Lamiaceae* [24] with bilocular ovary and a single anatropous, tenuinucellate unitegmic ovule on axile placentacion in each locale (Fig. 7). Like in the most Angiosperms innermost layer of the single integument differentiates into endothelium [28]. Within the still young ovule, unicellular archesporium forms hypodermally without formation of parietal cells (Fig. 8). The archesporium cell functions directly as a megaspore mother cell, which later on undergoes meiosis to produce a linear megaspore tetrad (Fig.9). The embryo sac (ES) development runs according to the *Polygonum* (monosporic)-type from the chalazal megaspore of tetrad that functions as an embryo sac mother cell. The other three megaspores degenerate progressively, during the advance of embryo sac development. After three successive mitoses, two-, four- and eight-nucleate ES forms. This type of embryo sac formation reported as typical for the *Lamiaceae* family [24, 25 and 26] and known as basical one for the Angiosperms (Poddubnaya-Arnoldi 1976) we also observed in *S. scardica* [23]. The mature ES consists of three-celled egg apparatus (usually pear-shape egg cell and two synergids), two polar nuclei (after their fusion a central cell of the ES forms) and three-celled antipodal apparatus in the chalazal part of the ES (Figs. 10, 11). The synergids degenerate after the fertilization. The antipodals are ephemeral and begin to degenerate before the fertilization (Fig.11).

The embryo and endosperm develop after porogamous double fertilization accompanied with a destruction of one synergid from the pollen tube penetrating in it through the mycropyle of the ovule. The first division of the zygote is transversal and usually runs before the beginning of endospermogenesis. The direction of the cell wall setting in young embryo after following mitoses indicates that the embryogenesis runs after the Onagrad-type reported as typical for the representatives of *Lamiaceae* [25, 26]. In the mature seed, the embryo is nearly straight with two equal cotyledons (Fig. 12). In *S. syriaca* apomixis was not registered as Poddubnaya-Arnoldi [28] noticed for whole family *Lamiaceae*.

Pollen and seed viability

After the application of acetocarmine stain technique for estimation of the pollen viability, cytoplasm and nuclei of viable pollen grains were stained in red while nonviable, empty and shrunken pollen remain colorless (Figs. 13-16). The results of the study show a high viability of the mature pollen in the studied population of *S. syriaca* over than 90% (Table 1).

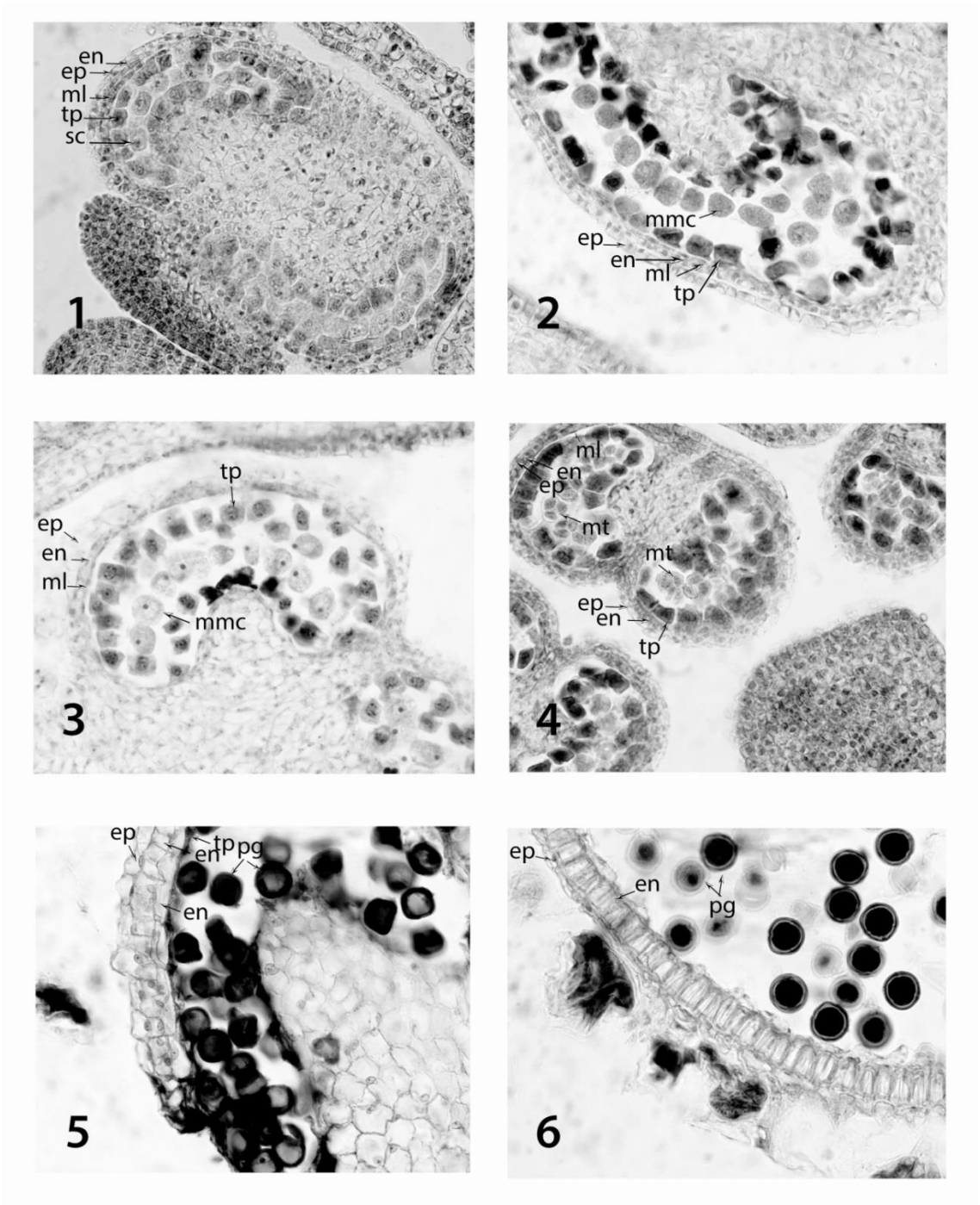
On the basis of results obtained after tetrazolium testing, the seeds (embryos) were differentiated in five classes (Figs 17-22): Class I – embryos stained 100 % (whole embryo stained in dark red); Class II – embryos stained 80 % (light red colored embryos); Class III – embryos stained 10 % (only the root of embryo stained in red); Class IV – colorless embryos; Class V – empty seeds. According to the criteria for interpretation of the results of tetrazolium test given by Moore [33], the viable embryos are represented by the color patterns of Classes I, II and III. Thus, in the studied population of *S. syriaca* the viable embryos were 62.36 % and the seed viability was considered as relatively high.

Table 1 Pollen viability

Number	Nonviable	Viable	Sum	Percent of viability
1	3	14	17	82,35
2	2	11	13	84,62

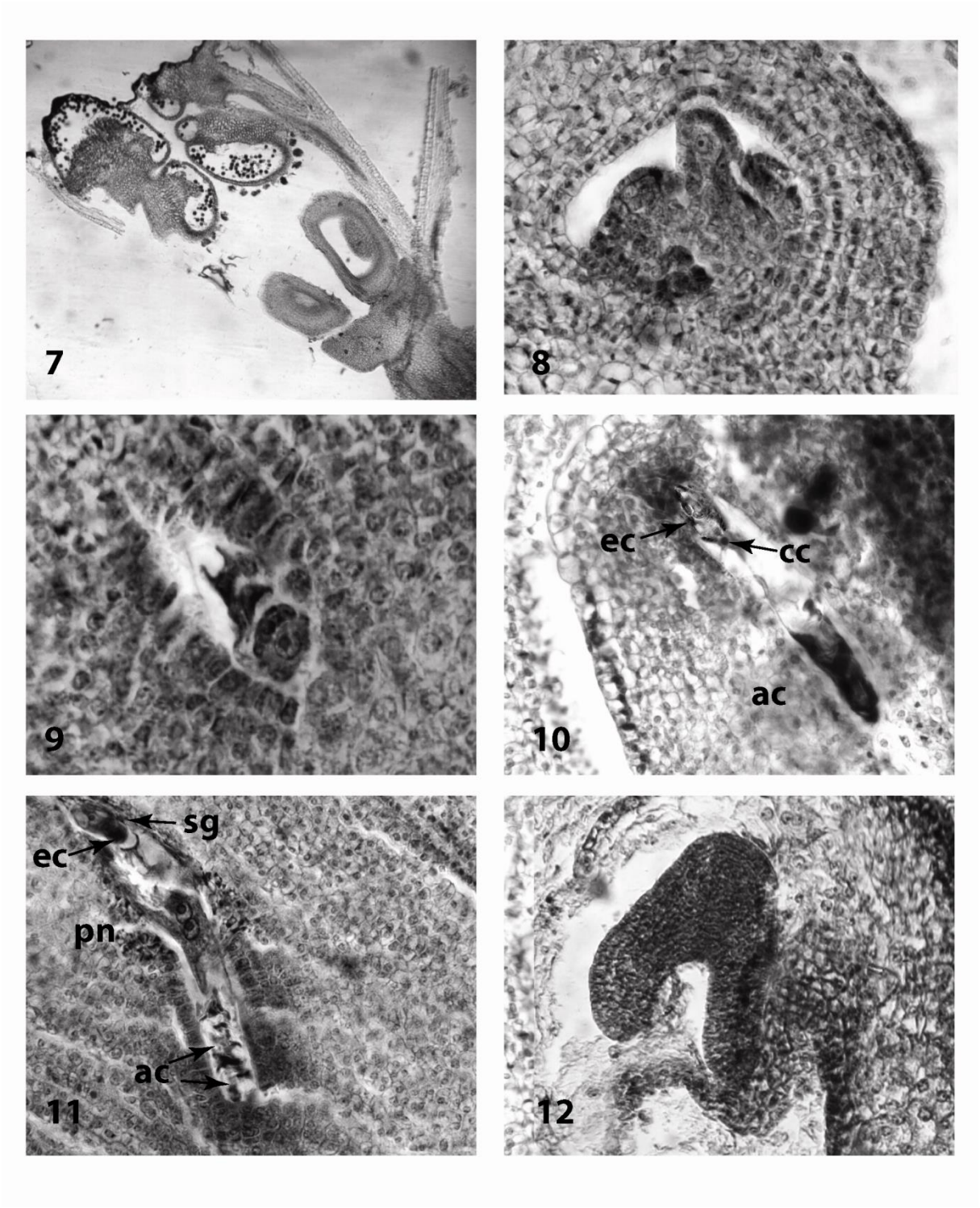
3	1	7	8	87,5
4	1	7	8	87,5
5	1	3	4	75
6	2	2	4	50
7	3	11	14	78,57
8	1	13	14	92,86
9	2	17	19	89,47
10	1	12	13	92,31
11	2	18	20	90
12	3	17	20	85
13	7	8	15	53,33
14	2	72	74	97,3
15	2	75	77	97,4
16	2	55	57	96,5
17	3	60	63	95,24
18	4	28	32	87,5
19	4	12	16	75
20	5	7	12	58,33
21	3	22	25	88
22	4	18	22	81,82
23	2	24	26	92,31
24	1	10	11	90,91
25	3	12	15	80
26	2	5	7	71,43
27	4	53	57	92,98
28	3	25	28	89,28
29	2	20	22	90,91
30	1	19	20	95
total	76	657	733	84,28

Results of statistical treatment	
Mean	84,2805
Standard error	2,25559
Standard deviation	12,3544
Coefficient of variation	14,6587
Minimum	50
Maximum	97,4026
Sum	2528,415



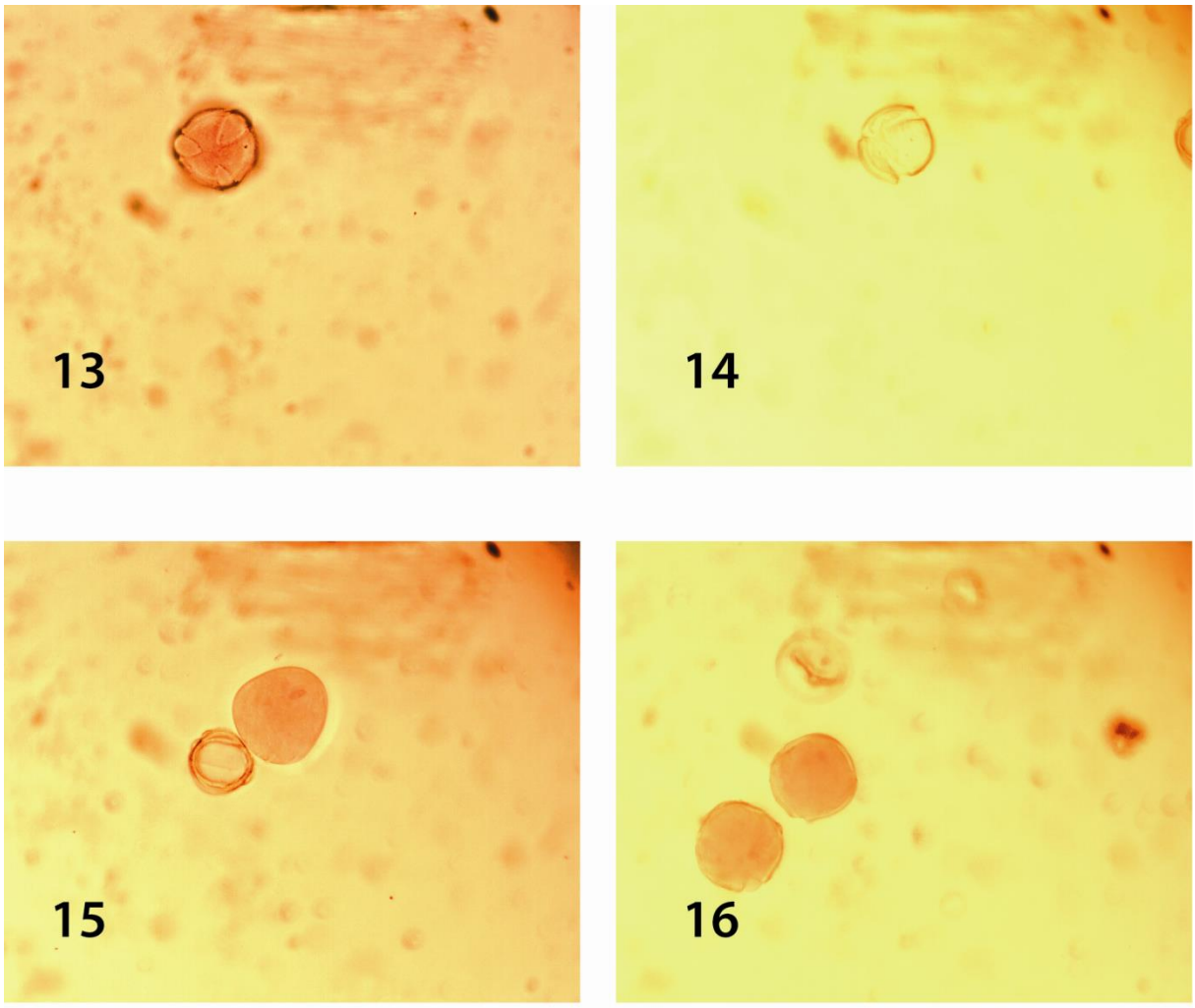
Figs 1-6. Anther and development of the male gametophyte:

1, Four-layered anther wall and sporogenous tissue; 2, Anther wall and microspore mother cells (MMCs); 3, Anther wall with two-nucleate tapetum cells and MMCs, 4, Anther wall with tetrahedral microspore tetrads; 5, Anther walll and one-nucleate pollen; 6, Anther wall and mature pollen; ep – epidermis, en – endothecium, ml – middle layer, tp – tapetum, mmc microspore mother cell, mt – microspore tetrad, pg – pollen grain; (x400).



Figs 7-12. Ovule and development of male gametophyte:

7, Flower with bilocular ovary and anthers; 8, One-celled archesporium in the ovule; 9, Megaspore tetrad whose chalazal cell function as embryo sac mother cell; 10, Mature embryo sac with egg cell, central cell and antipodal complex; 11, Mature embryo sac with egg apparatus, two polar nuclei and degenerating antipodal cells; 12, Mature embryo; ec – egg cell, cc – central cell, ac – antipodal cell, pn – polar nucleus (7x100; 8-12x400).



Figs 13-16. Pollen viability tested by acetocarmine staining:

13, Tricolpate viable pollen grain stained in red; 14, Nonviable colorless pollen grain; 15, 16,Viable pollen stained in red and nonviable colorless pollen (x400)

Figs 17-22. Seed viability according tetrazolium test:

17, Mature seeds not treated with tetrazol solution; 18, Empty seed; 19, Dark red colored viable embryo; 20, Ligth red colored viable embryo; 21, Viable embryo with root and part of cotyledons stained in red; 22, Nonviable colorless embryo.

CONCLUSION

As result of the present study, the mode of reproduction and reproductive capacity of *Sideritis syriaca* were established in connection to define the character and state of its populations. The observed embryological features and absence of apomixis characterize *S. syriaca* as a sexually reproducing species.

The established high pollen and seed viability reveals a high reproductive capacity of this species. The low plasticity of the male and female generative sphere (balanced processes and stable structures and only sexual reproduction that restrict the adaptive mechanisms), determined during this study, regardless of the high pollen and seed viability, very likely is a fundamental cause that defines the restricted distribution of *Sideritis syriaca* and fragmented characters of its populations.

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Original scientific paper

ETHNOBOTANICAL STUDY OF MEDICINAL PLANTS TRADITIONALLY USED IN FIERI DISTRICT, ALBANIA.

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ABSTRACT

Medicinal plants of the endemic flora are traditionally used in Albania as therapeutic means, especially in rural areas. This study aims to identify plants collected for medicinal purposes by the local people in Fieri district, to document their traditional therapeutically uses, in order to contribute into the preservation and enrichment of ethnobotanical knowledge. Research was carried out in the rural areas of Fieri district. The information on medicinal uses of plants was collected by interviewing native people, mainly farmers, elderly people, local herbal drugs sellers, traditional herbal medicine practitioners, housewives. A total of 120 inhabitants (average 65 years old) were interviewed. A comparative analysis with specific ethnobotanical literature was carried out in order to highlight particularly interesting aspects or uses not previously described in the specific ethnobotanical and ethnomedicinal literature. The study revealed 84 medicinal species belonged to 49 families. Lamiaceae, Asteraceae, Liliaceae, Rosaceae were the most dominant medicinal plants families. The uses of the recorded species relate to mild ailments, mainly in the treatment of wounds and burns, gastrointestinal disorders, urinary apparatus diseases etc. The species with the highest use value were *Hypericum perforatum* L., *Salvia officinalis* L., *Cichorium intybus* L., *Allium sativum* L., *Teucrium polium* L., *Acanthus spinosus* L. etc. Traditional medicine in Fieri district is nowadays practiced by some elderly people who resort to medicinal plants only for mild complaints. The study also highlights the uses of some plants, not previously described in the specific ethnobotanical and ethnomedicinal literature.

***Key words**: Ethnobotany, medicinal plants, Ethnomedicine, Fieri district.*

INTRODUCTION

Medicinal plants or plant derived medicines have always played a key role in world health including the maintenance of health as well as in the introduction of new treatment. Traditional knowledge about the medicinal plants has been the starting point for many

successful drug development projects over the last decades [2]. Nowadays, ethnobotany plays a crucial role in the study of traditional medicine [1].

Albania is rich in biological resources and particularly well known for medicinal and aromatic plants. Medicinal plants of the endemic flora are traditionally used in Albania as therapeutic means, especially in rural areas [3]. Until 1992, the Tirana-based "Institute of Traditional Medicine" investigated and promoted the traditional use of Albanian medicinal plants. This institute has contributed in collection and recording of folk treatment methods [4].

This study aims to identify plants collected for medicinal purposes by the local people in Fieri district, to document their traditional therapeutically uses, in order to contribute into the preservation and enrichment of ethnobotanical knowledge.

MATERIALS & METHODS

Research was carried out in rural areas of Fieri district, located in the south-west of Albania, during the year 2012. The information on medicinal uses of the plants was collected using open and semi-structured interviews of native people, mainly farmers, elderly people, local herbal drugs sellers, traditional herbal medicine practitioners, housewives [5, 6]. A total of 120 inhabitants (average 65 years old) were interviewed. The plants collected, indicated by the locals, have been identified according to "Flora of Albania"[7]. Voucher specimens of the collected plants are preserved in the herbarium of Faculty of Pharmacy, University of Medicine, Tirana. The plants are arranged in alphabetical order of their botanical names followed by the family, vernacular name, plant parts used and a brief note on its ethnomedicinal use, methods of preparation and application. The ethnobotanical information collected was analyzed to obtain the following data: number of useful plants mentioned; number of botanical families and the most common plants; plants to which most uses were attributed; parts of plants most frequently mentioned etc. The frequency of citations for each of the reported plants, was used as a basis to identify and to calculate the most frequently medicinal plants occurring in the study area. A comparative analysis with specific (available) ethnobotanical literature was carried out in order to highlight particularly interesting aspects or uses not previously described in the specific ethnobotanical and ethnomedicinal literature [3, 4, 8].

RESULTS & DISCUSSION

The data collected during the field study are listened in Table 1.

Table 1. Traditional medicinal uses of plants in Fieri district.

Botanical taxa, families	**Albanian folk names**	**Ethnomedicinal uses**	**Part (s) used**	**Preparation**	**Frequency of citation ***

Acanthus spinosus L., Acanthaceae	Gjëmbaçi, Drashta.	Prostatitis, cystitis, peptic ulcers.	Seeds	Decoction; Roasted, crushed and mixed with honey.	+++
Achillea millefolium L., Asteraceae	Bishtamithi	To treat hemorrhoids, epistaxis	Aerial parts	Infusion, Decoction Fresh juice in topical application.	++
Agrimonia eupatoria L., Rosaceaea	Podiqja e egër.	Headache.	Aerial parts	Infusion, Topical application	+
Allium cepa L., Liliaceae	Qepa	Wound healing, antihypertensive	Tuber (bulbs)	Beaten and then mixed with vinegar and salt: topical application; Maceration.	+++
Allium sativum L., Liliaceae	Hudhra	Minor antihypertensive, gastrointestinal tract disorder, for bacterial and fungal infection of mouth (gargle) and skin.	Tuber (bulbs)	-Tinctures, -Eaten raw, -Maceration (with water and salt in gargles); topical application	+++
Allium porrum L., Liliaceae	Prasi	To treat colitis.	Leaves (aerial parts)	Eaten fresh, Drink the fresh juice.	++
Althea rosea L., Malvaceae	Mullaga e bute	The common cold, bronchitis.	Leaves	Decoction	++
Arum maculatum L., Araceaea	Kelkaza, Kilikaza	Rheumatic pains	Fruits	Fried with olive oil, topical application	+
Asplenium trichomanes L., Aspleniaceae	Fier guri	Urinary calculi	Aerial parts	Decoction	+++
Bellis perennis L.,	Luledele	To treat inflammation of	Flowers	Infusion	+

Asteraceae		the upper respiratory tract.			
Brassica oleracea L., Brassicaceaea	Lakra	Used as blood purifier; to treat burns.	Leaves	Consumed fresh; Crushed and fresh juice applied topically	+
Bryonia dioica Jacq., *Bryonia alba* L., Cucurbitaceae	Sterkungulli	Headache, ear inflammation	Root	Decoction, filtered and applied as ear drops	+
Buxus sempervirens L., Buxaceae	Bushi, Shemshiri	Anxiety	Leaves, Bark(cortex)	Infusion	+
Capsella bursa pastoris L., Cruciferae	Shtrapëri	Inflammation of urinary tracts, prostatitis; amenorrhea	Leaves	Decoction	+
Carex paniceae L., Cyperaceae	Bar presje	Hemorrhage from wounds	Aerial parts	Beaten and then mixed with salt: topical application	+
Chamomilla recutica L., Asteraceae	Kamomil	Abdominal pains	Flowers	Infusion	+++
Chelidonum majus L., Papaveraceae	Tamblagjaku	To treat hepatitis, cholecystitis. Externally to treat verruca.	Aerial parts	Infusion, Tinctures	++
Centaurea cyanus L., Asteraceae	Kokoçelli	To treat hepatitis	Flowers	Infusion	++
Ceterach officinarum Lam., Polypodiaceae	Bar i gjarprit	To treat the inflammation of upper respiratory tract, cough.	Aerial parts	Decoction	+
Cichorium intybus L., Asteraceae	Çikore	Used as blood cleansers, hepatobiliary disorders.	Leaves, root	Eaten raw as salad; Decoction; Soup.	+++
Clematis viticella L., Ranunculaceae	Kulpuri i zi	Dermatitis, eczema.	Aerial parts	Ground and topically applied	++

Crataegus ssp. Rosaceae	Murrizi	Adjuvant for cardiac arrhythmias and hypertension. To treat insomnia during menopausal syndrome.	Fruits, Flowers	Infusion, Decoction	+++
Cuscuta campestris Yunker, Convolvulaceae	Kuskuta, Bar dreqi	Intestinal worms.	Aerial parts	Decoction	+
Cynodon dactylon Pers. Poaceae	Grami	Cystitis, urethritis, urinary gravel and nonobstructive stones	Aerial parts	Decoction, Infusion	+++
Ecbalium elaterium A. Rich., Cucurbitaceae	Kungulli i egër	To treat hepatitis (jaundice).	Fruits juice	Instilled in the nose for treating of hepatitis.	+
Echium vulgare L., Asteraceae	Bari i butëzës	To treat wounds.	Aerial parts	Ashes	+
Equisetum arvense L., Equisetaceae	Bishtkali;	Prostatitis, hemorrhoids, epistaxis.	Aerial parts	Decoction	++
Erica manipupuliflora Salisb., Ericaceae	Bar Drokthi	Nervousness, Anxiety,	Aerial parts	Decoction	+
Erythrea centaurium Pers., Gentianaceae	Kinëfusha	To treat anemia	Aerial parts	Decoction	+
Ficus carica L., Moraceae	Fiku	Constipation, to treat coughs. Externally in inflammation of the mouth (gargle).	Fruits	Eaten fresh or dried, Decoction	++
Fumaria officinalis L.,	Lule pëllumbi	To treat metrorrhagia.	Aerial parts	Infusion	+

Papaveraceae					
Galega officinalis L., Fabaceae	Ballbreshke, Kukurjaku	To promote the secretion of milk in nursing mother.	Aerial parts	Infusion	++
Genista tinctoria L., Fabaceae	Gjineshtrangjyruese	Inflammation of urinary tract.	Aerial parts	Infusion	+
Geum urbanum L., Rosaceae	Melakja	Gastritis, ulcerative colitis. Externally it used as gargle in gingivitis.	Rhizomes	Decoction	+
Hedera helix L., Araliaceae	Urthi	Nonobstructive gallstones.	Leaves	Decoction	++
Hypericum perforatum L., Hypericaceae	Lulebasani;	Internally in gastritis, ulcerative colitis. Externally in wounds, burns.	Aerial parts	Infusion Maceration with olive oil or alcohol.	+++
Inula helenium L., Asteraceae	Bari i plevitit	To treat hemorrhoids.	Aerial parts	Decoction topically applied	+
Juglans regia L., Juglandaceae	Arra	Internally it is used in diarrhea; externally in eczema.	Leaves	Infusion	+++
Juncus acutus L., Juncaceae	Zhuga	Inflammations of urinary tract (nephritis)	Aerial parts	Decoction	++
Lagenaria vulgaris Ser., Curcubitaceae	Susaku	To treat hepatitis	Fruits	Instilled in the nose for treating of hepatitis.	+
Laurus nobilis L., Lauraceae	Dafina	To treat the common cold, cough, rhinitis; edema.	Leaves, fruits	Infusion, Decoction	+++
Linum usitatissimum L., Linaceae	Lini	Kidney and urinary calculi.	Seeds	Maceration, decoction	++
Lythrum salicaria L., Lythraceae	Bargjaku	Metrorrhagia.	Aerial parts	Infusion	+

Marrubium vulgare L., Lamiaceae	Kapinoku	Inflammation of respiratory tract	Aerial parts	Decoction	+
Melilotus officinalis L., Fabaceae	Grunamadhi	Internally in headache; externally to treat wounds	Aerial parts	Infusion	++
Melissa officinalis L., Lamiaceae	Bar blete	To treat headache, intestinal cramps.	Leaves	Infusion	++
Ocinum basilicum L., Lamiaceae	Borziloku	To treat insomnia.	Aerial parts	Decoction, Smell inhaled	+
Olea europea L., Oleaceae	Ulliri	Hipertension, adjuvant in diabetes mellitus.	Leaves	Decoction	+
Ononis spinosa L., Fabaceae	Gjuhënusja, Kulmuth	Inflammation of urinary tract; eczema.	Root	Decoction	++
Origanum hirtum (Link.), Ietswaart, Lamiaceae	Rigoni i bardhë	Inflammatory and spastic conditions of upper respiratory tract, gastrointestinal disorders.	Aerial parts	Infusion, Spice	+++
Paliurus aculeatus Lam., Rhamnaceae	Driza	To treat diarrhea.	Fruits	Infusion	+
Phaseolus vulgaris L., Fabaceae	Fasulja	To treat bruises.	Fruit without seed	Boiled	+
Physalis alkekengi L., Aspleniaceae	Bari i lungës së zezë	Bacterial skin infection, wound infected, abscess.	Aerial parts	The fresh plant beaten and applied on the wound.	+
Pelargonium radula L. Her., Geraniaceae	Idershahu	Adjuvant in diabetes.	Leaves	Decoction	+
Plantago major L., *Plantago media* L., Plantaginaceae	Gjethe delli i madh, mesatar	Diuretic, haemostatic, anti-bacterial.	Leaves	Decoction; Externally, the fresh leaves (macerated in hot water) applied	+++

				directly in wounds.	
Polygonum avicularae L., Polygonaceae	Bar pate	Inflammation of urinary tracts, urinary calculi, eczema.	Aerial parts	Infusion	++
Polygonum bistorta L., Polygonaceae	Bar i nejçes së përdredhur	To treat diarrhea, ulcerative colitis; used as gargle in inflammation of the mouth.	Aerial parts	Decoction	+
Primula veris L., Primulaceae	Aguliçja	To treat stomach ache and flu.	Flowers	Infusion	+
Prunus avium L., Rosaceae	Qershia e eger	To treat kidney stones	Seeds	Tinctures (crushed seeds macerated in alcohol)	+
Punica granatum L., Punicaceae	Shega	Anti-diarrheal	Cortex	Decoction	+++
Quercus robur L., *Quercus petraea* Matt., Fagaceae	Rrënja dhe Bunga	To treat hemorrhoids	Roots	Decoction	+
Rosa canina L., Rosaceae	Trëndafil i egër	The common cold, digestive; used to prevent various illnesses	Fruits	Decoction	+++
Rhamnus frangula L., Rhamnaceae	Drunakuqi	To treat the constipation, anti-hemorrhoidal.	Bark (cortex)	Decoction	+++
Rhamnus catharticus L., Rhamnaceae	Pjerza dlirëse	To treat amenorrhea.	Fruits	Infusion	++
Rubus ulmifolius Schott., Rosaceae	Manaferra	To treat diarrhea; used as gargle in inflammation of the throat, pharyngitis.	Leaves, Aerial parts	Decoction	++
Salvia officinalis L., Lamiaceae	Sherebela	To treat diarrhea, sore throats, flu, tonsillitis and cough.	Leaves	Decoction	+++
Salix ssp.,	Shelgu	Externally it is	Bark	Topical	+

Salicaceae		used for wounds, ulcers.	(cortex)	application of ash.	
Sambucus nigra L., Caprifoliaceae	Shtogu	For common cold and feverish states. To treat diarrhea (fructus).	Flowers, fruits	Decoction	+
Sambucus ebulus L., Caprifoliaceae	Qingla	Inflamation of urinary tract; Bacterial skin infection, wound infected, abscess.	Fruits, root	Maceration Crushed fruits mixed with olive oil and nishader powder (ammonium chlorate).	+
Satureja montana L., Lamiaceae	Trumza, Shtermen	Minor inflammatory and spastic condition of the gastrointestinal tracts.	Aerial parts	Infusion	++
Sempervivum tectorum L., Crassulaceae	Bar veshi	To treat earache	Leaves	Fresh leaves, crushed and the juice applied in ear (drops ear)	++
Solanum niger L., Solanaceae	Bar plasje, Idhnaq	Bacterial skin infection, wound infected, abscess	Aerial parts	The ash (of plant) mixed with olive oil, topical applied.	+
Solanum tuberosum L., Solanaceae	Patatja	Externally to treat burns of the eyes.	Bulb	Fresh, cut and applied topically.	+
Teucrium polium L., Lamiacaea	Bar majaselli;	To treat colitis and hemorrhoids.	Aerial parts	Decoction	++
Tilia cordata L., Tiliaceae	Bliri	To treat sore throat, coughs and flu.	Flowers	Decoction with *Malva* ssp.	+++
Trigonella corniculata L., Fabaceae	Trëndelina	To treat high cholesterol and triglycerides, adjuvant in	Flowers	Infusion, Decoction	+

		diabetes.			
Tussilago farfara L., Asteraceae	Thundërmu shka	To treat coughs	Leaves, flowers	Decoction	+
Thymus serpyllium L., Lamiacaea	Zhumbrica	Inflammatory and spastic conditions of upper respiratory tract, gastrointestinal disorders.	Aerial parts	Infusion	++
Urtica dioica L., Urticaceae	Hithra	To treat cystitis and urinary gravel. Externally, in sciatica; in alopecia.	Leaves	Decoction; Externally, as an alcoholic extract.	++
Ulmus campestris L., Ulmaceae	Vidhi	To treat burns.	Bark (cortex)	Decoction, topically applied.	+
Verbascum ssp., Scrophulariaceae	Netulla, Bar peshku	To treat irritation dry cough.	Flowers	Infusion	++
Verbena officinalis L., Verbenaceae	Bar i shpretkës	To treat nonobstructive gallstones; antidyscratic.	Aerial parts	Infusion, Gargle, Topically applied	+
Viscum album L., Loranthaceae	Vjeshtulla e bardhe	To treat albuminuria during pregnance.	Leaves	Maceration	+
Vaccinium myrtillus L., Rosaceae	Boronica;	To treat intestinal inflammation, anti-diarrheic; "blood cleaner".	Fruits, leaves	Eaten fresh, Decoction	+++

* +++ use quoted by more than 40 % of informants; ++ use quoted by 12-40% of informants; + use quoted by less than 12 % of informants.

The study revealed 84 medicinal species belonged to 49 families. Lamiaceae, Asteraceae, Rosaceae, Fabaceae were the most dominant medicinal plants families. The uses of the recorded species relate to mild ailments, mainly in the treatment of wounds and burns, gastrointestinal disorders, urinary apparatus diseases etc. The medicinal plant preparations were applied through different routes of administration like oral, topical or dermal and nasal routes. However, oral application (up to 70%) was the highest and most commonly used route of application.The species with the highest use value were *Hypericum perforatum* L., *Salvia officinalis* L., *Cichorium intybus* L., *Allium sativum* L., *Teucrium polium* L., *Crataegus spp.*, *Acanthus spinosus* L., *Laurus nobilis* L. etc.

CONCLUSION

Present study shows that the study area is rich in various types of valuable medicinal plants. Phytotherapy in south-west Albania is nowadays practiced by some elderly people who resort to medicinal plants only for mild complaints. Easy access to modern medicines and less recognition of traditional plants are the main causes leading to decrease the interest of young generation in the use of traditional medicinal plants. So the emphasis should be given for the documentation of this knowledge. The study also highlights the uses of some plants, not previously described in the specific ethnobotanical and ethnomedicinal literature as example: *Paliurus aculeatus* L. as antidiarrheal; *Juncus acutus* L. in urinary apparatus diseases; *Echium vulgare* L. to treat wounds etc. These plants deserve to be taken into consideration for further phytochemical investigation.

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Review paper

RESEARCH AND DEVELOPMENT OF THE PLANT MEDICINE BLOSSOMS IN SLOVAKIA – NEW VARIETIES

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ABSTRACT

Modern phytotherapy is direct successor of the rich tradition of popular doctoring in Slovak Republic. Generally, research & development should contribute to the solution of following tasks: * monitor of the Slovak gene-pool of herbs, * collecting expeditions of genetic resources, *determination of chemotypes, * protection of genetic resources in the Gene Banks, * breeding of medicinal, aromatic and spices plants, * development of seed production, * introduction of new medicinal plant species into large-scale cultivation, * improvement of the methods of large-scale cultivation, harvest and post-harvest technologies, * creation of extension offices, * giving of the quality certificates of the all herb items, * introduction of the without waste technologies,* presentation of the results of research and development at the domestic and foreign actions and exhibitions, *marketing investigation of the world market and presentation of information review about this situation. The biodiversity of medicinal plant species, the use of natural drug resources and the experience of folk medicine has been a continuous subject of reaseach and development (R&D) at several universities and research institutes in Slovakia. The actual R&D results of chamomile (*Matricaria recutita* L.), peppermint (*Mentha ×piperita* L.) and poppy (*Papaver somniferum* L.) – the new varieties are presented.

Keywords: *medicinal plants, R&D, results, varieties, well-known success*

INTRODUCTION

Modern phytotherapy is direct successor of the rich tradition of popular doctoring in Slovak Republic. It is based on the years of experience of people having direct contact with nature. Over the years this experience has been verified in practice, supplemented and classified – folk herbalist wisdom formed and passed from generation to generation. The use of natural drug resources and the experience of folk medicine has been a continuous subject of reaseach and development (R&D) at several universities and research institutes in Slovakia (Salamon, 2000).

Chamomile, *Matricaria recutita* L. – the highest Bisabolol content

Chamomile, *Matricaria recutita* L., (Fig. 1) is something of a general physician. The board spectrum of its curative effects and uses could be compared to the healing power of the legendary root of ginseng. It is one of the few medicinal plants with an ever-increasing consumption in the world.

Slovakia is one of the European countries in which particular attention has been devoted to research of chamomile in all its aspect, including the propagating of this medicinal plant. Based on the study of chamomile´s pharmacodynamics properties, the sesquiterpenes: /-/α-Bisabolol, Chamazulene and /-/β-Farnesene are considered to be the most valuable constituents. Gradually, between the years 2008 – 2013 the chamomile variety "*Lianka*" were bred at the University of Presov, Slovakia (Fig. 2). The variety is characterized by its high percentage of sequiterpenes (/-/-α-Bisabolol [52 – 55 %], Chamazulene [18 – 19 %], the low contents of /-/-α Bisabololoxides A and B [< 3 %] and essential oil content is from 0.65 to 0.85 %).

After the improvement of better variety, chamomile flower drug production has been directed at special, large-scale cultivation. The majority of production areas, which grow about 500 hectares every year, are contracted in Slovakia (Salamon, 1992).

Chamomile plants are picked only in the stage of developed anthodia, using various types of harvesters. Sorting the chamomile biomass is performed by sorting machines. Drying is provided mostly on hot-air dries. The dry chamomile drug of the first quality is delivered directly to the processing enterprises. The remaining plant material and the waste are used to produce essential oil and extracts (Salamon, 2007).

In Slovakia more than 50 chamomile commercial phytotherapeutical preparations and variety of cosmetics products were produced. Today, the Slovak Drug Research Institute in Modra deals with the development of dosage (tinctures, extracts, solutions, gels and injections) of active substances isolated from the natural chamomile material.

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ROZHODNUTIE
o registrácii odrody

Ústredný kontrolný a skúšobný ústav poľnohospodársky v Bratislave - odbor odrodového skúšobníctva (ďalej len „kontrolný ústav"), ako orgán príslušný na konanie podľa § 4 písm. c) zákona č. 597/2006 Z. z. o pôsobnosti orgánov štátnej správy v oblasti registrácie odrôd pestovaných rastlín a uvádzaní množiteľského materiálu pestovaných rastlín na trh v znení zákona č. 467/2008 Z. z., v zmysle § 3 nariadenia vlády Slovenskej republiky č. 50/2007 Z. z. o registrácii odrôd pestovaných rastlín v znení neskorších predpisov (ďalej len „nariadenie") a v súlade s § 46 zákona č. 71/1967 Zb. o správnom konaní (správny poriadok), v znení neskorších predpisov rozhodol takto:

r e g i s t r u j e o d r o d u
Lianka

druh **Rumanček kamilkový** – **Matricaria recutica** L. pre **Prešovská univerzita v Prešove, 17. novembra 1, 081 16 Prešov, SK.**

Odôvodnenie:
Žiadateľ podal v zmysle § 3 ods. 7 písm. a) nariadenia žiadosť o registráciu odrody pod pracovným označením PO-MATRI_REC-1 dňa **2. 1. 2012,** pod číslom **212R141.** Štátne odrodové skúšky boli vykonané v roku **2012 - 2013.**
Kontrolný ústav vydáva rozhodnutie o registrácii hore uvedenej odrody podľa podkladov pre návrh rozhodnutia zo dňa **7. 10. 2013.**
Vzhľadom na to, že prihlásená odroda spĺňa podmienky na registráciu odrody podľa § 3 ods. 1 nariadenia, bolo potrebné rozhodnúť tak, ako je hore uvedené.
Na základe rozhodnutia kontrolného ústavu, kontrolný ústav zapíše odrodu do Štátnej odrodovej knihy pod evidenčným číslom **7058.**

Poučenie:
Proti tomuto rozhodnutiu možno podľa § 53 a 54 správneho poriadku podať odvolanie na Ústredný kontrolný a skúšobný ústav poľnohospodársky v Bratislave, Matúškova 21, 833 16 Bratislava a to v lehote 15 dní odo dňa jeho doručenia.
Toto rozhodnutie možno preskúmať súdom až po využití riadneho opravného prostriedku (odvolania).

Ing. Juraj Moško
generálny riaditeľ

Rozdeľovník:
1. Prešovská univerzita v Prešove, 17. novembra 1, 081 16 Prešov, SK
2. Spis

Fig. 1 and 2: The decision (October 2013) of the Slovak Ministry of Agriculture for the new chamomile variety "*Lianka*"

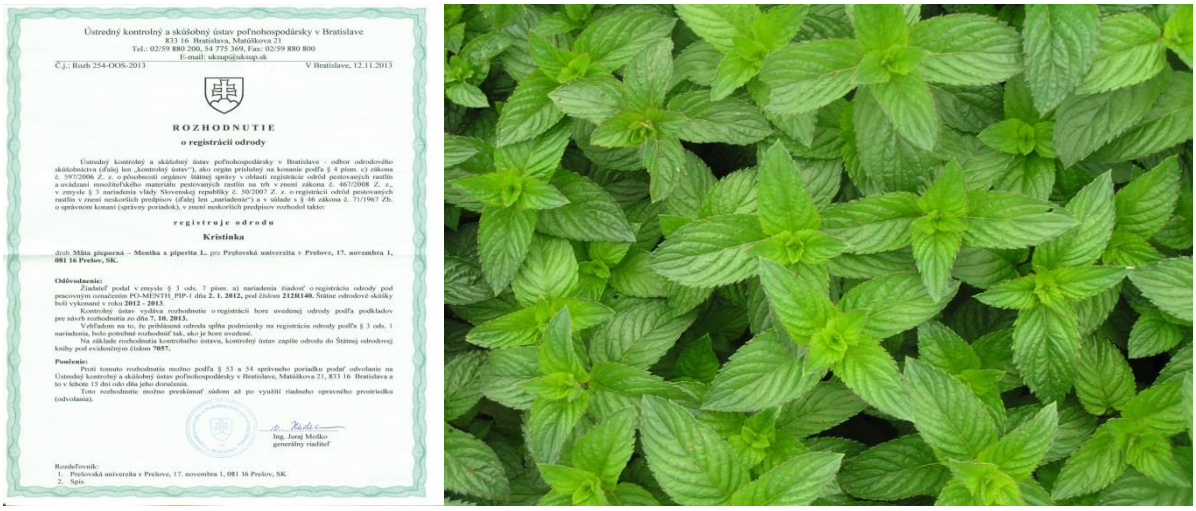
Peppermint, *Mentha ×piperita* L. – the highest Menthol content

Peppermint, *Mentha ×piperita* L., is a plant which represents the oldest and traditional medicinal herbs used both Eastern and Western traditions till recent time. The peppermint has a history of use in herbal medicine dating back to ancient Egypt, Greek and Roman times (Murray, 1995), although they may not be used for the same reasons. The wide therapeutic effects of peppermint dry drug but much more essential oil using in the human medicine has become peppermint precious applied in pharmaceutical industry. The entire herb is medicinal (peppermint dry leaves and leafed shoot tops are the pharmacopoeia material) usually used as component of herb teas and mixtures, but much more used is the peppermint essential oil (*Menthae piperitae aetheroleum*) applied at cosmetics, pharmaceutical and food industry.

Peppermint essential oil has unique therapeutic properties which have been documented by use throughout history as one of the most well-known essential oils. Peppermint oil is a very grateful agent to allay nausea and vomiting, indigestion, fevers, flatulence, headaches, migraine, liver problems and arthritis. It is stimulating to the nervous system, cooling to the body for fevers or in hot weather. A strong digestive aid and breathe freshener. Peppermint's strong antispasmodic action makes it useful in massage for sports injuries. It is anti-inflammatory action helps sciatica, neuralgia, and arthritis. The oil of peppermint, on account of the menthol present in it, is local anesthetics, and may be employed to relieve local pain, as in the inflamed joints of rheumatism, as a spray in painful inflammation of the throat, and in any painful condition where a direct application of the anesthetic can be made. It is stimulating ability helps mental concentration and memory. It is very stimulating to the mind, relieves mental fatigue and depression (Sustrikova and Salamon, 2004).

The world peppermint production is realized by the large – scale cultivation upon the suitable intensive practices. Study on the qualitative – quantitative characteristics of the peppermint essential oil produced under agro-ecological conditions of the Eastern Slovakia confirmed its high composition of the Menthol [70 – 75 % of herbs and 80 – 85 % of leaves] of essential oil [2.6 %] into the dry raw material. Suitable Menthol content of peppermint cultivated in Slovakian provenience destines this peppermint gene material for the breeding of new variety "*Kristinka"* (Fig. 4), which was registered by the Slovak Ministry of Agriculture in 2013.

Menthol activates coolness feeling on the skin by the specific irritation of nervous axons with the desensitization in the locality of application (Watson, 1978). It has an anesthetic, antiseptic, antibacterial affects, removes the itch and reduces gland secretion. It is suitable carminative and antiemethicum.



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r e g i s t r u j e o d r o d u

Kristinka

druh **Mäta pieporná – Mentha x piperita L.** pre **Prešovská univerzita v Prešove, 17. novembra 1, 081 16 Prešov, SK.**

Odôvodnenie:
Žiadateľ podal v znysle § 3 ods. 7 písm. a) nariadenia žiadosť o registráciu odrody pod pracovným označením **PO-MENTH_PIP-1** dňa **2. 1. 2012,** pod číslom **2120140.** Štátne odrodové skúšky boli vykonané v roku **2012 - 2013.**
Kontrolný ústav vydáva rozhodnutie o registrácii hore uvedenej odrody podľa podkladov pre návrh rozhodnutia zo dňa **7. 10. 2013.**
Vyhlásením na to, že prihlásená odroda spĺňa podmienky na registráciu odrody podľa § 3 ods. 1 nariadenia, bolo potrebné rozhodnúť tak, ako je hore uvedené.
Na základe rozhodnutia kontrolného ústavu, kontrolný ústav zapíše odrodu do Štátnej odrodovej knihy pod evidenčným číslom **7057.**

Poučenie:
Proti tomuto rozhodnutiu možno podľa § 53 a 54 správneho poriadku podať odvolanie na Ústredný kontrolný a skúšobný ústav poľnohospodársky v Bratislave, Matúškova 21, 833 16 Bratislava a to v lehote 15 dní odo dňa jeho doručenia.
Toto rozhodnutie možno preskúmať súdom až po využití riadneho opravného prostriedku (odvolania).

Ing. Juraj Moško
generálny riaditeľ

Rozdeľovník:
1. Prešovská univerzita v Prešove, 17. novembra 1, 081 16 Prešov, SK
2. Spis

Fig. 3 and 4: The decision (October 2013) of the Slovak Ministry of Agriculture and the peppermint variety "*Kristinka*"

Poppy, *Papaver somniferum* L. – the large spectrum of variety breeding

Slovakia belongs among countries with large-scale poppy cultivation, because it is a politically stable, hence a reliable supplier. The food poppy varieties contain from low to moderate content of morphine, so contamination is negligible and consumption is not harmful to human health.

The availability of poppy cultivars of a high chemical diversity offered a new challenge to clear up the regularity of the inheritance of alkaloid accumulation (Finetto, 2008). This type of knowledge might contribute to the construction of new cultivars of high practical importance as well as provide a good scientific background to make regulations and put the regulations into effect (Salamon and Fejer, 2011).

Selection of poppy plants in Slovakia dates from 1948 and is concentrated on generation of universal varieties. The main goal was cultivation of a variety with the capacity to accumulate high levels of morphine in dry capsules and high production of quality seed of blue color. The result of long-time attempts is a number of varieties with high production potential of poppy seed.

There have been several varieties of poppy cultivated and grown in Slovakia. At the present time, six varieties of poppy with blue-colored seed are registered "*Gerlach*", "*Opal*", "*Bergam*", "*Maraton*", "*Major*" and "*Malsar*". The variety "*Albin*" has white seed (Fig. 5). They are well adapted to central-European soil-climatic conditions. The yield of poppy seed has been varying from 0.28 $t.ha^{-1}$ to 0.73 $t.ha^{-1}$ in last ten years (Fig. 6). Genetic potential of seed yield is up to 2.0 $t.ha^{-1}$. These varieties are suitable primarily for food industry purposes. The poppy straw yield ranges from 300 to 500 $kg.ha^{-1}$. Morphine accumulation in dry capsules moves between 0.3 to 0.6 %. The straw of these varieties is used for morphine extraction, but it does not meet the rising demands of pharmaceutical industry. These varieties are valuable for their good tolerance to herbicides used. Therefore, they are suitable for large-scale production conditions. The quality of the varieties is reflected in their growing in Czech Republic – annual production of seed is about 120 tones (Vasak et al., 2010).

In regard to introduced requirements and international trends in cultivation of poppy, the breeding of the poppy crop focuses on creating two types of varieties: – industrial, with the capacity to accumulate high levels of morphine, or other alkaloids in dry capsules, and – food varieties with high production potential of quality seed suitable for food industry utilization, with low to moderate morphine content, eventually without morphine (Kapoor, 1995). The recent aim of extensive research for better poppy properties is carried out to hormone application, genetic engineering, controlled cross-pollination etc.

Fig. 5 and 6: Purple-white flowers of poppy and the harvest of dry plants

CONCLUSION

The worldwide demand for medicinal and aromatic plants and for products derived of them is permanently increasing. This is well documented in one of our old proverbs: „*There is not plant without use*“ and our prosperous work can decisively contribute the exploitation of the properties of the single plants to benefit mankind. In regard to this predication and the valuable experiences the production of medicinal and aromatic plants in Slovak Republic has a large perspective.

ACKNOWLEDGEMENTS

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Review Paper

TURKISH ANISE (*PIMPINELLA ANISUM* L.)

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ABSTRACT

Anise (*Pimpinella anisum* L.) belongs to the Apiaceae (Umbelliferae) family; it is an annual plant which has medicinal and aromatic fruits and is commonly used in confectionery, perfumes, pastries, beverages and pharmaceutical industry. *Pimpinella* is represented in Turkey by 23 species (five endemic), two subspecies and two varieties representing a total of 27 taxa. The fruit and the essential oil are known for antispasmodic, antioxidant, antimicrobial, insecticidal and also antifungal effects. Essential oil yield of anise fruits which are called aniseed is 1.5-5.0% and contains *trans*-anethole with around 90%. Due to the usage of anise in production of Turkish traditional beverage "raki", its cultivation is quite common in Turkey. Average cultivation area of anise is 24200 ha and the production is nearly 15 000 tones for last 25 years. In Turkey, 64% of anise production is cultivated in Western Mediterranean region (Burdur, Antalya) and the other cultivation provinces are Konya, Izmir, Denizli, Afyon, Muğla, Balıkesir and Eskisehir. Syria, Spain and the United States are among the countries which import anise from Turkey. In this study, it is aimed to emphasize anise cultivation and trade in Turkey.

Key Words: *Pimpinella anisum L., anise, trans-anethole, cultivation, trade.*

INTRODUCTION

In today's industry, synthetics are used extensively in many products. The over usage has a negative impact on human health. However, consumers have become more and more conscious and are demanding products made of natural raw materials. Nowadays, developed countries prefer herbal products for medical treatment and the demand for medicinal and aromatic plants is increasing with each passing day.

Traditional medicine has maintained its importance in the developing world, and its use is rapidly gaining in popularity in industrialized countries. Nearly 80% of the world population use traditional medicine, mainly medicinal plants, to cure illnesses and ailments. Moreover, the percentage of the people using alternative therapies at least once a year, has reached about 48.5% in Australia 33% in Finland, 10% in Denmark and 17% in Canada [1].

According to the World Health Organization, the number of medicinal and aromatic plants used in the world today is around 20000. Although, 4000 of them has still been widely used, only the 2000 of them in the world and 500 of them in Western Europe have a commercial potential today [2]. UNCTAD COMTRADE data in this report are given in Table 1 based on classification of HS1992 and commodities of 1211 (plants, plant parts for perfumery, pharmacy, etc.) "Plants and parts of plants (including seeds and fruits), of a kind used primarily in perfumery, in pharmacy or for insecticidal, fungicidal or similar purposes, fresh or dried, whether or not cut, crushed or powdered". As it can be seen from the tables; Turkey has exported nearly 14 million dollars of plants and plant parts to the world and has imported 5 million dollars from the world in 2013.

Table 1. Top export and import partners of Turkey for plants, plant parts for perfumery, pharmacy, etc. in (Uncomtrade, 2013)

EXPORTS			IMPORTS		
Partner	Trade Value ($)	NetWeight (kg)	Partner	Trade Value ($)	NetWeight (kg)
World	13.951.311	3.707.263	World	5.034.244	1.790.009
Free Zones	4.289.574	940.147	Albania	1.182.478	402.230
Germany	2.796.125	723.222	Bulgaria	604.987	109.865
USA	1.021.996	446.769	Egypt	453.931	326.344
Spain	773.692	327.354	Nigeria	435.180	228.881
France	760.262	223.638	China	317.506	60.267
Other	4.309.662	-	Other	2.040.162	-

ANİSE

Anise is an annual plant mostly grows naturally in Mediterranean countries. It is cultivated in many countries like India, Egypt, Bulgaria, Syria, Spain, China and Iran [3,4]. Anise is a plant cultivated for its fruit called "seed" and is commonly used in confectionery, perfumery, pastries, beverages and pharmaceutical industry. In many countries, people consume alcoholic beverages made from anise such as "Sambuca, Arak, Anisette, Pastis, Ouzo, Tsipouro, Mastika and Raki". Turkey is used seventy percent of produced aniseed in manufacturing of Turkish Raki and thirty percent of these are exported around the world [5]. *Pimpinella anisum* fruits have been used in Turkish folk medicine as carminative, appetizers, sedative, and agents to increase milk secretion [6,7]. Aniseed is an important agricultural crop of Turkey. Turkish anise is known as their cultivated region name like Çeşme Anise, Burdur Anise, Fethiye Anise and Denizli Anise . Anise is mostly cultivated in the provinces of Burdur, Denizli, Muğla, Antalya, in lesser amounts in Afyon, Bursa, Balıkesir, Uşak, Eskişehir and İzmir in Turkey [3,4,8]. Although anise is widely produced in our country, there is not any registered variety.

TRADE

Aniseed has an important place among the medicinal and aromatic plants due to it is used in industries of beverage, spices, pharmaceutical, perfumery, etc. Nowadays, there are certain countries such as India, Egypt, Bulgaria, Syria, Spain, China and Iran etc. According to FAO

2014 data, Turkey took second place after India with 24 856 tons in terms of quantity, while it was in sixth place in the spices exporting with $ 63 223000 in terms of value, in 2011 (Figure 1). In 2012, Turkey took eighth place in the anise, badian, fennel and coriander exporting with 9 260 tons, while it is in sixth place with $ 26 945 000 in 2011.

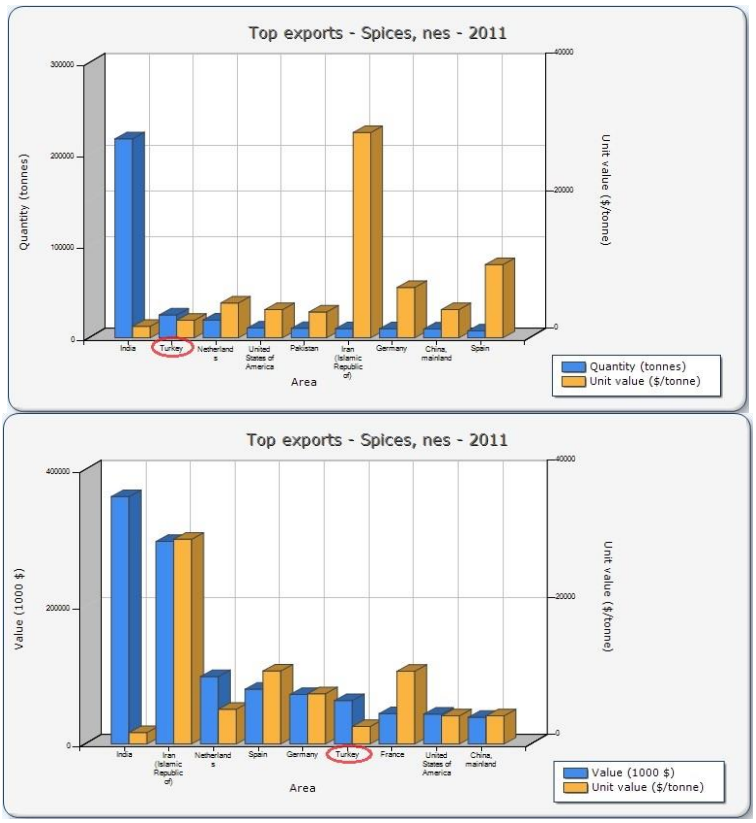
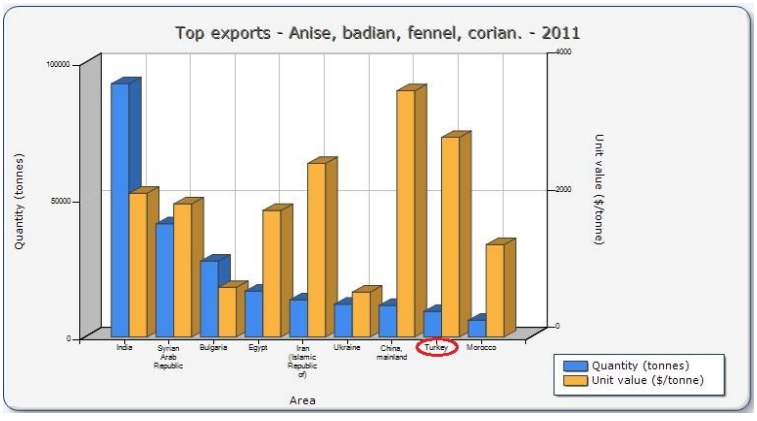


Figure 1. Exporting value and quantity of the countries by spices in 2011 (FAO, 2014)



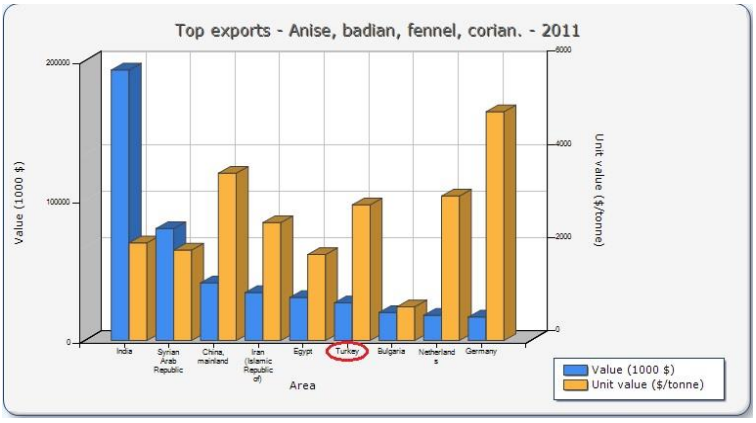


Figure 2. Exporting value and quantity of the countries by anise, badian, fennel, coriander in 2011 (FAO, 2014)

USAGE AND CONTENT

Aniseeds are mostly used for producing of traditional Turkish alcoholic beverage "raki" in Turkey. By fermentation and distillation of pulps of fresh grapes left over the wine production, distillate is obtained and after addition of aniseed into the suma, it is distilled second time and the traditional Turkish raki is obtained [5]. Some producers use raisins but in recent year, fresh grape raki has become more popular in Turkey. The amount of alcohol by volume must be at least 40% [8], but it varies from 40 to 50% and anethole content of aniseed essential must be at least 800 milligrams per liter of the product [9].

Anise fruits contain 40-60% carbohydrates, 15-25% protein, 58-16 fatty oil, 15-25% crude fiber, ash 7% [9]. The essential oil content is 1-4% [3] and the main constituent of the essential oil is *trans*-anethole (80-90) [11]. In addition, the essential oil of the anise fruit also contains a small proportion of estragol and anisaldehyde [4,12,13] γ-himachalene and cis-anethole [13].

CLIMATE AND SOIL REQUIREMENT

Anise grows well in hot, humid, sunny, yet rainy enough regions. It requires cool and rainy weather conditions until the florescence period. After that, specifically in maturity period it requires dry and hot weather conditions. Frost, excessive rainfall, dry and hot winds are quite harmful particularly in florescence period. It doesn't like climates that are under directly marine effect and high humidity.

Anise grows better in lime-rich, notr, lightly alkaline, transparent and sandy loam soil; it doesn't prefer humid and heavy ones.

CULTIVATION

Our country is located in a temperate climate. Mediterranean, Black Sea and Continental climate is observed. Anise cultivation is carried out in Mediterranean and Continental climate regions in Turkey. In Mediterranean climate region anise cultivation is made in spring and autumn, but in Terrestrial climate region only spring sowing is made. Burdur which has a

Continental climate and where 58% aniseed is produced, autumn ploughing is used twice before sowing. Aniseed is sowed between February 15 and March 15 according to weather conditions and 1-2 kg per decare via broadcast sowing. Some of the anise producers use 15-30 kg diamonium phosphate (DAP), 30 kg 20:20:0 and 10 kg urea per decare. During the vegetation period, flood irrigation is applied at least two times according to the needs of the plants. Weed controlling is carried out with herbicides and hoeing however no pesticide application is done. Aniseed is harvested from mid-July to mid-August. Alternation is applied biennially with other crop plants.

DIASEASES AND PESTS

Most common diseases are leaf spot disease (*Blumeriella jaapi*), powdery mildew. The most frequently encountered pests are anise moth (*Depressaria* cf. *daucivorella* Rag.), aphids (*Hyadaphis foeniculi* (pess.) and *Aphis fabae* Scop.) and cutworm (*Agrotis* spp.)

HARVEST

Harvest of aniseed is done in the period of fruits of the main branches become browning but not in fully maturity. For avoiding the fruit losses, early harvest should be preferred. Harvest is done at the bottom of the plant by uprooting in small fields or by harvester in large fields. The harvested plants are dried in the field before bunched and then threshing. The fruits get dark and the quality is reduced when the anise bunches exposed to the rainfall during the drying period. Threshing is done with thresher or harvester. The harvesting and threshing of anise is done by manpower are very costly and increases the costs.
The yield of the aniseed varies between 450 to 1100 kg per ha according to the seed quality, sowing date, plant density, water supply, harvesting time and method, ecological conditions and cultivation.

CONCLUSION

Anise cultivation has a great importance in Turkey due to high demand for the fruits used in kind of industry. Although aniseed is widely produced in our country; there is not any registered variety, yet. For obtaining of the high-quality standard products, registered varieties should be developed according to the suitable ecological conditions for the cultivation of anise. Developing of the new varieties, the landraces must be protected, since landraces are very important for breeding programs. Labor costs should be reduced in anise cultivation, otherwise production quantities will be reduced proportionally.

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Section II

" Pharmacology and biological effects of active MAP compounds"

Original scientific paper

ANTI-NOCICEPTIVE, ANTI-INFLAMMATORY AND ANTIPYRETIC EFFECTS OF *MORINGA OLEIFERA PLANT*

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ABSTRACT

Moringa oleifera leaves were screened for its phytochemical, toxicological effect and some pharmacological activities. The toxicological pattern of the ethanolic extract of *Moringa oleifera* leaves was studied by determination of LD_{50} in mice by oral administration of upgrading doses of 75% ethanolic extract with continuous monitoring. The oral anti-inflammatory (carrageenan induced edema), antipyretic (Brewer's yeast induced hyperthermia) and anti-nociceptive (Thermal and chemical induced pain) effects of 75% ethanolic extract of *Moringa oleifera* were carried out on experimental animal models. The obtained result showed that, *Moringa oleifera* leaves contain carbohydrates and/ or glycosides, tannins, alkaloids and /or nitrogenous bases, flavonoids, saponins and resins but not contain unsaturated sterols and /or tri-terpenes. Oral administration of the ethanolic extract of *Moringa oleifera*leaves was devoid of any toxicity in mice given a dose up to 6000 mg/kg orally. Treatment with ethanolic extract of *Moringa oleifera* (200, and 400 mg/kg, orally) and rats treated with Diclofenac (25 mg/kg) showed a significant decrease of rat's paw thickness after 1 h, 2 h and 3 h. compared control rats. Pyrexia model showed antipyretic activity of *Moringa oleifera* at 200 and 400 mg kg^{-1} / b.wt (body weight) which was compared to Paracetamol 50 mg kg^{-1} b.w. The pain inducing model showed good analgesic activity at both tested doses of 200 & 400 mg kg^{-1} b.wt., compared to respective standard analgesic drugs according to respective pain models. In conclusion Oral administration of ethanolic extract possessed significant anti-inflammatory, antipyretic and anti-nociceptive activities.

***Key words**: Moringa oleifera- phytochemical-toxicologica- Anti-inflammatory- Anti-nociceptive- antipyretic*

INTRODUCTION

Pyrexia and inflammation are the body defense mechanisms to create unfavorable conditions to infectious agent. Inflammation, pain and fever are modulated by prostaglandins as (PG E_2 is responsible for elevating the set point of thermoregulatory center leading to fever, PG E_2 also induce the release of substance P which lead to long lasting pain **White (1996)**, PG lead to vasodilatation leading to inflammation and pain by pressing of the inflammatory fluid on nerve ending. Appropriate inflammation and fever are required to overcome the unfavorable conditions but when they become inappropriate or exceed the desired level they produce dangerous conditions such as autoimmune disease and malignant

hyperthermia. Drugs having anti-inflammatory activity generally possess antipyretic activity as NSAIDs **Begum et al., *(*2011)**. Search for safe herbal remedies with potent anti-inflammatory and antipyretic received momentum recently as the available paracetamol and aspirin have toxic effect to various organ of the body. So at the present study the ethanolic extract of ***Moringa oleifera*** plant was tested for its anti-inflammatory, anti-nociceptive and antipyretic activity. ***Moringa oleifera***, an important medicinal plant is one of the most cultivated species of the family Moringaceae. Every part of this plant is said to have beneficial properties that can serve humanity **Mishra *et al.*, (2011)**.

MATERIAL AND METHODS

Materials

Plant material:

The fresh ***Moringa oleifera*** (Moringaceae) were collected during November to December, 2012 from private farm in the El Sadat city. The plant specimen was authenticated by the Department of Botany, Faculty of Science, Cairo University, Egypt.

Plant preparation:

The plant leaves were washed with tap water dried at room temperature then chopped into small fragments and pulverized by using grinder. Cold extraction was done with 75% ethanol for 72 hour at room temperature with intermittent shaking (**Handa 2008**). The extracts were concentrated in a rotatory evaporator at 40 ◦C and refrigerated until use. The yield was 38.83 %.

Animals:

1-Mice:
Mature mice of (20-25) grams body weight of either sex were used for the studying the toxicity and the analgesic activity.

2-Rats:
Mature female and males of (150-200) grams body weight were used for studying the antipyretic and anti-inflammatory effect of the tested extract.

A-Toxicological studies:

The toxicological pattern of the ethanolic extract of ***Moringa oleifera*** leaves was studied by determination of LD_{50} in mice. This study was of value before studying the pharmacological aspects of the tested extract of the studied plant.

Determination of LD_{50}:
LD_{50} of the studied extract was carried out in mice according to method described by **Kerber (1941).** For these purpose five groups of 5 mice in each, weighing 20-25 grams one of them served as control group. Other four groups were dosed orally the studied extract on ascending doses (30, 60, 90, and 120 mg/20g body weight). Animals were put under observation for 24

hours for recording the toxic symptoms, mortality rate and post-mortem findings in each group. LD_{50} of the tested extract was calculated according to the following formula:

$$LD_{50} = Dm - \frac{\Sigma(zxd)}{n}$$

Where:

Dm = The largest dose which kill the animals.

z = Mean of dead animals between 2 successive groups.

d = The constant factor between 2 successive doses.

n = Number of animals in each group.

$\sum$ = The sum of (a × b).

B-Pharmacological studies:

1-Anti- inflammatory:
Rats of both sexes weighing 150-200 g were randomly divided into four groups of six animals in each one. The group I served as control with induced inflammation only and the group II served as standard by oral administration of diclofenac sodium 30 mg/kg body weight whereas those of the group III andgroup IV were given orally 200and 400 mg/kg of ethanolic extract respectively.

Thirty minutes after drug or test compound administration, 0.1 ml of1% w/v Carrageenan suspension was injected subcutaneously in to the plantar surface of theright hind pawfor induction of edema or inflammation as described by *Winter et al.*, **(1962)**. The thickness of each rat paw was measured in mm using varniar caliber to determine the inflammation produced by carrageenan after 1, 2 and 3 hours.

2- Antipyretic effect:
Fasting rats of both sexes weighing 150-200 g were randomly divided into four groups of six animals in each. Basal rectal temperature was recorded for each rat then all rats were made hyperthermic by subcutaneous injection of Brewer's yeast; 1ml /100 g body weights, of 12.5% yeast suspension in normal saline. After 17 hours, the rectal temperature was recorded for all groups to be served as the basic line of elevated body temperature, to which the antipyretic effect will be compared (animals that showed elevation of 0.3-0.5 ºC in rectal temperature were selected).

The group I served as control non- treated whereas those of group II andIII were given orally the ethanolic extract on concentration of 200 and 400 mg/kg body weight respectively while the group IV were given orally paracetamol at dose level of 50 mg/kg.

The rectal temperature was recorded at 1, 2 and 3hours after extract or drug administration according to method described by **Teotino *et al.*, (1963).**

3-Analgesic effect:

Hot plate method

The analgesic effect was determined using the hot plate method as described by **Janssen and Jageneau (1957)** and modified by **Jacob and Bosovski (1961).**

For this purpose 40 mice of either sex weighing 20-25 g were divided into 4 equal groups each of 10 mice. The group I served as control non treated whereas those of group II and III were given orally the ethanolic extract on concentration of 200 and 400 mg/kg body weight respectively while the group IV were given orally paracetamol at dose level of 50 mg/kg.

The apparatus composed of water bath thermostatically controlled at 56 ± 0.5 ºC and beaker of 2 liter capacity which immersed in the water bath and form the hot plate.

After 30 minutes from the oral administration, each mouse was placed in the beaker until the mouse licks its paw or jump. The time elapsed until the mouse licks its paw or jumps was considered as reaction time for the analgesic. The reaction time of each group was determined at 30, 60,120, 180 minutes after oral administration of the tested extract and compared with that of the control non treated group and the standard one.

Writhing test:

An acetic acid –induced abdominal constriction in mice (writhing effect) was determined by the method described by **Collier *et al.*, (1968)**. For this purpose 20 mice of either sex weighing 20-25 g were divided into 4 equal groups each of 5 mice and pre-treated as following:

The group I served as control non- treated whereas those of group II and III were given orally the ethanolic extract on concentration of 200 and 400 mg/kg body weight respectively while the group IV was given orally acetyl salicylic acid at dose level of 100 mg/kg.

After 30 minutes, each mouse was injected by 0.7% of an aqueous solution of acetic acid (10 ml /kg b.wt.) and the mice were then placed in transparent boxes for observation.

The number of writhes was counted for 20 min after acetic acid injection. The number of writhes in each treated group was compared to that of the control non-treated group.

The number of writhes and stretchings was recorded and the percentage of protection was calculated as following:

$$\text{Percentage of protection} = \frac{\text{Control mean} - \text{treated mean}}{\text{Control mean}} \text{X100}$$

RESULTS AND DISCUSSION

A- Toxicological studies:

LD50:

Oral administration of the 75% ethanolic extract on ascending manner 30, 60, 90, and 120 mg/20g body weight showed no toxicological symptoms, mortalities and post-mortem changes. From this observation there is no LD50 recorded till oral administration of 6000 mg/kg body weight which corresponding to 15.5 g of air dried powdered leaves.

B- Pharmacological studies:

1- Anti-inflammatory effect:

The ethanolic extract of ***Moringa oleifera*** leaves was evaluated for its anti-inflammatory activity using Carrageenan-induced rat paw edema and the data was compared with that of control in table (1) and graph (1). Carrageenan injected subcutaneously in the plantar surface of rat's paw in all group rapidly induced a significant increase in the paw thickness.

Treatment with ethanolic extract of ***Moringa oleifera*** (200, and 400 mg/kg, orally) and diclofenac sodium (30mg/kg) had a significant effect on paw thickness after, 1, 2 and 3 hours compared to control group. It was observed that both doses of the ethanolic extracts of ***Moringa oleifera*** (200 and 400 mg/kg) exhibited an anti-inflammatory activity against carrageenan-induced hind paw edema as produced by standard diclofenac sodium (30 mg/kg) at 1, 2 and 3hours.

2-Antipyretic Activity:

Effect of ethanolic leaf extract of ***Moringa oleifera*** on rectal temperature of rats is presented in Table (2) and graph (2). The subcutaneous injection of brewer's yeast suspension markedly elevated the rectal temperature after 17h of administration. Treatment with ***Moringa oleifera*** extract in doses of 200 and 400 mg/kg decreased the rectal body temperature of the rats. Antipyretic activity of the high dose and the standard was nearly similar and better than the lower dose at 1hour but the antipyretic activity of both tested dosage (200and 400 mg/kg) and the standard were nearly similar at 2 and 3 hours. Significance was indicated by lowering the body temperature after their administration at 2 hour (37.56 ± 0.30 and 37.20 ± 0.37 and 37.44 ±0.71) of 200, 400 mg/kg of tested extract and 50 mg/kg of standard paracetamol respectively and at 3hour (37.52 ± 0.37, 37.12 ± 0.58 and 37.12 ±0.57) of 200, 400 mg/kg of tested extract and 50 mg/kg of standard paracetamol respectively.

The antipyretic effect started as early as 1hour and the effect was maintained for 3hour after their administration. Both the standard drug Paracetamol 50 mg/kg and tested drug ***Moringa oleifera*** extract were significantly reduced the yeast-elevated rectal temperature, at 1st, 2nd and 3rd hour compared to control group.

3-Analgesic effect:

Hot plate method:

The effects of ethanolic extract of ***Moringa oleifera*** on hot plate test are shown in Table (3) and graph (3). Oral administration of ethanolic extract of ***Moringa oleifera*** at different dose levels resulted in significant prolongation of the reaction time in the hot plate test. Higher dose of ***Moringa oleifera*** (400 mg/kg) producing its analgesic effect starting from 30 minutes till the end of experiment. Maximum latency time of 400 mg/kg ***Moringa oleifera*** was observed in 120 and 180 minutes (16.50 ± 2.88 and 16.67±1.86) respectively in comparison with standard paracetamol (13.50 ± 1.87 and12.00 ± 1.67) respectively and control non treated (7.00 ± 0.89 and 6.00 ± 1.26) respectively. The lower dose (200 mg/kg) starting its

effect after 1 hour and produce significant increase in latency time at 1, 2, 3 hour (13.50 ± 2.51, 13.50 ± 1.87 and 13.00 ± 3.35) respectively in comparison with control non- treated (7.50 ± 2.43, 7.00 ± 0.89 and 6.00 ± 1) respectively. On the other hand the standard parcetamol (50 mg/kg) significantly increased the reaction time in mice with maximum effects obtained at 30 min after treatment.

Writhing method:

The peripheral anti-nociceptive activity of ***Moringa oleifera*** extract was evaluated using acetic acid-induced writhing test and illustrated in table (4) and graph (4). The tested extract exhibited analgesic activity in dose dependent manner. The extract has reduced number of writhes by oral dose of 400 mg/kg with percentage inhibition of 64.09which is superior to that group treated with Acetyl salicylic acid (100 mg/kg) standard with percentage inhibition of 57.98%.

The result of acute oral toxicity (LD50) study of ethanolic leaf extract of *Moringa oleifera* showed no mortality at the maximum dose of 6000 mg/kg/body weight. In an acute oral toxicity study by Awodele *et al.,* (2012),*Moringa oleifera* leaf extract was documented to be non-lethal in animals at 6400 mg/kg body weight. In addition, Adedapo *et al.,* (2009) found that *Moringa oleifera* leaf extract was documented to be non-lethal in animals at 2000 mg/kg body weight. More so, the report of Diallo *et al.,* (2009) revealed that the aqueous extract of *Moringa oleifera leaf* is safe at dosage as high as 5000 mg/kg. These results may indicate safety of ethanolic or aqueous leaf extract of *Moringa oleifera* when administrated orally as the acute administration of 2 g/kg dose was reported to be the ceiling point for acute oral toxicity of medicinal plants. (Lu *et al.,* 1965).

This study investigated the potential activity of ethanolic extract of *M. oleifera*for its anti-inflammatory, antipyretic and analgesic effect.

Induction of edema in rat's paw by different agents as carrageenan or formalin is a biphasic response, in which the first phase is mediated by histamine, serotonin, and kinins, whereas the second phase is mediated by prostaglandins (cyclo-oxygenase product of arachidonic acid metabolism) and production of reactive oxygen species [Chen (1993) Panthong (2004)]. The ethanolic extract of *M. oleifera* at both 200 and 400 mg/kg b. wt. possessed significant reduction in paw edema at 1 and 3 hours when its potency is compared to standard diclofenac sodium. The effectiveness of the ethanolic extract to reduce edema at 1and 3 hours may attributed its antagonist effect to first phase products (histamine, serotonin, and kinins) or its antagonist effect to second phase products (prostaglandins) or its synthesis by inhibition of cyclooxygenase enzyme leadind to subsequent reduction in prostaglandins production and may attributed to inhibit liberation of the reactive oxygen species (second phase mediator) from phagocytes invading the site of inflammation and leading to tissue damage [Cross *et al.,* (1987); Winrow *et al.,* (1993) ; Parke and Sapota (1996)].

The antipyretic activity of ethanolic extract was also studied for both 200 and 400 mg/kg b. wt. doses and the potency of ethanolic extract was compared to standard paracetamol. Dewan *et al.,* (2000) reported that plants showing the antipyretic effect also possess analgesic activity. Hyperthermia is due to the infected or damaged tissue promots the formation of pro-inflammatory mediators (cytokines like interleukin 1β, α, β and TNF-α) which increase the synthesis of PGE2 near pre-optic hypothalamus area thereby triggering the hypothalamus to elevate the body temperature (Spacer and Breder, 1994). Its effect may be due to antagonizing the prostaglandins effect on hypothalamus.

This study found the ethanolic extract of the plant possesses both central and peripheral analgesic activity at both doses 200-400mg/kg.

Peripheral effect was determined by acetic acid induced writhing test, acetic acid induced writhing syndrome is due to release of endogenous substance by cyclooxygenase and lipooxygenase enzyme to the peritoneal fluid which sensitize the nerve ending in abdomen leading to its conistriction (Deraedt *et al.*, 1980); (Collier *et al.*, 1968). As it is found that the intensity of *M. oleifera* is 64.09 % when compared to standard acetyl salicylic acid 57.98% we suggest that the ethanolic extract possess it is effect by convergent mechanism to acetyl salicylic acid and NASIDs, they produce their effect by blocking the action of endogenous substance which produce pain in nerve endings by inhibiting cyclooxygenase or lipooxygenase in preiphral tissue responsible for the production of this endogenous substance.

Central effect was detected by measuring the reaction time till the mice jump or lick their paws in hot plate test. It is found that the extract produce its effect in a dose dependent manner.

The anti-inflammatory and anti-nociceptive effect of the ethanolic extract may attribute to the presence of flavonoids. As flavonoids isolated from some medicinal plants have been proven to have anti-inflammatory and anti-nociceptive effect (Duke 1992). That is related to several mechanisms produced by flavonoids as inhibiting the phosphodiesterases involved in cell activation (Duke 1992), or inhibiting the biosynthesis of prostaglandins, which are involved in various immunologic responses and are the end products of the cyclooxygenase and lipoxygenase parthways (Moroney *et al.*, 1988) or inhibiting protein Kinases (class of regulatory enzymes) leading to inhibit inflammatory processes (Manthey *et al.*, (2001) andRajnarayana*et al.*, (2001).

Table (1): The effect of *Moringa Oleifera* extract on Carrageenan induced edema (n=6).

Groups	**Dose (mg/kg bwt)**	**Thickness before induction**	**Time after measurement**			
			Zero hr	**1hr**	**2hr**	**3hr**
Control non treated	___	0.33 ± 0.02^{a}	0.67 ± 0.03^{a}	0.66 ± 0.02^{a}	0.67 ± 0.01^{a}	0.67 ± 0.01^{a}
Moringa oleifera	200	0.35 ± 0.03^{a}	0.67 ± 0.04^{a}	0.49 ± 0.07^{b}	0.48 ± 0.07^{b}	0.45 ± 0.07^{b}
	400	0.37 ± 0.04^{a}	0.70 ± 0.07^{a}	0.55 ± 0.03^{b}	0.47 ± 0.04^{b}	0.46 ± 0.04^{b}
Standard (diclofenac sodium)	30	0.38 ± 0.02^{a}	0.67 ± 0.04^{a}	0.49 ± 0.04^{b}	0.44 ± 0.06^{b}	0.45 ± 0.06^{b}

Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 (Bonferroni test)

Table (2): The Effect of *Moringa Oliefera* on Brewer yeast induced hyperthermia on rat (n=6):

Groups	Dose (mg/kg) body weight	Initial body temperature	After 17 hour of yeast	Time of measurement		
				1hr	2hr	3hr
Control Non treated	——	37.18 ± 0.88 [a]	39.52 ± 0.33 [a]	39.13 ± 0.53 [a]	38.82 ± 0.81[a]	38.67 ± 0.59 [a]
Moringa oleifera	200	37.38 ± 1.42 [a]	39.14 ± 0.65 [a]	38.20 ± 0.33 [b]	37.56 ± 0.30 [b]	37.52 ± 0.37 [b]
	400	37.56 ± 0.68 [a]	39.26 ± 0.61 [a]	37.06 ± 0.55 [c]	37.20 ± 0.37 [b]	37.12 ± 0.58 [b]
Standard (paracetamol)	50	37.20 ± 0.93 [a]	39.72 ± 0.58 [a]	37.12 ± 0.57 [c]	37.44 ± 0.71 [b]	37.12 ± 0.57 [b]

Means with different letters (a, b, c) within the same column are significantly different at P value ≤ 0.05

Table (3): The effect of *Moringa Oleifera* extract on the hot plate reaction time in mice (n=10):

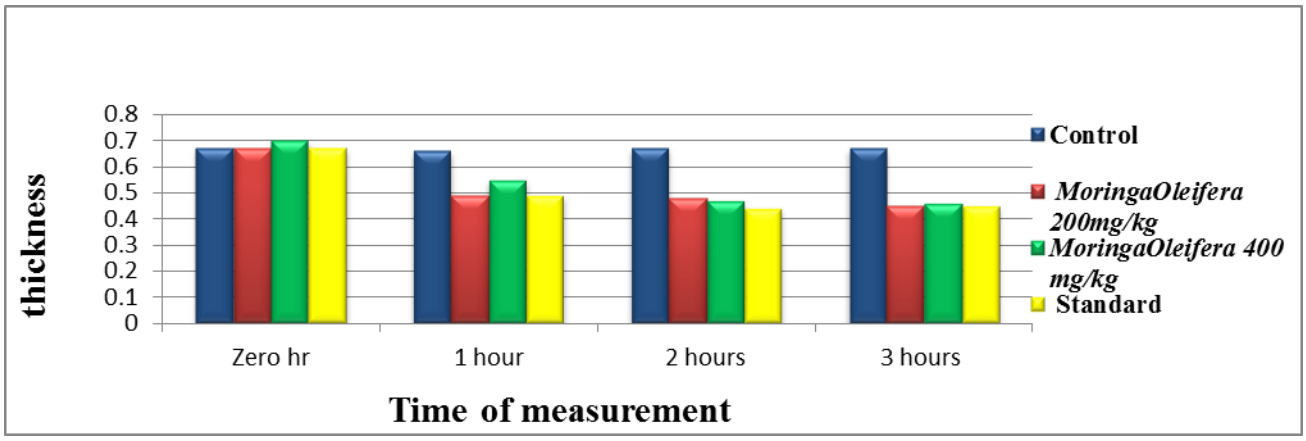
Groups	Dose in mg/kg body weight	Time of measurement			
		30 minutes	1 hour	2 hours	3 hours
Control non treated	—	9.00 ± 1.26 [c]	7.50 ± 2.43 [b]	7.00 ± 0.89 [c]	6.00 ± 1.26 [c]
Moringa oleifera	200	10.33 ± 1.37 [c]	13.50 ± 2.51 [a]	13.50 ± 1.87 [b]	13.00 ± 3.35 [b]
	400	13.33 ± 1.63 [b]	15.17 ± 4.49 [a]	16.50 ± 2.88 [a]	16.67 ± 1.86 [a]
Standard Paracetamol	50	16.00 ± 1.41 [a]	14.67 ± 1.75 [a]	13.50 ± 1.87 [b]	12.00 ± 1.67 [b]

Means with different letters (a, b, c) within the same column are significantly different at P value ≤ 0.05

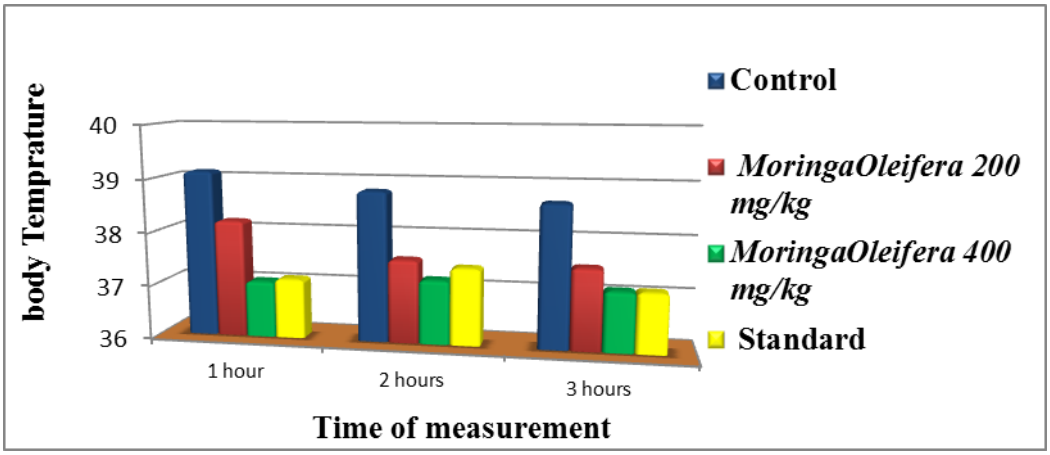
Table (4):The effect of Moringa oleifera on an acetic acid-induced writhing in mice (n=5):

Groups	Dose in mg/kg body weight	No. of writhes/20 minute	Percent of inhibition %
Control non treated	___	25.7±1.64	0
Moringa oleifera	200	14.5±0.79	43.58
	400	9.23±0.41	64.09
Standard acetyl salicylic acid	50	10.8±0.57	57.98

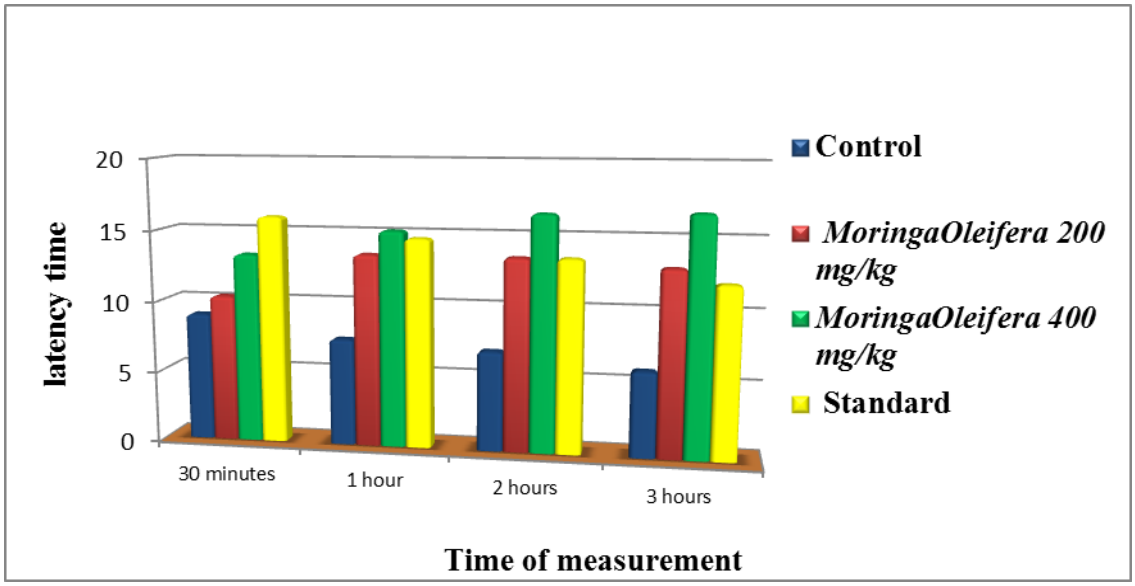
Graph (1): The effect of *Moringa Oleifera* extract on Carrageenan induced edema.



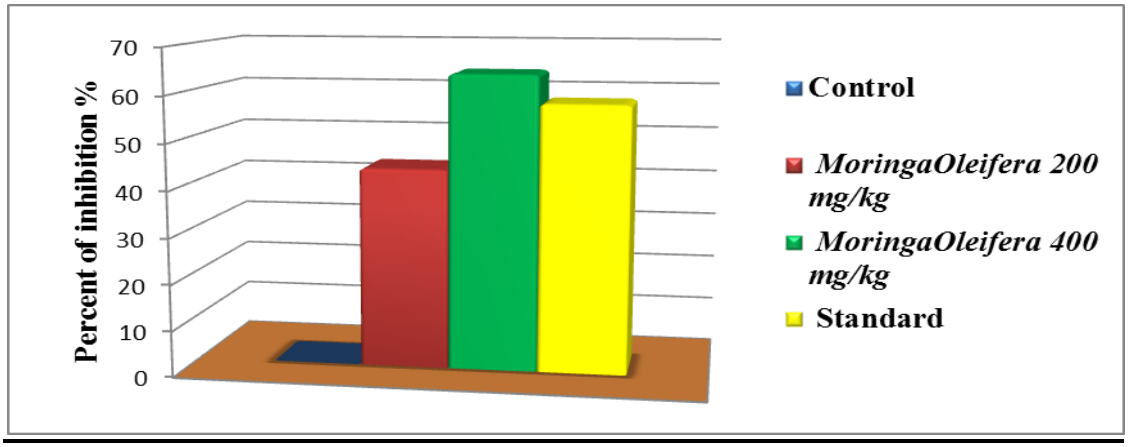
Graph (2): The Effect of *Moringa Oliefera* on Brewer yeast induced hyperthermia on rat.



Graph (3): The effect of *Moringa oleifera* extract on the hot plate reaction time in mice.



Graph (4): The effect of *Moringa oleifera* on acetic acid-induced writhing in mice.



CONCLUSION

Based on these results, we can conclude that 75 % ethanolic extract of ***Moringa oleifera*** leaves possesses anti-inflammatory, analgesic and antipyretic effects. These activities support its use in traditional medicine for the treatment of painful inflammatory conditions. This offers a new perspective for the treatment of inflammation, pain and fever. The study also recommends further investigation to isolate the most bioactive compounds responsible for each activity.

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Original scientific paper

ANTIOXIDANT CAPACITY AND PHENOLIC CONTENT OF *SALVIA* L.

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ABSTRACT

Total antioxidant capacity (TAC) and Total phenolic content (TPC) of 37 samples of sage were investigated. Values obtained by FRAP method ranged between 1.64 - 33.21 mM TE.100 g-1 dried weight (d.w.), while those obtained by DPPH method ranged between 3.40 - 32.84 mM TE.100 g-1 d.w. The total amount of phenolics ranged between 990.79 - 5136.70 mg GAE.100 g-1 d.w. among species. A positive linear correlation was observed between TAC and TPC of the extracts.

Key words: *sage, TAC, DPPH, FRAP, phenolic compounds*

Introduction

Salvia L. is a largest genus of *Lamiaceae* family, which include nearly 900 species throughout the world [1]. Sage is one of the oldest medicinal plants used by human. It is known that sage have been used since ancient times and is considered as universal panacea [2]. Many sage species are used as herbal tea and food flavouring agents, as well in cosmetics, perfumery and the pharmaceutical industries throughout world [3]. Curative properties of sage are particularly recognized since earliest times and it is used for tonic, stimulant, carminative, antiseptic and antihydrotic properties [1].

Antioxidants are considered as important nutraceuticals on account of their many health benefits and are widely used in the food industry as inhibitors of lipid peroxidation [4]. Recently, it has also been found that sage have an antioxidant effect by several researches [5, 6]. It was reported many times, that sage has excellent activities in scavenging radicals, reducing metal ions and inhibiting lipid peroxidation [7, 8].

It was found that antioxidant effects of sage are based mainly on presence of phenolic compounds. The major phenolic compounds identified in extracts of sage are rosmarinic acid, carnosic acid, salvianolic acid and its derivatives - carnosol, rosmanol, epirosmanol, rosmadial and methyl carnosale [9]. Due to the variability of environmental factors, phenolics extracted from different sage samples shown great differences in composition and consequently, differences in antioxidative power [9, 10].

The goal of present study is to determine the antioxidant activity in extracts obtained from *Salvia* species by two methods (namely DPPH and FRAP) as well as determination of total phenolic content by Folin-Ciocalteu method.

MATERIAL AND METHODS

Plant material

Assortment of *Salvia* L. is cultivated at experimental fields of Mendel University in Brno, Faculty of Horticulture in Lednice (altitude 176 m, annual average temperature 9 °C, annual average rainfall 516.6 mm, soil type loam, soil group black) and Agricultural Research, Ltd. Troubsko (altitude 277 m, annual average temperature 9.3 °C, annual average rainfall 516.6 mm, soil type loam to clay-loam, soil group black).

In 2012, 37 samples of *Salvia* in stage of full flowering cultivated in Lednice and Troubsko were harvested. Aerial parts were dried in bundles by natural air, then were all them stored in paper bags at room temperature until further analysis.

Chemicals

DPPH (2,2-difenyl-1-picrylhydrazyl), methanol, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tris(2-pyridyl)-s-triazin, HCl, acetate buffer (sodium acetate, acetic acid), $FeCl_3.6\ H_2O$, Folin-Ciocalteu reagent, sodium carbonate, destilled water

Extract preparation

Dry and pulverized plant material (5 g) was extracted by 75 % methanol. Extracts were filtered after 24 hours of extraction in room temperature with filter paper [11] and stored in refrigerator until further analysis.

DPPH method

The total antioxidant capacity was determined by DPPH method. The test is based on the capability of antioxidants to quench the radical cation DPPH+ (2,2-diphenyl-picrylhydrazyl). The purple colored radical changes to yellow-colored reduced DPPH after the reaction with a radical scavenger. DPPH solution (100 μM.l-1) was prepared in methanol. Then 3.8 ml of the DPPH solution was pipetted into tube and 200 μl of the tested extracts was added. After 30 minutes of incubation at room temperature was measured absorbance at 515 nm. The standard curve was prepared by using different concentration of Trolox. Result are expressed as mM Trolox equivalent per 100 g of d.w.

FRAP method

Principle of this method is based on capability of antioxidants contained in sample reduce a ferric complex. To prepare the FRAP reagent is needed to mix: acetate buffer (pH 3.6), TPTZ and ferric chloride in ratio 10:1:1. Then 25μl of Trolox was added to 2 ml of reagent and finnaly 100μl of sample. The absorbace was measures after 10 minutes at 593 nm. The

standard curve was prepared by using different concentration of Trolox. The results were expressed as mM Trolox equivalent per 100 g of d.w.

Determination of total phenolic content (TPC)

Total phenolics content were determinated using Folin-Ciocalteu method. Methanol extract was mixed with 9 ml of distilled water and 1 ml of Folin-Ciocalteu reagent and allowed to stand at room temperature for 5 min. Sodium bicarbonate solution (10 ml, 7%) was added to the mixture and incubated at room temperature for 90 min and the absorbance was measured at 765 nm. The total phenolic content was expressed as mg Gallic Acid equivalent per 100g of d. w.

Statistical Analysis

PC software Statistica Cz v. 12 (StatSoft) was used for statistical evaluation of the results, specifically one-way analysis of variance followed by ANOVA and Tukey's test were used. Data are expressed as means ± SD (standard deviation). The differences between individuals were significant at level $p<0.05$.

RESULTS AND DISCUSSION

TAC and TPC values shown wide variation among the samples.

The antioxidant capacity of sage extracts was determined through two spectrophotometry methods (specifically DPPH and FRAP) and results are clearly presented in Table 1 and Fig. 1, 2. Values obtained by FRAP method ranged between 1.64 and 33.21 mM TE.100 g-1 d.w., while those obtained by DPPH method ranged between 3.40 and 32.84 mM TE.100 g-1 d.w. Also many other authors [12, 13] indicate high antioxidant capacity of sage extracts.

The results of evaluation of TPC are presented in Table 1 and Fig. 3. The total amount of phenolics ranged between 990.79 to 5136.70 mg GAE.100 g-1 d. w. among species. The highest TPC was determined in extract from *Salvia tomentosa* (5136.70 mg GAE.100 g-1), while the lowest was observed in *Salvia jurisici* extract (900.79 mg GAE.100 g-1). The results obtained from TAC (both methods) and TPC showed a high degree of positive correlation, but we can say that the highest degree of correlation was observed between TPC and FRAP values. Many other authors [11, 14] reported high correlation between TPC and antioxidant capacity. The results suggest that phenolic compounds are key contributors to the antioxidant capacity of the sage extracts.

CONCLUSION

In conclusion, methanol extract of *Salvia* L. species cultivated in Lednice and Troubsko had high antioxidant capacity and good content of phenolic compounds. Extract of *Salvia jurisicii* exhibied the lowest antioxidant capacity (FRAP method 1.64 mM TE.100 g-1 d.w. and DPPH method 3.40 mM TE.100 g-1 d.w.) and also the lowest phenolic content (990.79 mg GAE.100 g-1 d.w.). Extract of *Salvia tomentosa*, in opposite, had the highest antioxidant

capacity (FRAP method 33.21 mM TE.100 g-1 d.w. and DPPH method 32.84 mM TE.100 g-1 d.w.) and TPC (5136.70 mg GAE.100 g-1 d.w.). Mainly extracts from *Salvia tomentosa*, *Salvia fruticosa*, *Salvia triloba*, *Salvia officinalis* 'Extracta', *Salvia officinalis* could be used as natural antioxidants.

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Original scientific paper

COULD MEDICINAL PLANTS OFFER ALTERNATIVES TO IMPROVE THE HUMAN IRON POOL?

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ABSTRACT

Iron deficiency remains one of the commonly occurring problems of modern nutrition, and an adequate content of this element in the diet is mandatory. It is estimated that non-haem Fe from plant-based foods accounts for over 75% of iron in the diet. The percentage is even higher for vegetarians, for which high-iron content plants must be regularly available. In our study, we proceeded to the investigation of Fe content in medicinal plants – a selection of vegetables particularly rich in various health-promoting organic micronutrients (antioxidants, chemoprotectants, regulators of digestive functions and enzyme activities). The presence of these compounds has an important additional value to any potential Fe-rich plant foodstuff proposed as natural iron supplement. Over 50 species used in modern and traditional phytotherapy were researched in the current survey. The Fe content was measured by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES). On an average, roots and rhizomes are the richest in iron (349 $\pm$ 221 mg/kg), followed by the flowering aerial parts (120 $\pm$ 110 mg/kg), leaves (107 $\pm$ 64 mg/kg), fruits (46 $\pm$ 14 mg/kg) and flowers (45 $\pm$ 19 mg/kg). Contents of over 500 mg Fe/ kg dry plant were recorded for marshmallow (*Althaea officinalis*) and dandelion (*Taraxacum officinale*) roots. The aerial parts (*herbae*) of common silverweed (*Potentilla anserina*), dandelion and vervain (*Verbena officinale*) contain over 300 mg Fe/ kg plant. Chicory herb (*Cichorium intybus*), sweet clover (*Melilotus officinalis*), linden flowers (*Tilia* sp), golden rod (*Solidago virgaurea*) are iron-poor, containing less than 50 mg Fe/ kg plant. Our study substantiates the traditional indication of vervain in the auxiliary treatment of anemia and adds both roots and upper parts of dandelion as a natural alternative for Fe supplementation. This plant is highly available from nature, does not contain significant amounts of tannins nor highly active secondary metabolites.

***Key words**: iron, medicinal plants, ICP-AES*

INTRODUCTION

Iron is an essential trace element for living organisms, due to versatile features like the ability to adjust its oxidation state, redox potential and electron spin state. Numerous proteins and enzymes containing this element have been discovered and characterized up to the present. Through hemoglobin and myoglobin, iron takes part in the distribution and utilization of oxygen; through cytochrome c, it is implicated in oxidative phosphorylations. The Fe-dependent cytochromes b5 and P450 participate in protein synthesis, catalyze the metabolism of xenobiotics, and contribute to both steroid and vitamin D3 biosynthesis. Fe-dependent enzymes, engaged in DNA synthesis, neurotransmitter formation and metabolism of fatty acids are also known [1, 2].

Taking into account the multiple metabolic implications of iron, the supply of the human organism with this element is imperative. Still, iron deficiency is the most widespread micronutrient deficiency besides vitamin A and iodide, especially affecting children [3] (WHO, 1996). The normative iron requirement at a bioavailability of 10% was estimated by the World Heath Organization to be 8 mg/day for young women, 6.5 mg/day for women aged over 50, respectively 6 mg/day for men [3]. The daily Fe intake recommended by the German Nutrition Society [4] and the National Research Council of the USA [5] represents an amount twice as large as the normative requirement: 15 mg/day for women and 10 mg/day for men. However, researches carried out in European countries show that the iron intake through food only covers the normative requirement. On an average, women have a Fe intake of 6,3-9,5 mg/day, while men take in 7,7-12 mg Fe/day [6, 7].

These findings suggest the necessity to improve the iron status. Although the pharmaceutical industry markets several preparations containing Fe salts, iron supplementation through foodstuffs has the advantage of providing a long-term, well balanced intake. While meat is an important source of dietary iron in its highly bioavailable heam form, it is estimated that non-haem Fe from plant-based foods accounts for over 75% of iron in the diet [8]. The importance of an equilibrated Fe supply becomes even more evident when considering the findings of two large epidemiological studies, reproducing the correlation between high iron stores and increased cardiovascular risk [9, 10], attributed to increased oxidative stress mediated via Fenton chemistry.

In this concept, we proceeded to the investigation of Fe content in medicinal plants, as their content in various organic micronutrients (flavones, anthocyans etc.) adds a particular value to a potential utilization for Fe supplementation. Our aim was to point out some plant products that could be employed as natural Fe supplements with high bioavailability; over 50 species used in modern and traditional phytotherapy were researched to this purpose.

MATERIAL AND METHODS

Plant material. Plant parts were gathered from the wild-growing flora of the Western Carpathians (Romania), Banat region. For each species, 8 samples were collected (n=8).The plants originated from soils with different geologic substrate: lime, granite and phyllite. Voucher specimens were deposited in the Herbarium of the Faculty of Pharmacy, Timisoara.

Analysis of iron content. The concentration of iron was assessed by Inductively Coupled Plasma - Atomic Emission Spectrometry (ICP-AES). Samples of 2-3 g plant material were heated to 105˚C in order to establish the dry mass. Matrix destruction was done after heating at

550˚C for 6 hours, until the plant material was transformed into white ash. The ash was dissolved after boiling in hydrochloric acid 10%, followed by the quantitative transfer of the content in a test-tube. The acid solutions were made up with bidistilled water to 25 mL, filtered and measured by ICP-AES without further dilution. The apparatus employed was IRIS Interprid II ICP-AES (Thermo Electron, Dreieich, Germany). The parameters of the measurement were as follows: power 1150 W; frequency 27.12 MHz; nebulizing pressure - 2.6 bar; pumping rate 1.8 L/min; number of measurements - 3; reading wavelength 240.4 nm, detection limit: 0.05 μg/L. As a first step, a semi-quantitative analysis was performed, allowing the estimation of the concentration ranges in the digestion solutions of the plant materials. The quantitative determinations were carried out based on a calibration curve established with ICP Multi Element Standard Solution XXI CertiPUR Merck, diluted to obtain optimal measurement range.

Accuracy of the data has been verified by a parallel analysis of two certified reference materials: Peach Leaves 1547 and Oriental Tobacco Leaves CTA-OTL-1.

RESULTS AND DISCUSSION

Following the analysis of 56 medicinal species, an iron content ranging from 6 mg/kg dry mass (birch tree leaves) to 993 mg/kg (marshmallow root) was measured through ICP-AES. The iron-richest organs proved to be roots and rhizomes, containing Fe amounts from 11 to 993 mg Fe/kg (table 1). Among subterranean parts, dandelion (Taraxacum officinale), primrose (Primula officinalis) and marshmallow possess the highest Fe concentration, surpassing on an average 500 mg Fe/kg.

The herbs and leaves contain significantly less ($p<0.05$) Fe than roots. In the flowering aerial parts, the median Fe concentration is 120 + 110 mg/kg. The extreme values were found in samples of sweet clover (12 mg/kg) and goose grass (512 mg/kg). In addition to the latter species, high Fe contents are displayed by Verbena officinalis, Lysimachia nummularia, Thymus pulegioides, Galium verum and Epilobium parviflorum. In leaves, the Fe concentration attains its minimum value in birch samples and its maximum in ribwort; the median iron concentration is 107 + 64 mg/kg. Constantly high Fe amounts were determined in the leaves of wild strawberries (236 + 46 mg/kg), hazel (193 + 16), wood garlic (187 + 29) and ribwort (183 + 70). On the contrary, stinging nettle, ash and blueberry leaves are relatively poor in the researched element.

The difference in the Fe content of fruits and flowers in comparison with other aerial parts is significant. The reproductive organs hold only small iron amounts: 46 + 14 and 45 + 19 mg/kg, respectively. The flowers of Filipendula ulmaria, Sambucus nigra and Verbascum phlomoides contain a higher Fe amount than the average, while hawthorn fruits are the Fe-richest products from their category.

When compared to data in the literature, the results emerged during our research on wild-growing plants from Banat region show an analogous Fe content to that of other locations: Data summarized by Kabata-Pendias and Pendias [11] point out average concentrations of 76-136 mg Fe/kg (Poland), 118-535 mg/kg (Hungary), 64-85 mg/kg (Ireland) respectively 116-253 mg/kg (Germany) in herbaceous species that make up the cover of pastures. This

situation is consistent with the good iron supply of soils from Banat region (2,5-3,0% - [12], relatively to the proportion of Fe in soils on a global average (0.5-5,0% - [11]).

The iron content of the investigated plants shows significant differences between various species, between plant organs, but also high intraspecific variations in function of the site where the samples were gathered. This is mostly related to the type of geologic substrate which influences the pH value of the soil and thus the bioavailability of Fe to the plants [13]. In our study, samples gathered from soils on limestone contain significantly less Fe ($p<0.05$) than plants originating from granite and phyllite weathering soils as the latter are more acidic. This observation is consistent with previous researches [8], measuring iron contents lower by 87% in plants grown on lime weathering soils than in those grown on granite. High intraspecific variations are best evident in case of horsetail, birch and wild thyme. On the contrary, species like Taraxacum officinale, Althaea officinalis, Geum urbanum, Primula officinalis, Allum ursinum, Coryus avellana, Fragaria vesca, Plantago lanceolata display a constantly high Fe content, which argues in favor of an iron-accumulating capacity of these plants. Surprisingly, the leaves of stinging nettle, known in literature for their high Fe content [14], proved to have only a modest Fe concentration (67+11 mg/kg GU), significantly lower than the Fe average of leaves (107+64 mg/kg).

Table 1. The iron content of medicinal plants (mg Fe/kg dry mass) (n=8)

Species	$\overline{x}$	s_x	**Min**	**Max**	**Species**	$\overline{x}$	s_x	**Min**	**Max**
Althaea officinalis	514	235	388	993	*Ononis spinosa*	91	30	59	142
Angelica archangelica	152	28	11	189	*Primula officinalis*	536	36	492	579
Cichorium intybus	118	29	85	163	*Saponaria officinalis*	287	45	236	366
Geum urbanum	403	1.3	335	460	*Taraxacum officinale*	689	62	608	782
Average Fe content of roots and rhizomes: 349 ± 221 mg/kg									
Agrimonia eupatoria	76	14	56	95	*Lysimachia nummularia*	242	46	198	304
Anthyllis vulneraria	47	8	36	57	*Lythrum salicaria*	115	21	89	135
Artemisia absinthium	126	23	93	153	*Melilotus officinalis*	18	5.7	12	25
Centaurium erythraea	61	17	42	89	*Mentha longifolia*	83	15	62	96
Chelidonium majus	82	17	68	106	*Mentha pulegium*	78	15	59	93
Cichorium intybus	42	17	21	65	*Origanum vulgare*	78	27	57	123
Echium vulgare	49	10	32	61	*Potentilla anserina*	460	52	389	512
Epilobium parviflorum	155	17	131	179	*Solidago virgaurea*	31	6	25	39
Equisetum arvense	69	60	18	81	*Taraxacum officinale*	348	87	233	430
Galium verum	180	24	154	215	*Thymus pulegioides*	133	119	31	359
Genista tinctoria	39	12	26	58	*Trifolium arvense*	70	7	62	78
Hypericum perforatum	40	10	25	53	*Verbena officinalis*	361	14	336	389
Leonurus cardiaca	69	24	45	93	*Viola tricolor*	81	21	53	103
Lycopus europaeus	113	22	85	136					
Average Fe content of herbs: 120 ± 110 mg/kg									
Allium ursinum	187	29	145	258	*Rubus idaeus*	81	9	71	91
Althaea officinalis	72	14	59	89	*Tussilago farfara*	54	10	45	67
Betula pendula	81	66	6	83	*Urtica dioica*	67	11	53	75
Corylus avellana	193	16	179	216	*Vaccinium myrtillus*	42	6	35	49
Fragaria vesca	236	46	185	237	*Viscum album*	49	9	38	57
Fraxinus excelsior	52	4	47	56	*Crataegus monogyna*	99	15	85	125

Plantago lanceolata	183	70	129	282	*Malva sylvestris*	98	11	85	113
Average Fe content of leaves: 107 ± 64 mg/kg									
Achillea millefolium	31	8	21	44	*Tilia cordata*	15	6	8	22
Filipendula ulmaria	61	6	54	70	*Tilia tomentosa*	44	7	35	53
Sambucus nigra	60	13	49	75	*Verbascum phlomoides*	58	9	46	68
Average Fe content of flowers: 45 ± 19 mg/kg									
Cerasus avium(stipites)	31	4	25	35	*Juniperus communis*	46	8	39	56
Crataegus monogyna	60	5	54	66					
Average Fe content of fruits: 46 ± 14 mg/kg									

$\overline{x}$: average iron content per species; s_x: standard deviation; Min: minim Fe content; Max: maxim Fe content; n: the number of analyzed samples for each plant part.

The distribution of iron in different organs indicates a significant difference between subterranean parts and herbs, as well as between herbs and reproductive organs (fruits and flowers). Its accumulation in roots is not mentioned by the literature, only the low mobility of the element in the plant organism is specified [15]. Highly significant differences between the Fe content of leaves and fruits have been described previously [7, 11] and can be partly explained through the involvement of Fe in photosynthesis-related reactions [16].

The present research could point out several medicinal species that accumulate larger Fe amounts. Some of these are suitable for an intake at higher amounts, as they do not contain intensely active principles like volatile oils or alkaloids. Examples in this regard are: ribwort leaves, wood garlic leaves, hazel leaves, wild strawberry leaves, dandelion herb, goose grass herb, vervain herb. However, if a plant product is evaluated as means for iron supplementation, the matter of the low Fe bioavailability from plants becomes a significant issue, due especially to the presence of tannins that precipitate this element and render it non absorbable. Administrated either as herbal teas or encapsulated powder, the bioavailability of Fe remains reduced: 10-30% in case of infusions [8], respectively of about 30% in the second case [17]. However, the bioavailability of Fe can be augmented through the concomitant administration of ascorbic and/or citric acid (lemon juice) [18].

In general, plant foods are Fe-poor: vegetables contain 3-6 mg/kg dry mass, fruits 2-4 mg/kg. Exceptions are black tea (170 mg/kg) and spices (parsley, dill, marjoram); however their impact upon the daily iron intake of humans is reduced as they are consumed in very little amounts [7]. Also, their content in other active principles make them unsuited to be consumed in higher amounts. On the contrary, plants like dandelion and wood garlic could represent good alternatives for the completion of iron intake on a natural basis, on account of their high Fe content, insignificant tannin content, absence in strongly active organic substances [19], and not at least their wide availability from nature. These findings could prove particularly useful for vegetarians, as their diet does not contain meat, the major Fe-supplying source of mixed diets [7].

CONCLUSIONS

The analysis of the iron content of 56 medicinal species shows a high Fe concentration in comparison to that of foodstuffs like vegetables and fruits. Subterranean parts are richest in iron (349 ± 221 mg/kg dry weight), followed by flowering aerial parts (120 ± 110 mg/kg),

leaves (107 ± 64 mg/kg), fruits (46 ± 14 mg/kg) and flowers (45 ± 19 mg/kg). Thirteen medicinal plants that accumulate larger Fe amounts (>180 mg/kg) could be pointed out; some of these (dandelion, wood garlic) are suitable for a consummation in higher amounts as they do not contain intensely active principles like volatile oils, alkaloids etc, nor tannins that would reduce the bioavailability of iron. Their use as encapsulated powders for food supplementation is especially recommended to vegetarians, as this type of diet is devoid of the major Fe-supplying aliments.

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Original scientific paper

FATTY ACID COMPONENTS OF SOME ENDEMIC *SIDERITIS*

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ABSTRACT

In this study, fatty acid components of some endemic *Sideritis* species seeds were investigated. These species are very important for its economical value and utilization for human health. According to the results, the most important fatty acid components in seeds of these species were linoleic acid, oleic acid and palmitic acid. The linoleic acid ratios were 57.1% by *S.condensata*; 63.9% by *S.leptoclada*; 58.3% by *S.libanotica* ssp. *linearis* and 60.4% by *S.tmolea*. The oleic acid ratios were 26.4% by *S.condensata*; 24.7% by *S.leptoclada*; 29.4% by *S.libanotica* ssp. *linearis* and 29.4% by *S.tmolea*.

Keywords: *Sideritis, endemic, seed, fatty acid, Turkey*

INTRODUCTION

The genus Sideritis (Fam. Lamiaceae) comprises about 140 species distributed in several countries of the Mediterranean region [1].

Sideritis species are a group of plants known as “mountain tea” in Turkey. Some species are used as tea, flavoring agents, and for medicinal purposes in several regions of Turkey. Infusion of aerial parts of a number of Sideritis species are used as tonics, carminatives, as anti-inflammatory agents, antispasmodics, diuretics, digestives, and in the treatment of colds [2].

In Mediterranean folk medicine, aqueous preparations of plants of this genus are considered to have antioxidant, anti-ulcer and anti-inflammatory activity [3].

Many reports are devoted to the chemical composition, as well as to the pharmacological activities of plants from this genus [4].

The value of fatty acid patterns in deducing systematic relationships among plants is becoming increasingly apparent. Many recent studies in which phylogenetic and taxonomic

aspects are considered in relation to fatty acid composition suggest fatty acids have evolutionary implications and taxonomic significance in higher plant systematics [5].

In this paper the results of analysis of fatty acid composition of nutlet lipids from the some endemic *Sideritis* species are presented.

MATERIAL AND METHODS

This study was carried out at the laboratories of the Selcuk University, Faculty of Agriculture, Department of Food Engineering, Konya, in 2013. The nutlet samples (seeds) were collected primarily in Çumra, Konya, during the summer of 2013, under cultivation conditions. The seed materials were represented by: *Sideritis condensata* Boiss. et Heldr., *Sideritis leptoclada* O. Schwarz et. P.H. Davis, *Sideritis tmolea* P.H. Davis and *Sideritis libanotica* Labill. ssp. *linearis*. The species of the collected samples are represented in table 1, below.

Table 1. The studied species, their habitat and characteristics.

Species	**Characteristics**	**Altitudes**
S. condensata Boiss. & Heldr.	branched and adpressed white-silky greyish tomentose perennial herb, up to 100 cm, growing on the pine forest and roadsides of Akseki (Antalya)	up to 1600 m
S. tmolea P.H. Davis	little-branched and shortly adpressed-tomentose perennial herb, up to 55 cm, growing on the rocky slopes of Bozdag (Izmir)	up to 1900 m
S. leptoclada O. Schwarz & P.H. Davis	simple or little branched and densely adpressed white tomentose perennial herb, up to 60 cm, growing on the Pinus brutia forest and serpentine rocks of Mugla	up to 800m
S. libanotica Labill. ssp. *linearis*	simple or branched and adpressed white-tomentose perennial herb, up to 100 cm, growing Bozkır (Konya)	up to 1500 m

From Gümüşçü (2014)

The seed samples were analysed for fatty acid components at the laboratory of Department of Food Engineering, Faculty of Agriculture, Selçuk University.

Extraction of oils

The oil extraction of dried and powdered seeds (10 g) was carried out at boiling point (34 °C) for 6 h using a Soxhlet extractor and diethyl ether as a solvent. The solvent was evaporated by rotary evaporator. The oil was esterified to determine fatty acid composition.

Fatty acids methyl esters (FAMEs) preparation

The fatty acids in the oil were esterified into methyl esters by saponification with 0.5 mol/L methanolic NaOH and transesterified with 14% BF 3 (v/v) in methanol [9].

Determination of fatty acids composition

Fatty acids in the seed oils were converted to their corresponding methyl esters according to the AOCS method [6]. In brief, 0.4 g seed oil of each variety were dissolved in 2 ml 0.5% (w/v) sodium hydroxide in methanol (KOH-MeOH) in a 25 ml flask with a ground-glass stopper, then saponification was carried out at 60°C for 15 min. After the solution was cooled to room temperature, 2 ml of 14% boron trifluoride-methanol (BF_3-MeOH) was added and esterification at 60°C was performed for 5 min. The solution was cooled again and 2 ml hexane (C_6H_{14}) and 2 ml saturated sodium chloride (NaCl) were then added to the samples. The fatty acid methyl ester (FAME) was recovered by centrifugation [7].

RESULTS AND DISCUSSION

According to the analyses, the fatty acid content of the *Sideritis* samples are represented in table 2.

Table 2. Fatty acid contents of studied seed samples.

	Samples			
Fatty acid content (%)	*Sideritis condensata*	*Sideritis leptoclada*	*Sideritis libanotica* ssp. *linearis*	*Sideritis tmolea*
Myristic acid (14:0)	0,07	0,06	-	0.06
Palmitic acid (16:0)	4,95	3,11	3.79	3.55
Stearic acid (18:0)	2,84	1,43	1.74	1.97
Oleic acid (18:1)	26,39	24,65	29,40	29.44
Linoleic acid (18:2)	57,15	63,85	58,33	60.44
Arachidic acid (20:0)	0,14	0,07	0,08	0.08
Linolenic acid (18:3)	1,67	1,35	1,47	1.37
Erucic acid (22:1)	0,23	0,05	0,09	0.06
Arachidonic acid (20:4)	0,05	0,09	0,10	0.07

According to the results (table 2), the most important fatty acid components in seeds of these species were: linoleic acid, oleic acid and palmitic acid. The linoleic acid ratios were 57.15% by *S.condensata*; 63.85% by *S.leptoclada*; 58.33% by *S.libanotica* ssp. *linearis* and 60.44% by *S.tmolea*. The oleic acid ratios were 26.39% by *S.condensata*; 24.65% by *S.leptoclada*; 29.40% by *S.libanotica* ssp. *linearis* and 29.44% by *S.tmolea*. Besides these components, there were found in the samples other fatty acids, such as palmitic acid, stearic acid, linolenic acid which are above 1%; myristic acid, arachidic acid, erucic acid and arachidonic acid which are below 1%.

The fatty acids, which are above 1% represented in figure 1; and which are below 1% represented in figure 2.

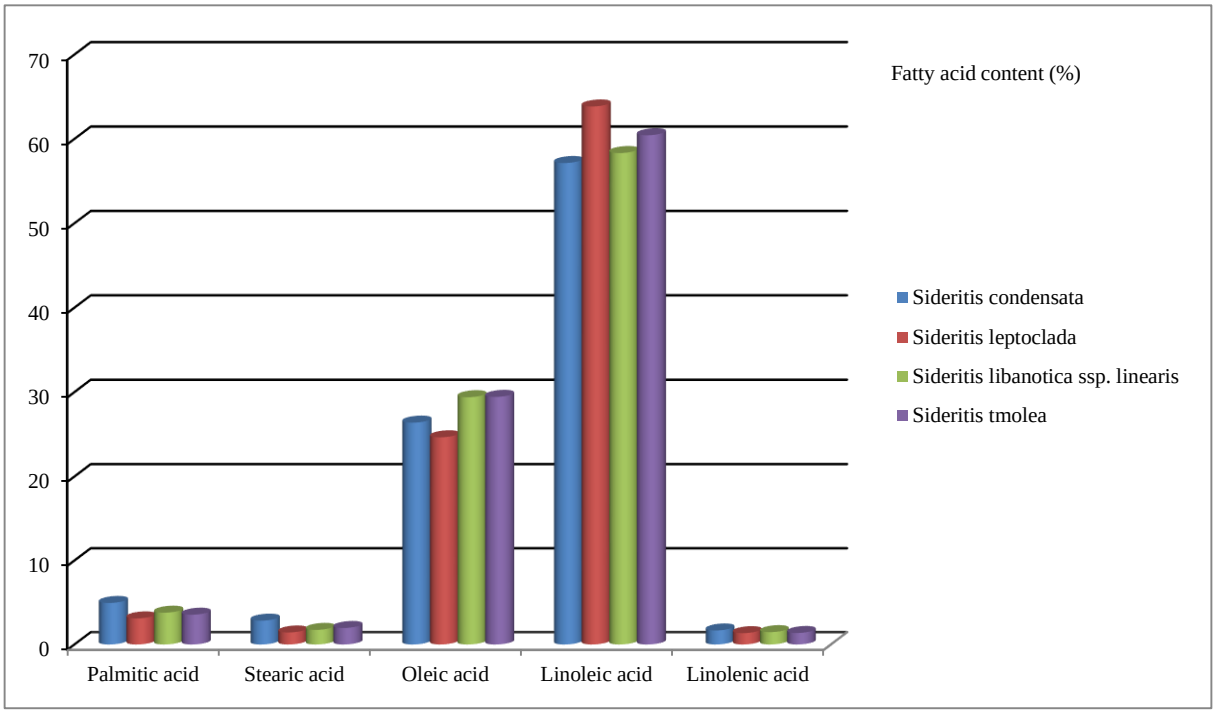

Figure 1. Some fatty acid contents of seed samples above 1% .

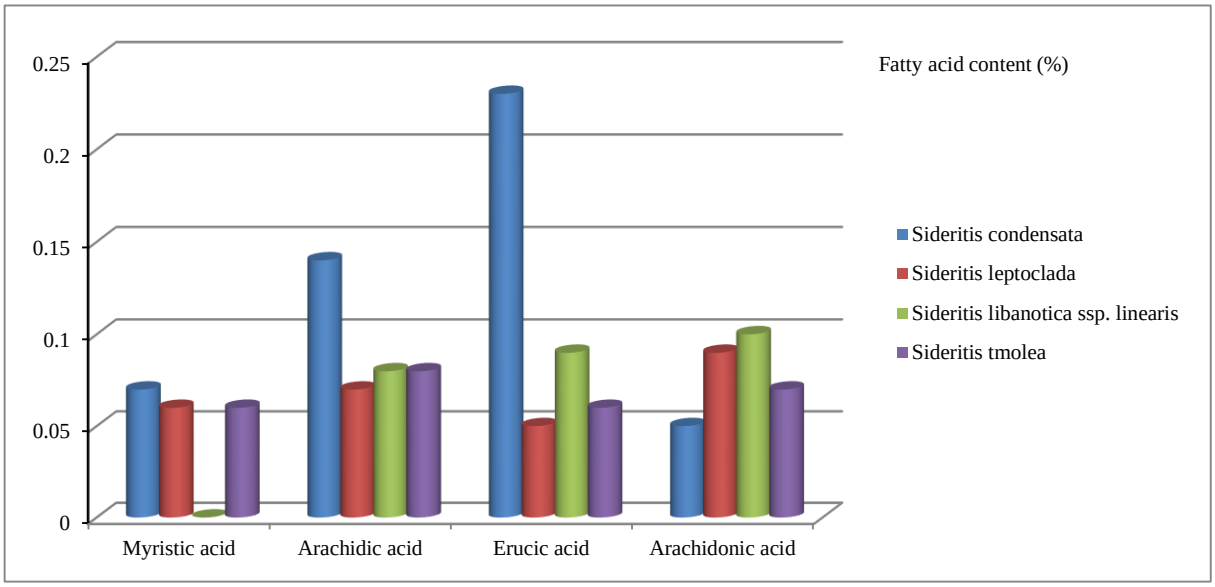

Figure 2. Some fatty acid contents of seed samples below 1% .

The dominant fatty acid in *Marrubium* and *Sideritis* (tribe Marrubieae) was octadecadienoic acid (18:2) (Linoleic acid). *Marrubium* spp. have a higher content of octadecenoic acid (18:1) (oleic acid) than *Sideritis* spp. [5]. Marin et al. [5] determined that the fatty acid components of *Sideritis hyssopifolia* were as; palmitic acid 3.0%, stearic acid 1.1%, oleic acid 11.6%, linoleic acid 83.3% and linolenic acid 1.0%; of *Sideritis montana* palmitic acid 2.9%, stearic acid 1.3%, oleic acid 13.1%, linoleic acid 81.6% and linolenic acid 1.1%.

Aboutabl et al. [1] recorded in their study that was determined the fatty acids in the aerial parts of *Sideritis taurica*; such as capric acid 42.72%, stearic acid 30.64%, myristic acid 4.41%, oleic acid 2.01%, lauric acid 1.99%, linolenic acid 1.80% and linoleic acid 1.38%.

Ertan et al. [7] *collected seeds of some Sideritis species such as S. athoa, S. brevidens, S. caesarea, S. condensata, S. congesta, S. dichotoma, S. erythrantha* var. *cedretorum, S. germanicopolitana* ssp. *germanicopolitana, S. hololeuca, S. lanata, S. libanotica* ssp. *violascens, S. lycia, S. niveotomentosa, S. perfoliata, S. phrygia, S. pisidica from different regions in Turkey. They obtained seed oils of 15* Sideritis *species by a Soxhlet apparatus using hexane. Fatty acids in the oils were converted to methyl esters and their compositions were determined by GC/MS. The main fatty acid components of the oils from all the species are linoleic (45.4-64.0%), oleic (12.3-26.5%), 6-octadecynoic (4.5-26.8%), palmitic (0.3-9.4%), and linolenic (0.8-2.0%) acids.*

Demirtas et al. [8] *found the total fatty acid content in aerial parts of Sideritis libanotica ssp. linearis as myristic 1.43%, palmitic 13.48%, linoleic 7.71%, oleic 9.23%, stearic 1.37%, arachidic 1.30% and lignoceric 2.30%. They found that* the main fatty acid content in the aerial part of *S. libanotica* ssp. *linearis* was palmitic acid.

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Original scientific paper

THE CONTENT OF SATURATED, MONOUNSATURATED AND POLYUNSATURATED FATTY ACIDS IN THE SEEDS OF DIFFERENT CANOLA VARIETIES

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ABSTRACT

Canola is a name applied to edible oilseed rape. This plant belongs to the *Brassicaceae* (mustard) family along with 3,000 other species. Close relatives of this crop have been cultivated for food since the earliest recordings of man. The name "canola" was registered in 1979 by the Western Canadian Oilseed Crushers Association to describe "double-low" varieties. Double low indicates that the processed oil contains less than 2% erucic acid ($C_{22:1}$). Like soybean, canola contains both high oil content as well as high protein content. It contains about 40% oil and 23% protein compared to 20 and 40%, respectively, for soybean consumption.

Commercial varieties of canola were developed from three species: *Brassica napus* L. (Argentine type), *Brassica campestris* L. (Polish type) and *Brassica juncea* L. (canola quality brown mustard). There are considerable differences in agronomic characteristics, yield, and fatty acid (FA) composition of seed oil between species and between varieties.

The main objective of this work was identification and determination the FA composition of the seed oil of the two canola varieties grown in the Republic of Macedonia, during 2012. For that purpose, a total of hundred samples of the seeds of the two types of canola varieties were analyzed for the presence of total saturated fatty acid (SFA), total monounsaturated fatty acids (MUFA), and total polyunsaturated fatty acids (PUFA).

The results of the study, showed different FA content among the two canola varieties. The canola variety type 2, was found to be high linolenic with the average content of linolenic acid ($C_{18:3}$) 44.0% ± 2.02. The canola variety type 1, was found to be high oleic with the average content of oleic acid ($C_{18:1}$) 59.5% ± 1.91. The average content of erucic acid ($C_{22:1}$) was below 0.2% in the both varieties.

The canola variety type 1 contained lower mean value of the total SFA (9.6% ± 0.56) in comparison with canola variety type 2, which had higher mean value of the total SFA (17.4% ± 0.67). The canola variety type 1, had higher content of the total MUFA unlike the canola variety type 2. The differences in the FA composition, as well as, the total SFA, MUFA, and

the PUFA content, the both canola varieties had similar values of polyunsaturated/saturated indexes (P/S), which were found to be 3.2, and 3.4, respectively. This means that the both varieties of the oil had the same nutritional value.

***Keywords**: Canola, fatty acid, gas chromatography, polyunsaturated / saturated index*

INTRODUCTION

Canola was developed in the early 1970s using traditional plant breeding techniques by Canadian plant breeders to remove the anti-nutritional components (erucic acid and glucosinolates) from rapeseed to assure its safety for human and animal consumption. The canola plant also produced seeds with a very low level of saturated fat, seven percent or below [1]. There is an internationally regulated definition of canola that differentiates it from rapeseed, based upon its having less than two percent erucic acid ($C_{22:1}$) and less than 30 μmol/g glucosinolates [2]. Oilseed products that do not meet this standard cannot use the trademarked term "Canola." Like soybean, canola contains both high oil content as well as high protein content. It contains about 40% oil and 23% protein compared to 20 and 40%, respectively, for soybean consumption [3].

Commercial varieties of canola were developed from three species: *Brassica napus* L. (Argentine type), *Brassica campestris* L. (Polish type) and *Brassica juncea* L. (canola quality brown mustard). There are considerable differences in agronomic characteristics, yield, and fatty acid (FA) composition of seed oil between species and between varieties. In order to develop herbicide tolerance of the canola plant, and to improve quality of the canola seed, some different innovations have been established. *Roundup Ready* and *Liberty Link* canola varieties were developed using a traditional plant breeding technique called mutagenesis. Another innovation is the development of hybrid canola varieties. Hybrids can increase yields and are increasing in acreage [4].

In the Republic of Macedonia, two different hybrids of canola varieties have been developed in the past decade. The main objective of our work was identification and determination the FA composition of the seed oil of the two canola varieties, which were grown in the Republic of Macedonia, during 2012. For that purpose, a total of hundred samples of the seeds of the two types of canola varieties were analyzed for the presence of total saturated fatty acid (SFA), total monounsaturated fatty acids (MUFA), and total polyunsaturated fatty acids (PUFA). The values of polyunsaturated / saturated indexes (P/S) were calculated for the both canola varieties.

MATERIAL AND METHODS

Materials

A total of hundred seed samples of two different hybrids of canola varieties were collected from the local producers during the 2012 (canola variety type 1, n=45; canola variety type 2, n=55).

Methods

Sample preparation

The total of 5 g of grinded samples was mixed with 5 g of anhydrous sodium sulphate (Na_2SO_4). An aliquot of 2 g was placed in Soxlet extractor system, and extraction was performed with petroleum – ether (40-69) within 24 hours. After cooling at room temperature, the solvent was evaporated to dryness in the stream of nitrogen.

Preparation of fatty acid methyl esters (FAMEs)

Fatty acid (FA) composition of the oils and fats was determined as their corresponding methyl esters. Preparation of FAMEs was carried out according to the modified ISO method (5). 0.1 - 0.2 g of oil was dissolved in 10 mL 0.2 mol/L H_2SO_4 prepared in anhydrous methanol. Esterification was performed by refluxing for 30 minutes at 100 ^{0}C in tightly sealed Pyrex tubes. After cooling at room temperature, 10 mL of petroleum ether (40 - 60) was added followed by 10 mL of deionized water, mixed gently and allowed to settle until the upper petroleum ether layer becomes clear. The distinct upper layer of methyl esters in petroleum ether was separated carefully in a capped vial and used for analysis. 2 µL of the petroleum ether aliquots were injected into the chromatographic column and peaks were recorded for their respective retention times and areas by the data processor unit of the GC. Identification of each individual fatty acid methyl ester was achieved by comparison with authentic reference standards. All solvents and standards were of analytical grade (Merck, Fluka).

Chromatography

HP model 5890 series II (plus) gas chromatograph equipped with an HP automatic liquid sampler and a flame-ionization detector (FID) was used either with a nonpolar fused silica capillary column (30 m x 0.32 mm id. x 1 µm film thickness) coated with 100% poly (dimethylsiloxane), commercially available as SPB^{TM-1} obtained from Supelco (USA). The carrier gas (nitrogen) flow rate was 1.5 mL/min and the split ratio was 1:10. The injection port was maintained at 250 ^{0}C and the FID at 280 ^{0}C. Oven temperature was set at 200 ^{0}C (1 minute) increasing for 5 ^{0}C/min. The final oven temperature was maintained at 250 ^{0}C (20 minutes). For confirmation of identified and determined FAMEs in oils and fats, a polyethylene glycol TPA modified polar column commercially available as HP-FFAP (25 m x 0.32 mm id x 0.52 µm) was used with the same HP model 5890 series II (plus) gas chromatograph. The carrier gas (nitrogen) flow rate was 1.5 mL/ min and the split ratio was 1:10. The injection port was maintained at 230 ^{0}C and the FID at 260 ^{0}C. Oven temperature was set at 180 ^{0}C increasing for 2 ^{0}C/ min. The final oven temperature was maintained at 230 ^{0}C (4 minutes).

RESULTS AND DISCUSSION

A total of 100 seed samples of two different hybrids of canola varieties were analyzed on the composition of fatty acids using gas chromatographic method. The content of following saturated and unsaturated fatty acids was tested in the samples: caproic acid ($C_{6:0}$), caprylic acid ($C_{8:0}$), capric acid ($C_{10:0}$), lauric acid ($C_{12:0}$), myristic acid ($C_{14:0}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), arachidic acid ($C_{20:0}$), behenic acid ($C_{22:0}$) , lignoceric acid ($C_{24:0}$), oleic acid ($C_{18:\ 1}$), linoleic ($C_{18:\ 2}$), linolenic acid ($C_{18:3}$), and erucic acid ($C_{22:1}$). The fatty acid percent composition of tested seeds is shown in Table 1 and Table 2, respectively. The mean of total saturated fatty acid (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and the values of polyunsaturated/saturated indexes (P/S) are shown in Table 3.

The results of the determination of FA composition (Table 1) indicate that the seeds of both canola varieties have low content of saturated FA with the predominant presence of $C_{16:0}$ and $C_{18:0}$. The level of $C_{18:0}$ in the seeds of the canola variety type 1 was 4.4%±1.4 of the total FA, and 6.9%±1.6 of the total FA in seeds of canola variety type 2. Although it is classified as a SFA, data accumulated during the past years indicate that $C_{18:0}$ is unique among the SFAs in the food supply [6]. Unlike other predominant long-chain SFA: $C_{16:0}$, $C_{14:0,}$ and $C_{12:0}$ which increase blood cholesterol levels, $C_{18:0}$ has been shown to have a neutral effect on blood total and low density lipoprotein (LDL) cholesterol levels [6,7].

Table 1. Saturated fatty acid composition of canola seeds of two varieties

Type of Canola variety	Mean ±SD									
	$C_{6:0}$ (%)	$C_{8:0}$ (%)	$C_{10:0}$ (%)	$C_{12:0}$ (%)	$C_{14:0}$ (%)	$C_{16:0}$ (%)	$C_{18:0}$ (%)	$C_{20:0}$ (%)	$C_{22:0}$ (%)	$C_{24:0}$ (%)
Canola type 1 (n=45)	<0.1	<0.1	<0.1	<0.1	<0.1	5.2±0.6	4.4±1.4	<0.2	<0.2	<0.1
Canola type 2 (n=55)	<0.1	<0.1	<0.1	<0.1	<0.1	10.5±2.5	6.9±1.6	<0.2	<0.2	<0.1

The obtained results for the unsaturated FA (Table 2) composition of the seed oil, showed the predominant presence of $C_{18:1}$ in the seed oil of canola variety type 1 which was found to be 59.5% ± 1.91 of the total FA. It is known that the consumption of $C_{18:1}$ is effective in lowering LDL cholesterol level. The predominant presence of $C_{18:3}$ (44.0% ± 2.02) was found in the seed oil of canola variety type 2. One of the most interesting yet controversial dietary approaches has been the possible prophylactic role of dietary γ-linolenic acid (GLA) in treating various chronic diseases states. This strategy is based on the ability of diet to modify cellular lipid composition and eicosanoid (cyclooxygenase and lipoxygenase) biosynthesis [8].

$C_{22:1}$ is a MUFA which is a major constituent of certain oils, such as rapeseed oil. Because it has been linked to cardiac muscle damage, oil such as canola oil that are low in $C_{22:1}$ was developed. In our studies, the seeds of the both canola varieties had low content of $C_{22:1}$.

Table 2. Unsaturated fatty acid composition of canola seeds of two varieties

Type of Canola variety	Mean ±SD			
	$C_{18:1}$ (%)	$C_{18:2}$ (%)	$C_{18:3}$ (%)	$C_{22:1}$ (%)
Canola type 1 (n=45)	59.5 ± 1.91	18.8 ± 3.5	11.9 ± 1.1	0.11±0.05

Canola type 2 (n=55)	23.2 ± 2.9	15.2 ± 3.6	44.0 ± 2.02	0.18±0.09

All samples presented total SFA content less than one fourth of the total FA content (Table 3). It is known that the excessive consumption of SFA is related to the increase of the plasmatic cholesterol and the obesity [9]. On the other hand, the consumption of PUFA and MUFA has been recommended to improve the lipid profile in relation to the saturated SFA. Yu-Poth *et al*. indicate that the rich diets in PUFA may provoke an increase in the LDL -cholesterol oxidation and the reduction of the HDL -cholesterol levels [10]. There is a tendency in increasing the recommendations of MUFA consumption, that seems not to affect the HDL levels, and also it may reduce the LDL and triacylglycerols blood levels, that make it more effective in prevention of hearth diseases. Canola oil variety 1 showed high content of MUFA (59.5 ± 1.91) and canola oil variety 2 showed hight content of PUFA (59.2 ±1.1).

Table 3. The content of SFA, MUFA, PUFA and the values of P/S indexes in two types of canola varieties

Type of Canola variety	Mean ±SD			P/S index
	SFA (%)	MUFA (%)	PUFA (%)	
Canola type 1 (n=45)	9.6 ± 0.56	59.5 ± 1.91	30.7 ± 1.7	3.2
Canola type 2 (n=55)	17.4 ± 0.67	23.2 ± 2.9	59.2 ± 1.1	3.4

The relationship between saturated and polyunsaturated FA content is expressed as P/S index. This value is an important parameter for determination of nutritional value of certain oil. Oils and fats with higher value of P/S index than 1 are considered to have nutritional value. Several studies indicate that higher value of P/S index means a smaller deposition of lipids in the body [11]. The results of our investigations (Table 3) showed that the both canola varieties had similar values of polyunsaturated/saturated indexes (P/S), which were found to be 3.2, and 3.4, respectively. This means that the both varieties of the oil had the same nutritional value.

CONCLUSIONS

The canola plant was developed in order to produce edible oil that contains much lower levels of erucic acid ($C_{22:1}$) than rapeseed oil. Canola oil derived from the seeds of the canola plant, the genetically engineered rapeseed plant or the canola hybrids, has been considered for human consumption. The types of fatty acids determine whether a vegetable oil is used for edible or industrial purposes.

In our investigations the FA composition of the seed oil obtained from two types of canola hybrids was analyzed. The canola variety type 2, was found to be high linolenic with the average content of linolenic acid ($C_{18:3}$) of 44.0% ± 2.02. The canola variety type 1, was found to be high oleic with the average content of oleic acid ($C_{18:1}$) 59.5% ± 1.91. The average content of ($C_{22:1}$) was below 0.2% in the both types.

The canola variety type 1 contained lower mean value of the total SFA in comparison with canola variety type 2, which had higher mean value of the total SFA. The canola variety type 1, had higher content of the total MUFA unlike the canola variety type 2. Besides the differences in the FA composition, as well as, the total SFA, MUFA, and the PUFA content, the both canola varieties had similar values of polyunsaturated/saturated indexes (P/S). This means that the both varieties of the oil had the same nutritional value.

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Original scientific paper

VOLATILE COMPOUNDS AND ANTIOXIDANT ACTIVITY OF PEEL ESSENTIAL OILS AND METHANOLIC EXTRACTS OF FOUR GREEK CITRUS SPECIES: *CITRUS BERGAMIA, CITRUS MEDICA, CITRUS AURANTIUM* AND *FORTUNELLA JAPONICA*

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ABSTRACT

Citrus essential oils, especially bergamot, are mainly utilized in perfumery, cosmetic, food and pharmaceutical industries, due to their flavor and bioactive compounds. The peel essential oils of four different Citrus species, namely *C. bergamia, C. medica* L. Diamante, *C. aurantium* L. and *Fortunella japonica* (Kumquats) growing in Greece were investigated. The hydrodistilled essential oils were analyzed by GC/MS. The main components of *C. bergamia* cultivars were: limonene (40.79-63.45%), linalool (4.16-32.60%) and linalyl acetate (4.34-10.36%). Limonene (51.09%) and γ-terpinene (20.26%) were the major compounds of *C. medica* peel oil, while limonene (93.06, 94.55%) and myrcene (2.76, 2.64%) were found in higher amounts in *C. aurantium* L. cv. Galata and *Fortunella japonica* essential oils.

Moreover, the total phenolic (TPC), total flavonoid (TFC) and ascorbic acid (ASC) content of peel methanolic extracts and juices were examined, in order to estimate the nutritional value of peel and juice. The antioxidant activity of the essential oils and peel extracts, was evaluated by testing their ability to scavenge 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical. In *C. bergamia* oils the DPPH scavenging activity ranged from 71.19 to 98.06%, while in *C. aurantium* L. cv. Galata and *Fortunella japonica* oils it was determined at 20.02 and 42.83%, respectively.

Key words: *Citrus, essential oils, antioxidant activity, phenols, flavonoids*

INTRODUCTION

Citrus fruits (Rutaceae family) such as oranges (*C. sinensis*), lemons (*C. limon*) and mandarins (*C. reticulate*) are some of the main crops of Greece. Except from these common citrus species that intended for fresh consumption and juice export, there are less known kinds of citrus, either as native or cultivated, such as bergamot, sour orange, citron and

kumquat, which are used for the preparation of jams, liqueurs, blends of teas, spoon sweets as condiments.

The residues derived from citrus fruits, represents the half of the whole grain [1]. They consist mainly of the bark (albedo and flavedo), which is almost a quarter of the fruit mass, the seeds, the flesh that remains after the extraction of juice and the essential oils [2], which are used as animal feeds because of their pectin content.

The fibers derived from plant tissues, are not only desirable for their nutritional value but also for their functional and technological properties [3]. In addition, the citrus fruits are considered to have better quality of these fibers from other sources, because of the presence of bio-active compounds (flavonoids and vitamin C), with antioxidant properties [4, 5].

Citrus essential oils and extracts are known for their antioxidant, antimicrobial, antiparasitic and anti-inflammatory properties, while they have been shown to possess significant bactericidal and antifungal properties as food additives [6].

The recognition of their biological activities makes them particle standards for use in the pharmaceutical industry as antibiotics, and as insecticides and pesticides as well [7, 8].

Citrus essential oils are extracted from fruit peel, leaves and flowers. The main constituent of almost all Citrus essential oils is limonene [9], with bergamot oil the only exception, where linalool and linalyl acetate are the major compounds [10]. The essential oils constitution is varying significantly among the different citrus types and varieties and is affected by the method of extraction, the season and the origin of the species.

It is well documented that citrus tissues (edible or not) are great source of flavonoids. In addition to ascorbic acid, the genus contains flavanones glycosides, such as naringin, hesperidin which are from the most important water-soluble phenols [11]. Moreover, other compounds, such as limonoids (derivatives of triterpenes), some flavones, and phenyl-propanoids, have a high antioxidant activity and affect positively human health [12]. Their ability: a) to protect the integrity of blood vessels [13], resulting in their use as supplements in patients with fragile blood vessels [14], b) to inhibit the processes of carcinogenesis [15, 16], c) to act against symptoms of menopause, similar to estrogen [17] and to reduce levels of cholesterol [18], and their pharmaceutical properties, such as anti-inflammatory and analgesic actions [19], justify the interest for these compounds. Recent studies report the effect of flavonoids against hepatitis C virus [20], burns and swellings, photoaging and carcinogenesis of the skin, caused by UVB radiation [21].

The main objective of the present study is to investigate the composition and the antioxidant activity of the essential oils of some less known Citrus species cultivated in Greece, and to determine the content of some antioxidant compounds from peel extracts and juice.

MATERIAL AND METHODS

Plant material and growth conditions

Ripe fruits were harvested from *Citrus bergamia* (cv. Du Reggio, cv. Chaniotiko and an unknown cultivar), *Citrus medica* L. cv. Diamante, *Citrus aurantium* L. cv. Galata and

Fortunella japonica (Kumquats) healthy, well-grown plants in the area of Arta (Mainland Greece), Chania (Crete) and Kerkira island. They were transported immediately to the laboratory, where they slightly washed with deionized water. The most of the fruits were used for the essential oil extraction. A part of them was cut into quarters, and split into separate parts: flavedo and juice, minted in very small particulates and transferred to a freezer (-20 °C) until their further analysis.

Essential oil content

The essential oil content (ml 100 g^{-1} of fresh peel) was determined using the European Pharmacopoeia apparatus (Clevenger-type). The fresh peel (flavedo part) was subjected to hydrodistillation for 3 h with a distillation rate of 3 to 3.5 mL/min. The essential oils obtained were dried over anhydrous sodium sulphate and stored at 4-6ºC until analyzed.

Gas Chromatography/Mass Spectrometry (GC/MS)

The essential oils were analyzed by GC/MS on a fused silica DB-5 column, using a Gas Chromatograph 17A Ver. 3 interfaced with a Mass Spectrometer Shimadzu QP-5050A supported by the Class 5000 software. Injection temperature: 260ºC, interface heating: 300ºC, ion source heating: 200ºC, EI mode: 70 eV, scan range: 41 – 450 amu, and scan time 0.50 s. The oven temperature programs were as follows: a) 55 – 120ºC (3ºC/min), 120 – 200ºC (4ºC/min), 200 – 220ºC (6ºC/min) and 220ºC for 5 min; and b) 60 – 240ºC at 3ºC/min, carrier gas He, 54.8 kPa, split ratio 1: 30. The percentage composition of the oils was computed after 3 GC runs of each sample from the peak areas without correction factors.

Identification of the GC/MS components

Identification of the constituents was based on comparison of their Relative Indices (RI) relative to n-alkanes with corresponding literature data, and by matching: a) their spectra with those of the MS libraries (NIST 98, Willey) [22] and b) the retention time (RT) of co-eluting reference compounds – peak enrichment technique (authentic samples by Roth and Sigma Aldrich).

Antioxidant compounds

Extraction

0.3 g frozen peel material (the colored part) was homogenized with 2 mL methanol 80% (v/v) in a cooled mortar at 4ºC. Then it was centrifuged at 4ºC and relative centrifugal force (RCF) at 12.000 for 20 min. The supernatants obtained were used for the assays.

Total phenol and flavonoid content

Total phenol concentration of fresh peel and juice was determined with Folin-Ciocalteu reagent using gallic acid as a standard, as described by Scalbert *et al*. [23]. Total phenolic content was expressed in mg gallic acid g^{-1} of peel fresh weight (FW) and mg of gallic acid ml^{-1} of juice. The total flavonoid content of *Citrus* peel extracts and juice was determined colorimetrically as described by Zhishen *et al.* with some modifications [24]. Rutin was used as the standard compound for quantification of total flavonoids. All values were expressed in mg of rutin g^{-1} of peel fresh weight (FW) and mg of rutin ml^{-1} of juice. Each measurement was repeated five times.

Antioxidant activity

Ferric Reducing/Antioxidant Power (FRAP) assay

The antioxidant activity was determined according to Benzie and Strain. The absorbance change was converted into a FRAP value by relating the change of absorbance of the test sample to that of a standard solution of L-ascorbic acid (1 mM) and the results were expressed in μM ascorbic acid g^{-1} of juice [25].

DPPH radical scavenging activity assay.
The total antioxidant activity was determined using the method described by Su *et al.* [26]. The antioxidant activity (%) was determined using the following formula:

Scavenging Activity (%) = {(Abs control - Abs sample) /Abs control} x100
where Abs. is the absorbance at 517 nm.

Statistical analysis
All samples were analyzed in triplicate and the results are expressed as the means. The data were analysed by Analysis of Variance (ANOVA), using the statistical package SPSS 11 17.0 (SPSS Inc. Chicago, Illinois, USA). For the correlations, the Pearson Product Moment was used.

RESULTS AND DISCUSSION

Chemical composition of Citrus essential oils
Table 1 presents the qualitative and quantitative constitution of the essential oils obtained through the hydrodistillation process, from the investigated *Citrus* species. The essential oil content was determined at 1.80, 1.52 and 3.25% for the Du Reggio, Chaniotiko and the unknown cultivars, while the peel of *C. medica* L. Diamante, *C. aurantium* cv. Galata and *Fortunella japonica* yielded 0.83, 2.66 and 1.12%, respectively.

Although bergamots are not consumed as fresh fruit, except in desserts, their essential oil is the most valuable byproduct for the perfumery. In the different cultivars of bergamot 13-15 constituents accounted for the 96.60-97.81% of the oil, with major compounds limonene (40.79-63.45%), linalool (4.16-32.60%) and linalyl acetate (4.34-10.36%), demonstrating thus two different chemotypes. The variety Du Reggio, in the oil of which the accumulation of monoterpene hydrocarbons is promoted (totally 84.11%), with limonene and γ-terpinene the main compounds and linalyl acetate in lower amounts. The essential oil of cv. Chaniotiko and the unknown cultivar, is characterized by higher amounts of oxygenated monoterpenes (totally 44.11-50.10%), with major constituents linalool and linalyl acetate, while γ-terpinene is not detected at all. Previous reports on *C. bergamia* also indicate variations in quantitative composition of peel essential oils, especially on the major compounds linalyl acetate, limonene and linalool, according to the genotype tested, while the concentration of the components is also affected by the isolation method of the essential oils [9, 27, 28]. Moreover, it has been previously reported, bergamot essential oils received by hydrodistilation, are qualitatively inferior because of the decomposition of linalyl acetate and linalool isomerization [29].

In *C. medica* L. cv. Diamante essential oil 21 components were indentified (which accounted for the 97.44% of the total oil) the main ones being: limonene (51.09%) and γ-terpinene

(20.26%). It seems that in citron essential oil higher amounts of monoterpene hydrocarbons are accumulated (totally 89.18%), while α- and β-pinene, sabinene, tr- and cis-β-ocimene, Z and E citral were detected in considerable concentrations (> 2%). Our data are in agreement with previous studies [30, 31], which reported that limonene and γ-terpinene are the major compounds of citron peel oil and consisted mainly from monoterpene hydrocarbons (72.9-95.9%), in proportion depending on the variety [32]. In a study on *C. medica* peel oil, limonene was also found to be the main component [33].

As can be seen in Table 1, the essential oils of both sour orange cv. Galata and kumquat consist almost exclusively by monoterpene hydrocarbons, with main component limonene, at concentrations 93.06 and 94.55%, respectively, while in kumquat oil the sesquiterpene hydrocarbon germacrene (1.16%) was also detected. Similar findings in kumquat peel oil was also noticed by Choi; Kontaratou et al. [34, 35] and Umano et al. [36]. In another study, Boussaada and Chemli indicated that the content of limonene of *C. aurantium* fruits essential oil from Tunisia ranged from 87 to 92.2% [37]. In addition, Caccioni et al. showed that limonene (58.6%), myrcene (1.88%), linalool (0.78%) and α-pinene (0.2%) are the main components of sour orange oil from Italy [38]. Most recently, Moraes et al. and Sarrou et al., in their research on local varieties of sour orange from Brazil and Greece, found that the major compounds of the peel oil are limonene and myrcene [39, 40].

Generally, it has been reported that limonene, despite being the main component of essential oils of citrus, does not exert an important role in their smell, while other components, although they found at very low concentrations contribute significantly in their characteristic aroma [41-43].

In summary, most of the above studies on the composition of essential oils showed qualitative and quantitative differences depending on the genotype (varieties), the age, maturity, the isolation method and bioclimatic factors. Such differences can be attributed to variations in the biosynthetic pathway, which can lead to the accumulation of a component at the expense of another [44]. However, this hypothesis needs further examination.

Antioxidant compounds

The antioxidant compounds (total phenolic, flavonoid and ascorbic acid content) and the antioxidant activity of methanolic extracts of peel and juice of the examined Citrus species are presented in Table 2. As can be seen, the peel is designated as better source of all antioxidant compounds compared to the juice. *C. bergamia*, cv. Du Reggio peel extracts presented the higher phenolic and flavonoid content (4.41 mg GAE g^{-1} FW and 2.05 mg RUT g^{-1} FW), while cv. Chaniotiko the greater amount of ascorbic acid in both parts of ~~the~~ fruits. According to our data, the phenolic and flavonoid content *of C. aurantium* cv. Galata was the highest and differed significantly from all the investigated citrus genotypes. Concerning the *Fortunella japonica,* it seems that either the peel extracts or juice have lower amount of phenolics and flavonoids, while in peel extract a remarkable concentration of ascorbic acid was observed (8.18 mM ASC g^{-1} FW). Based on the results presented in table 2, the juice of cv. Chaniotiko had the higher antioxidant activity, while kumquat had the lower one. Moreover, the results show that the total phenolic and the ascorbic acid content of the juice correlate significantly with the antioxidant activity measured by the FRAP method with a value ~~of~~ r= 0.960 and r=0.916 ($P< 0.01$), respectively. The above data indicate that phenolics and ascorbic acid may be the main antioxidant compounds of the examined Citrus species.

It has been previously reported that the content of ascorbic acid in citrus fruits can be influenced by the: cultivation techniques, maturation stage, climatic conditions, the position of the fruit on the tree, processing of fruits in different products, genotype (species-variety) and the storage conditions [45, 46]. Thus, the cultivation in tropical climates appears to limit the accumulation of ascorbic acid in fruits [47]. On the other hand, fruits in shady places contain lower concentrations of ascorbic acid than those exposed to the sun, the rootstock of the grafted variety affects significantly the ascorbic acid accumulation [48] and flavedo contains higher concentrations than the other parts of the fruit [49, 50].

The ability of peel extracts and the essential oils to act as hydrogen or electron donors in the transformation of DPPH into its reduced form DPPH-H was examined in the studied species. All the peel extracts were able to reduce the stable radical DPPH at an intermediate level (52.23-60.12%) with *C. bergamia* cv. Chaniotiko having the greatest value (Table 2). However, the results were different regarding the DPPH scavenging activity of the essential oils. The oil of *C. bergamia* cv. Du Reggio showed the highest antioxidant activity (98.06%), followed by the *C. bergamia* unknown cultivar (82, 87%), the *C. bergamia* cv. Chaniotiko (71,19%), the *Fortunella japonica* (42,83%) and the *C. aurantium* cv. Galata (20.02%).

Several reports have shown the close relationship between the antioxidant capacity and the content in phenolic compounds [51-53]. Thus, the high phenolic, flavonoid and ascorbic acid content in the fruits of some citrus genotypes, could be the main reason for its high antioxidant activity. Similar results have been reported by [9], who studied the DPPH scavenging activity of 34 citrus essential oils and reported values ranged from 11.0-39.8%, while geraniol, terpinolene and γ-terpinene showed the highest antioxidant capacity (52.6 54.3, and 87.7, respectively). In addition, according to Wei and Shibamoto, limonene has high antioxidant activity [54]. Therefore the differences observed in the antioxidant activity of citrus essential oils in our study may be the result of both qualitative and quantitative composition.

Table 1. Essential oil content (%) of 3 *C. bergamia* cultivars, *C. medica*, *C. aurantium* L. cv. Galata and Fortunella japonica.peel oils and their volatile components.

				C. bergamia			***other Citrus species***		
				% Concentration[d,e]					
Nr	**Compounds[a]**	**KIL[b]**	**KI[c]**	**Du Reggio**	**Chaniotiko**	**Unknown cultivar**	**C. medica**	**C. aurantium Galata**	**Fortunella japonica (Kumquat)**
1	α-phellandrene	931	926	-	-	-	0.83 ± 0.04	-	-
2	α-pinene	939	932	1.63 ± 0.10	0.45 ± 0.02	0.44 ± 0.03	2.01 ± 0.09	0.74 ± 0.00	0.54 ± 0.01
3	sabinene	976	971	1.09 ± 0.09	0.64 ± 0.03	0.57 ± 0.07	2.01 ± 0.09	0.74 ± 0.00	0.54 ± 0.01
4	β-pinene	980	975	6.07 ± 0.07	4.22 ± 0.24	3.51 ± 0.38	2.33 ± 0.09	0.47 ± 0.02	-
5	myrcene	991	989	2.43 ± 0.10	1.51 ± 0.01	1.58 ± 0.03	1.85 ± 0.00	2.79 ± 0.01	2.64 ± 0.03
6	α-terpinene	1018	1016	-	-	-	0.53 ± 0.00	-	-
7	cymene	1022	1021	-	-	-	0.75 ± 0.01	-	-
8	limonene	1031	1032	63.45 ± 0.67	40.79 ± 0.57	47.60 ± 0.45	51.09 ± 0.57	93.06 ± 0.04	94.55 ± 0.09
9	cis-β-ocimene	1040	1040	-	-	-	2.80 ± 0.05	-	-
10	tr-β-ocimene	1050	1048	-	-	-	3.70 ± 0.06	0.20 ± 0.02	-
11	γ-terpine	1062	1061	8.94 ± 0.33	-	-	20.26 ± 0.55	-	-
12	α-terpinolene	1088	1086	0.50 ± 0.03	-	-	1.02 ± 0.02	-	-
13	linalool	1104	1102	4.16 ± 0.47	32.60 ± 0.15	30.03 ± 0.69	-	-	-
14	citronellal	1153	1151	-	-	-	0.41 ± 0.04	-	-
15	4-terpineol	1177	1173	-	-	-	0.20 ± 0.03	-	-
16	α-terpineol	1189	1186	1.19 ± 0.04	2.50 ± 0.07	1.93 ± 0.07	-	-	-
17	nerol	1228	1226	0.19 ± 0.00	0.62 ± 0.00	0.68 ± 0.06	0.84 ± 0.22	-	-
18	citronellol	1228	1228	-	-	-	0.61 ± 0.06	-	-
19	citral-Z (neral)	1240	1238	0.15 ± 0.00	0.89 ± 0.05	0.65 ± 0.04	2.43 ± 0.15	-	-
20	geraniol	1255	1255	-	-	-	1.04 ± 0.17	-	-
21	linalyl acetate	1261	1259	4.34 ± 0.27	10.36 ± 0.14	8.53 ± 0.08	-	-	-
22	citral-E (geranial)	1270	1269	0.17 ± 0.01	1.24 ± 0.09	0.94 ± 0.05	3.49 ± 0.15	-	-

23	neryl acetate	1365	1367	0.55 ± 0.03	0.64 ± 0.02	0.53 ± 0.03	-	-	-
24	geranyl acetate	1383	1385	1.74 ± 0.14	1.25 ± 0.04	0.82 ± 0.04	-	-	-
25	germacrene	1480	1474	-	-	-	0.25 ± 0.04	-	1.16 ± 0.01
26	β-bisabolene	1509	1504	-	-	-	0.62 ± 0.03	-	-
	Total percentage[d]			96.60%	97.71%	97.81%	97.44%	97.47%	98.89%
	Essential oil content (%)			1.80 ± 0.04	1.52 ± 0.08	3.24 ± 0.13	0.83 ± 0.06	2.66 ± 0.02	1.12 ± 0.05

[a] order of elution on DB-5 column; [b] Literature Retention Indices on similar column; [c] Calculated relative to C7-C40 n-alcanes, on DB-5 column.
[d] Percentage of the total peak area. Components with percentage ≥ 0.1% are presented; [e]: ± St error.

Table 2. Total phenolic (TPC), flavonoid (TFC), ascorbic acid (ASC) content and antioxidant potential of peel essential oils, juice and peel extracts, using FRAP and DPPH methods, of *C. bergamia*, *C. aurantium* L. cv. Galata and *Fortunella japonica* fruits.

Genotype	**TPC**		**TFC**		**ASC**		**FRAP**	**% DPPH Scavenging activity**	
C. bergamia	peel (mg GAE[a] g^{-1}FW[b])	juice (mg GAE ml^{-1}J[c])	peel (mg RUT[d] g^{-1}FW)	juice (mg RUT ml^{-1}J)	peel (mM g^{-1}FW)	juice (mM ml^{-1}J)	juice (mM ASC ml^{-1}J)	peel extract	Essential oils
Du Reggio	4.41 ± 0.16	0.14 ± 0.01	2.05 ± 0.06	0.18 ± 0.01	7.33 ± 0.05	0.51 ± 0.05	38.24 ± 0.48	55.10 ± 1.37	98.06 ± 0.42
Chaniotiko	3.02 ± 0.07	0.18 ± 0.01	0.99 ± 0.05	0.07 ± 0.00	8.24 ± 0.11	1.10 ± 0.01	43.34 ± 1.82	60.12 ± 1.73	71.19 ± 1.23
Unknown cultivar	2.38 ± 0.15	0.08 ± 0.00	0.90 ± 0.03	0.09 ± 0.00	8.22 ± 0.13	0.32 ± 0.02	15.77 ± 0.33	56.23 ± 0.54	82.87 ± 1.56
other Citrus species									
C. aurantium cv. Galata	7.87 ± 0.27	0.15 ± 0.00	3.06 ± 0.03	0.15 ± 0.00	7.48 ± 0.09	1.62 ± 0.05	8.07 ± 0.07	54.48 ± 0.26	20.02 ± 0.13
Fortunella japonica (Kumquat)	1.65 ± 0.08	0.02 ± 0.00	0.09 ± 0.01	0.02 ± 0.00	8.18 ± 0.06	0.14 ± 0.00	0.66 ± 0.04	52.23 ± 0.25	42.83 ± 1.15

[a] GAE : Gallic acid; [b] FW: Fresh weight; [c] Juice; [d] RUT : Rutin; Columns represent means (n=5) ± St. Errors

CONCLUSION

The study of peel essential oils from three Greek bergamot *(C. bergamia)* genotypes demonstrated that different chemotypes occur, with one chemotype accumulating more monoterpene hydrocarbons and another one promoting the synthesis of oxygenated monoterpenes. Moreover, citron peel oil is a great source of monoterpenes hydrocarbons as well as Z- and E-citral, while limonene is the main component of *C. aurantium* L. cv. Galata and *Fortunella japonica.* The essential oils of all the bergamot cultivars examined, are characterized by a higher scavenging activity comparing those of peel methanolic extracts. The antioxidant activity of sour orange and kumquat essential oils were lower, indicating probably that other compounds, except from the volatiles, may affect the DPPH scavenging activity of the peel extracts.

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Original scientific paper

EVALUATION OF THE ANTIFUNGAL ACTIVITIES OF MACEDONIAN WILD MUSHROOM EXTRACTS AGAINST SELECTED FUNGAL STRAINS

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ABSTRACT

With the isolation of an array of active metabolites with antibacterial, antioxidant, anticancer and immunomodulatory properties from mushrooms, they have proved to be an essential asset in the search for new natural products with pharmacological activity. In this study, the antifungal activities of fifteen mushroom extracts from three Macedonian wild mushrooms: *Russula cyanoxantha*, *Suillus fluryi* and *Tricholoma acerbatum* were evaluated against *Saccharomyces cerevisiae ATCC 10231*, *Aspergillus niger I.N. 1110*, an osmophilic food spoilage mold isolated from home-made jam (fruit preserve) designated as FM4 and a xerophilic food spoilage mold isolated from white bread designated as FM5. Each mushroom was subjected to extractions with four different solvents with increasing polarity (hexane, dichloromethane, ethyl acetate and methanol), and the final concentration of all the extracts under investigation was adjusted to 10 mg/mL. The antifungal activity of each extract individually as well as the synergistic activity of a combination of the extracts were determined. A variation of the microtiter plate-based assay was employed to assess for the antifungal activity; and the minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) were calculated. 7 out of 15 extracts demonstrated mild inhibitory activity against the selected fungal strains, 4 of which proved to possess fungicidal effects as well. The most effective antifungal activity was displayed by the extracts of *Russula cyanoxantha* which inhibited the growth of *S. cerevisiae*, *A. niger* and FM5 at MICs lower or equal to 5 mg/mL, and further demonstrated fungicidal activity against *S. cerevisiae* and FM5 at MFCs lower or equal to 5 mg/mL. *Tricholoma acerbatum* showed inhibitory and fungicidal effects only against FM5, at MICs and MFCs of 5 mg/mL, while *Suillus fluryi* did not display any inhibitory or fungicidal effects against any of the investigated microorganisms. None of the extracts could inhibit the growth of the osmophilic food spoilage mold FM4. Even though the results obtained in this study may suggest that these mushrooms have limited antifungal effects, their secondary metabolites should be further investigated and may demonstrate a stronger effect against other microorganisms.

***Keywords**: antifungal activity, mushrooms, microtitre-plate assay*

INTRODUCTION

By the definition of Chang and Miles, the term mushroom entails ‘a macrofungus with a distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand’[1]. Since ancient times, mushrooms, meaning Basidiomycota, have been widely used for medicinal purposes due to their profound health promoting benefits without negative side-effects. Even though modern scientific studies on medicinal mushrooms have expanded exponentially during the last two decades, mushrooms still comprise a vast and largely untapped source of powerful new biologically active compounds. It is estimated that there are about 140,000 species of mushrooms on earth and of these only 22,000 which is around 10% constitute as known species [2]. Even among the known species, the proportion of well investigated mushrooms is very low [3]. Therefore, there is still much to explore about mushrooms properties and their potential applications as yet unstudied species hold the promise of providing new natural products. So far, there are at least 270 species of mushrooms that are known to possess various therapeutic properties [4] and hence the term “medicinal mushroom” has been coined. Among these the genera Lentinula, Hericium, Grifola, Flammulina, Coprinus and Pleurotus have demonstrated the best biological activities [5].

Mushrooms grow in highly competitive environments and need natural protective substances with antibacterial and antifungal properties to survive and protect themselves from attacking microbes. Since fungi are eukaryotes and share similar metabolic pathways with humans, it is not surprising that antimicrobial compounds can be isolated from many mushrooms that could be beneficial for humans [6]. Whereas most fungi produce very similar primary metabolites, secondary metabolites are more species–specific and products are often unique to a particular species [7]. Fungal fruiting bodies, fungal mycelium, spores or the culture fluid in which the mycelium has been cultivated may all be explored for secondary bioactive metabolites [8-12]. Investigations on the secondary metabolites of mushrooms so far have demonstrated that mushrooms accumulate a variety of pharmacologically active compounds including phenolic compounds, polyketides, terpenes and steroids. The spectrum of the detected pharmacological activities of these compounds is very broad, including immunomodulatory, antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, antiparasitic and hepatoprotective.

Considering that microscopic fungi are well known for the production of important antibiotic compounds such as penicillin, griseofulvin and fusidic acid, macromycetes should be an excellent choice in the search for new natural products with antimicrobial properties. However, even though there is some early reference to the antimicrobial activities exhibited by fungi that belong to the division Basidiomycota [12-14], it is within the last twenty years that a broader range of genera, species and isolates from within this division have been explored in more detail for antimicrobial properties [15-20].

Here, we report the antifungal activities of fifteen mushroom extracts from three Macedonian wild mushrooms: *Russula cyanoxantha*, *Suillus fluryi* and *Tricholoma acerbatum* against four fungal strains *Saccharomyces cerevisiae, Aspergillus niger,* an osmophilic food spoilage mold isolated from home-made jam (fruit preserve) designated as FM4 and a xerophilic food spoilage mold isolated from white bread designated as FM5.

MATERIAL AND METHODS

Mushrooms: Three mushroom species - *Russula cyanoxantha, Suillus fluryi* and *Tricholoma acerbatum* were used in this study, which were collected from different areas on the territory of Republic of Macedonia during the period of 2009 and 2012. The determination of the species was done during the field research and at the Mycological Laboratory within the Institute of Biology, Faculty of Natural Science, Skopje, microscopically by using reagents (Melzer`s reagent, KON, sulfovanilin etc.). Parts from the samples have been preserved in the National Mycological Collection (FUNGI MACEDONICI), while all indispensable data about the species are entered in the MACFUNGI database.

Preparation of Mushroom Extracts: The fresh fruit bodies of the mushrooms were dried in an oven and the dried materials were pulverized in a blender to get coarse powder. The extracts were obtained by continuous Soxhlet extraction with four different solvents with ascending polarity: starting with hexane, through dichloromethane, ethyl acetate and finishing with methanol. The ratio of powder to solvent was 10 g of mushroom powder to 150 mL of each solvent. These preliminary extracts were concentrated to dryness in a rotary evaporator under reduced pressure and controlled temperature (40 C°). The dried extracts were then resolved in pure dimethyl sulfoxide (DMSO), adjusting the concentration to 100 mg/mL. All the pure DMSO extracts were further diluted with sterile distilled water to a final working concentration of 10 mg/mL and these extracts were used in the evaluation of the antifungal activity of the mushrooms. Each mushroom produced five different extracts, four derived from each solvent used in the Soxhlet extraction, and a fifth extract which was a combination of the four different solvent extracts in order to account for additive, synergistic or antagonistic effects.

Fungal Strains and Cultures: The test microorganisms used in this study included four strains of fungi: *S. cerevisiae ATCC 10231, A. niger I.N. 1110,* an osmophilic food spoilage mold isolated from home-made jam (fruit preserve) designated as FM4 and a xerophilic food spoilage mold isolated from white bread designated as FM5. The *Saccharomyces cerevisiae ATCC 10231* and *Aspergillus niger I.N. 1110* fungal strains were derived from stock cultures, property of the Institute of Biology at the Faculty of Natural Sciences in Skopje, Macedonia. All the strains were identified according to their macroscopic and microscopic morphological properties. The mediums for growth and maintenance of the fungal cultures were Sabouraud Dextrose Broth (SDB) and Sabouraud Dextrose Agar (SDA). The cultures were incubated at room temperature and were transferred to fresh media every 3-5 days for the yeasts and every 5-7 days for the molds.

Preparation of Fungal Suspensions: For preparation of the fungal suspensions, inoculum of the culture was suspended into sterile normal saline solution (0.90% w/v of NaCl). The turbidity of the fungal suspension was matched to a McFarland standard and the concentration of the working fungal suspension was adjusted to $1{,}5x10^6$CFU/ml.

Resazurin solution: The resazurin solution was prepared by dissolving 270 mg of resazurin powder (Sigma-Aldrich GmbH, Germany) in 40 ml sterile distilled water.

Microtiter plate based assay: The antifungal activities of the mushroom extracts were assessed using a modified version of the microdilution techniques described by Drummond and Waigh (2000). The antifungal assay was performed by using a sterile 96-well plate and the Minimal Inhibitory Concentration (MIC) and the Minimal Fungicidal Concentration

(MFC) values were determined. All the assays were prepared using aseptic conditions. Resazurin was used as an indicator of growth for the yeast assays, while the growth in the mold assays was inspected visually. Sterile Sabouraud Dextrose Broth (SDB) was used as a growth medium in the assay. A positive and a negative control were used in each plate in order to ascertain the viability of the fungal culture and the sterility of the working conditions and solutions. Each extract was subjected to serial dilutions in descending concentrations starting from a concentration of 5 mg/mL and finishing with a concentration of 2.5 μg/mL. The microtiter plates were wrapped in sterile tinfoil in order to prevent contamination and then were incubated at room temperature for 3-5 days for the yeast assays and 5-7 days for the mold assays.

- MIC: A blue colored solution indicated growth inhibition in the test wells, while pale pink to colorless solution indicated microbial growth or absence of inhibition. The mold assays were inspected visually: a clear solution indicated absence of growth while visual indication of mycelia indicated microbial growth or absence of inhibition.
- MFC: All the extracts that demonstrated inhibitory activities were further tested for fungicidal activity. Namely, a sample from each well that tested positive for inhibitory activity was inoculated on fresh sterile Sabouraud Dextrose Agar (SDA) plates and incubated an additional 3-5 days for the yeast and 5-7 days for the molds. Absence of colonies/mycelia was regarded as positive for fungicidal activity, while growth of colonies/mycelia was regarded as negative.

All the tests were performed in triplicate.

RESULTS AND DISCUSSION

The antifungal activities of fifteen mushroom extracts of three Macedonian wild mushrooms were studied by a variation of the microtiter plate based method. The minimal inhibitory concentration (MIC) and the minimal fungicidal concentration (MFC) were used as a measure of the antifungal activity of the extracts under investigation. The results of the study demonstrated that all the mushroom extracts possess either very weak or none at all antifungal activity. Seven out of fifteen extracts demonstrated mild inhibitory effects against the selected fungal strains, four of which demonstrated fungicidal effects as well. The data relating to the antifungal activities of the extracts of each mushroom specifically is presented in Tables 1-3.

Table 1.Antifungal activity of different organic solvents of *Russula cyanoxantha* (in mg/mL)

Russula Cyanoxantha	**Hexane extract**		**Dichloromethane extract**		**Ethyl acetate extract**		**Methanol extract**		**Combination extract**	
Test Organism	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Saccharomyces cerevisiae	5	5	5	-	-	-	5	5	-	-
Aspergillus niger	-	-	-	-	5	-	-	-	5	-

FM4	-	-	-	-	-	-	-	-	-	-
FM5	-	-	-	-	-	-	-	-	-	-

*The symbol (-) means that no antifungal activity was observed

Table 2.Antifungal activity of different organic solvents of *Suillus flurryi* (in mg/mL)

Suillus fluryi	**Hexane extract**		**Dichloromethane extract**		**Ethyl acetate extract**		**Methanol extract**		**Combination extract**	
Test Organism	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Saccharomyces cerevisiae	-	-	-	-	-	-	-	-	-	-
Aspergillus niger	-	-	-	-	-	-	-	-	-	-
FM4	-	-	-	-	-	-	-	-	-	-
FM5	-	-	-	-	-	-	-	-	-	-

*The symbol (-) means that no antifungal activity was observed

Table 3.Antifungal activity of different organic solvents of *Tricholoma acerbatum* (in mg/mL)

Tricholoma acerbatum	**Hexane extract**		**Dichloromethane extract**		**Ethyl acetate extract**		**Methanol extract**		**Combination extract**	
Test Organism	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Saccharomyces cerevisiae	-	-	-	-	-	-	-	-	-	-
Aspergillus niger	-	-	-	-	-	-	-	-	-	-
FM4	-	-	-	-	-	-	-	-	-	-
FM5	5	5	-	-	-	-	5	-	5	5

*The symbol (-) means that no antifungal activity was observed

The most effective antifungal activity was displayed by the extracts of *Russula cyanoxantha* which inhibited the growth of *S. cerevisiae*, *A. niger* and FM5 at MICs of 5 mg/mL, and further demonstrated fungicidal activity against *S. cerevisiae* and FM5 at MFCs of the same concentration. *Tricholoma acerbatum* showed inhibitory and fungicidal effects only against FM5, at MICs and MFCs of 5 mg/mL, while *Suillus fluryi* did not display any inhibitory or fungicidal effects against any of the investigated microorganisms. None of the extracts could inhibit the growth of the osmophilic food spoilage mold FM4. Moreover, no synergistic activity was noticed by the combination extracts.

It is only recently that mushrooms have become of interest due to their production of biologically active secondary metabolites exhibiting a wide range of antimicrobial activities. So far, numerous mushrooms have been screened for antimicrobial activity in the search for the new antimicrobial agents. It was found that the intensity of the antimicrobial effects depended on the species of the mushroom, the concentration and the tested organism [21-25]. Generally, the fungi kingdom is considered to have weak antifungal activities [26] and, therefore, fungi have rarely been investigated for their bioactivity as antifungal agents. Numerous studies so far have demonstrated that bacteria are more sensitive to the metabolically active compounds isolated from fungi then fungi themselves. The reason for different sensitivity between the fungi, Gram-positive bacteria and Gram-negative bacteria can be due to the structural differences of the cell wall [21, 27]. The cell wall of the gram-positive bacteria consists of peptidoglucans (mureins) and teichoic acids, the cell wall of the gram-negative bacteria consists of lipopolysaccharides and lipopoliproteins, whereas, the cell wall of fungi consists of polysaccharides such as hitchin and glucan.

Generally, the observed values for all the investigated extracts against the selected fungal strains were low, which supports the hypothesis of [28] that antifungal agents are not commonly present in Basidiomycetes. [29] found in their study that *Auricularia polytricha, Corilopsis occidentalis, Daldinia concentrica, Daedalea elegans* and *Tricholoma lobayensis* exhibited various degrees of antagonistic effects against *Aspergillus niger, Aspergillus flavus, Candida albicans* and *Microsporum boulardii.* This finding is in contrast to our result where *Tricholoma acerbatum* did not exhibit any antifungal activity against *Aspergillus niger*, but this could be due the different mushroom species which possess different constituents in different concentrations. According to [30], extracts from *Lentinus crinitus* contained antimicrobial sesquiterpenes, desoxyhypnophilin and hypnophilin that were active against the fungus spores of *Aspergillus niger*, *Aspergillus flavus* and *Mucor rouxii*. Antifungal compounds have also been found in the liquid cultures of *Lentunula edodes* [28] including lenitamicin, β-ethyl phenyl alcohol, and lentin, an antifungal protein.

From the results, it is clear that the bioactive components of medicinal mushrooms differ in their affinity towards the extractive solvent used and accumulate in different organic solvents according to their polarity. It should be pointed out that the total yield of the crude extracts obtained from each of the mushroom species were relatively low and concentrations could not be adjusted to more than 100 mg/mL for the crude extracts and this could probably be due to the extraction methods employed. Furthermore, the concentration of the fungal inoculum was high as well, $1x10^6$ CFU, hence further analysis should be conducted with lower inoculum concentrations. In our study, the strongest antifungal effects were demonstrated by the hexane mushroom extracts which is in accordance to the study of [31] which reported that the most active components are generally water insoluble, hence it is expected that low polarity organic solvents would yield more active extracts.

CONCLUSION

The antifungal activities of fifteen mushroom extracts from three Macedonian wild mushrooms: *Russula cyanoxantha, Suillus fluryi* and *Tricholoma acerbatum* were screened against four selected microorganisms: *Saccharomyces cerevisiae ATCC 10231, Aspergillus niger I.N. 1110,* an osmophilic food spoilage mold isolated from home-made jam (fruit preserve) designated as FM4 and a xerophilic food spoilage mold isolated from white bread

designated as FM5. Seven out of fifteen extracts displayed weak inhibitory effects at concentrations of 5 mg/mL, four of which showed fungicidal effects as well. The remaining eight extracts did not show any antifungal activity and did not inhibit the growth of the fungal mycelia at all. The most effective antifungal activity was displayed by the extracts of *Russula cyanoxantha* against *S. cerevisiae, A. niger* and FM5, followed by *Tricholoma acerbatum* which showed inhibitory and fungicidal effects only against FM5, while *Suillus fluryi* was completely ineffective against all the tested microorganisms. Having in mind that the production of pharmacologically active secondary metabolites by mushrooms is species and method dependent, the utilization of a different extraction method or the evaluation of the mushroom extracts against different microorganisms should be further investigated and may demonstrate a stronger effect.

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Original scientific paper

CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF ESSENTIAL OILS FROM ALBANIAN MEDICINAL PLANTS.

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ABSTRACT.

Plants contain different secondary metabolites which can function as growth inhibitors of fungi. Aromatic compounds of essential oils may be used to control postharvest pathogens since they can be effective and posses no negative effects. Their volatility makes these compounds appropriate for use in their volatile phase. The aim of this work was to study the chemical composition of six commercial essential oils and their antifungal activity against *Penicillium expansum* pathogen. The chemical profiling of the essential oils was studied by GC/MS and allowed identification of different compounds. The antifungal activity was conducted on inhibition of the colony growth (0.28g/L air). *In vitro* experiments demonstrated that essential oils of *Thymus* spp, *Origanum vulgare* and *Satureja montana* inhibited the colony growth of the pathogen (100% inhibition) after seven days at 24°C, while *Salvia officinalis*, *Laurus nobilis* and *Juniperus communis* showed low antifungal activity. The activity of essential oils was fungistatic. *Thymus* spp, *O. vulgare* and *S. montana* are rich in phenolic compounds (thymol, *p*-cymene and carvacrol) and these can explain their higher antifungal activity compared with other oils.

Keywords*: essential oil, phenolic compounds, antifungal, P. expansum.*

INTRODUCTION

Blue mould caused by *Penicillium expansum* Link. is one of the main diseases of apple and other pome fruits in postharvest. Developmet of *P. expansum* in fruits can produce the mycotoxin patulin which is cytotoxic [1] and can cause immunotoxic [2] and neurotoxic effects in animals [3]. The main method to control this pathogen is the application of fungicides. *P. expansum* has a high frequency to develop resistant strain to thiobendazole [4] one of the main pesticides used to control the pathogen. Some other fungicides seem to have promotion activity of the mycotoxin production by some isolates of *P. expansum* [5]. A possible residue level of this fungicides and problems related to reduced effectives due to resistant strains are the main constrains to the use of the fungicides. Alternative methods to pesticide control have been tested against postharvest pathogens of fruits and vegetables and *P. expansum* in particular.

Essential oils are a safe alternative to control *P. expansum* in postharvest since they are effective in volatile and contact phase, biodegradable and more acceptable from the consumers [6]. Essential oils are complex volatile compounds produced from plants [7] which can have fungicidal, bactericidal, viricidal and insecticidal properties [8,9]. Complex chemical constituents of essential oils can reduce the possibility of the pathogen to develop resistant strains.

The objective of this research was to study the chemical composition of essential oils obtained by steam distillation from different Albanian medicinal plants and their potential antifungal activity on *P. expansum*.

MATERIAL AND METHODS

Essential oils from *Thymus* spp., *Origanum vulgare, Salvia officinalis, Laurus nobilis, Juniperus communis* and *Satureja montana* extracted by steam distillation, were furnished by "Xherdos Herbs" company Maminas, Durrës. The pathogen used in this study *P. expansum* was isolated from infected apple with typical symptoms of the disease (blue masses of spores) on PDA (Potato dextrose agar) medium. The isolates were identified morphologically and grown in pure culture (PDA) for 5-7 days prior to *in vivo* experiments.

Gas Chromatography-Mass Spectrometry (GC-MS).
Essential oil analyses were performed on a Shimadzu GC-2010-GCMS-QP2010 system operating at 70eV. This was equipped with a split/splitless injector (230^{o}C) and a fused silica HP-5 MS capillary column (30m x 0.25mm i.d., film thickness 0.25μm). The temperature program was from 50^{o} C to 290^{o} C, at a rate of 4^{o} C/min. Helium was used as a carrier gas at a flow rate of 1.0 mL/min. Injection volume of each sample was 1 μL. Retention indices for all compounds were determined according to Van den Dool and Kratz [10], using n-alkanes as standards. The identification of the components was based on comparison of their mass spectra with those of NIST21 and NIST107 [11], and by comparison of their retention indices with literature data [12].

In vitro antifungal activity test.
The antifungal activity of *Thymus* spp., *O. vulgare, S. officinalis, L. nobilis, J. communis* and *S. montana* essential oils were tested on colony growth using the methods given by Soylu *et al*. [13] and Shao *et al*. [14] with some modifications. Sterile plastic Petri dishes (90x15mm) with PDA medium were inoculated with mycelial discs (4mm) obtained from the edges of actively growing colony. Sterile filter paper discs (14 mm diameter) were attached to the inner surface of each Petri dish lid. The amount of 20 μl (0.28g/L) of each essential oil was added onto the filter paper, and the dishes were quickly covered. The Petri dishes were wrapped with parafilm along the rim to inhibit the release of volatile components. The compounds were allowed to volatilize inside the Petri dishes spontaneously at 24°C for three hours before the parafilm was removed. Controls were prepared similarly with the exception of the volatile treatment. Treatments were carried out with three replications. The efficacy of the treatment was evaluated by measuring the average of two perpendicular diameters of each colony.

Percentage mycelial inhibition = [(*dc* − *dt*)/*dc*] × 100, where *dc* is the mean colony diameter for the control sets and *dt* is the mean colony diameter for the treatment sets was calculated. All tests were repeated two times.

Statistical analysis.
One-way analysis of variance (ANOVA) was used to determine the statistical significance; P values ≤ 0.05 were considered significant. The percentage data were Arcine-square-root transformed prior to statistical analysis. The means were separated by Duncan's multiple range test. The data were statistically analyzed using the software package STATISTICA 6, StatSoft Inc, Tulsa, USA.

RESULTS AND DISCUSSION

Chemical analysis.
GC/MS analysis of essential oils identified different compounds. In **table 1** are shown the main compounds and their retention times of each essential oil. The main compounds of *O. vulgare* essential oil were p-cymene, carvacrol and γ-terpinene. *Thymus* spp. and *S. montana* essential oil have as main compound p-cymene and thymol. The oil of *S. montana* in addition had also carvacrol as main compound. The essential oil of *S. officinalis* have as main compound cis-thujone, trans-thujone while the essential oil of *L. nobilis* have 1,8-cineole and linalool as major compound. α-pinene and limonene were the main compounds of *J. communis* essential oil.

Table 1. Main compounds identified in each essential oil.

Compounds	RI[a]	*O. vulgare*	*Thymus* spp.	*S. montana*	*S. officinalis*	*L. nobilis*	*J. communis*
α-pinene	932						+
p-cymene	1025	+	+	+			
1,8-cineole	1026					+	
limonene	1028						+
γ-terpinene	1045	+					
linalool	1090					+	
cis-thujone	1100				+		
trans-thujone	1115				+		
thymol	1288		+	+			
carvacrol	1299	+		+			

[a]RI = Kovats index on HP-5 columm

In vitro antifungal activity.
The colony diameter (mm) of artificially inoculated *P. expansum* was measured after three, four, five, six and seven days on Petri dishes treated with essential oils and control (water) incubated at 24°C. Also three plates infected with mycelium of the fungus and not treated (without essential oil and water) served as control. In **figure 1**, the inhibition of colony diameter in percentage of essential oils is shown. Essential oils of *Thymus* spp., *O. vulgare* and *S. montana* gave 100% inhibition of mycelial growth after all the days that they were

measured. Essential oils of *S. officinalis* and *L. nobilis* showed low mycelial inhibition, with 27% (38.7 mm) and 27.7% (38.3 mm) respectively, compared to control (53 mm). The oil of *J. communis* showed low mycelial inhibition 5.3 % (52.3 mm).

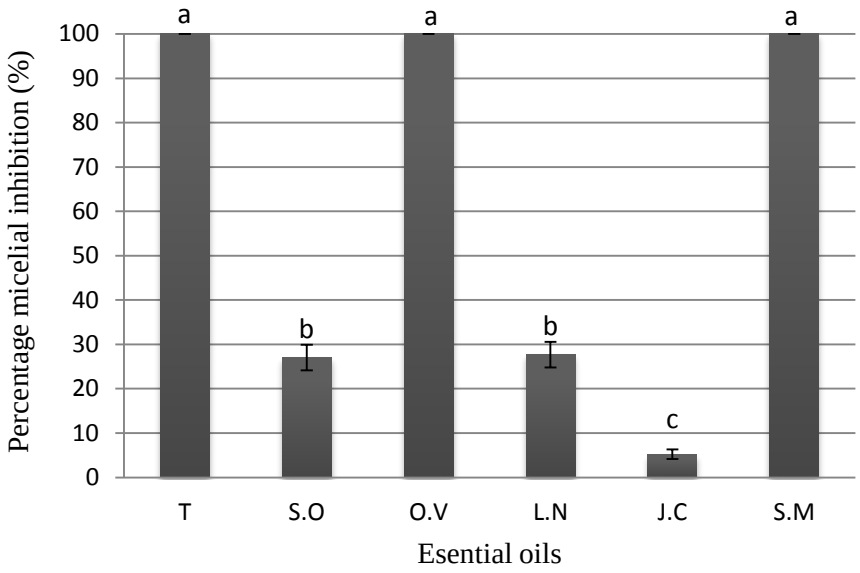

Figure 1. Inhibition of mycelial growyh from essential oils after seven days. *Thymus* spp. (T), *S. officinalis* (S.O), *O. vulgare* (O.V), *L. nobilis* (L.N), *J. communis* (J.C), *S. montana* (S.M). Bars in graphic represent the mean value of three replicates (± standard deviation). Different letters indicate significant differences at $P \leq 0.05$ according to Duncan's multiple range test.

In **figure 2**, the mycelial growth treated with essential oils after seven days from inoculation is shown.

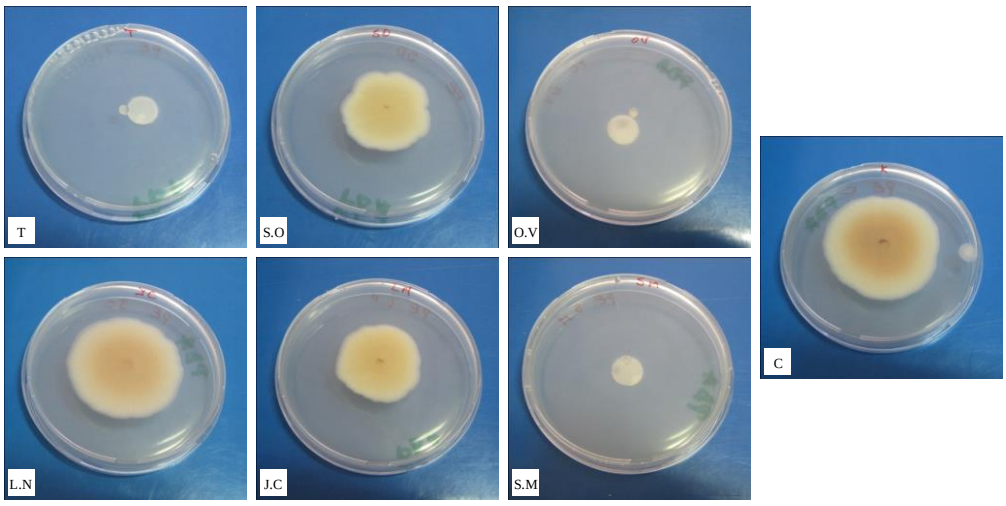

Figure 2. Growth of *P. expansum* at 24°C treated with essential oils and control (C). *Thymus* spp. (T), *S. officinalis* (S.O), *O. vulgare* (O.V), *L. nobilis* (L.N), *J. communis* (J.C), *S. montana* (S.M).

To determine whether the activity was fungitoxic or fungistatic, the mycelial plugs of each Petri plate was transferred after seven days in new PDA medium Petri dishes without oil. Two days later, the fungus started to grow so the essential oils of *Thymus spp., O. vulgare* and *S. montana* are considered as fungistatic in volatile phase.

Essential oil of *Thymus* spp. has been widely studied and they are shown to be fungicidal and fungistatic. *T. spathulifolius* essential oil inhibited the growth of different plant pathogens including *Penicillium* spp. [15]. Thymol and essential oil of *T. vulgaris* [main components *p*-cymene (36.5%) and thymol (33.0%)] showed fungicidal and fungistatic activity in different fungal species [16]. Different studies attribute the antimicrobial activity of essential oils to their main phenolic components [17-19]. Our chemical analysis of essential oil shows that the main compounds are p-cymene and thymol and the antifungal activity can be attributed to these compounds.

Salvia officinalis oil has been studied for its antifungal properties against different *Fusarium* isolates [20]. In *in vitro* experiments, this oil altered the hypae morphology and inhibited the growth of hyphae tips of *Alternaria alternata* [21]. However experiments with this essential oil on *P. digitatum* showed a promotion of the colony growth [22]. In our experimental conditions this oil gave fair results in controlling the growth of the *P. expansum* colony.

The main components of *Origanum vulgare* essential oil showed antifungal activity on various fungal pathogens [23,24]. Tested against green mould of citrus fruits caused from *P. digitatum* this oil have blocked the growth of germinative tube and altered the morphology of the hyphae [25]. An increasing quantity of carvacrol and γ-terpinene is correlated with high antifungal activity on pathogenic fungi [26]. In our studies carvacrol, γ-terpinene and p-cymene were the main compounds of this oil.

The essential oil of *Laurus nobilis* extracted with CO_2 in supercritical phase, only at high doses inhibited the growth of *P. digitatum* in contact phase [27]. The main antifungal inhibitory compound of *L. nobilis* essential oil was determined as 1,8-cineole [28]. However in both these experiments a low antifungal activity was observed and higher doses were required to reduce the fungal growth. In our experimental condition the low antifungal activity is in accordance with [27, 28] moreover the mode of application (volatile phase) can explain this fair activity of *L. nobilis* essential oil.

Juniperus communis essential oil tested in *in vitro* conditions has shown varying degree of antifungal activity [29, 30]. This oil in volatile phase didn't show antifungal activity on postharvest pathogens growth [24]. Also in the present study this oil gave the lowest antifungal activity against the blue mould.

The essential oil of *Satureja montana* is tested against different field plant pathogens [31] and postharvest pathogens like *Monilinia laxa*, *Botrytis cinerea* [32] and is shown to have antifungal activity.The antimicrobial activity of this essential oil is attributed to phenolic compounds like thymol; especially carvacrol and his precursors *γ*-terpinene and *p*-cymene [*33, 34*]. Thymol, carvacrol and p-cymene are the main compounds identified in our essential oil of *S. montana.*

CONCLUSION

In this study the steam distilled essential oils from Albanian medicinal plants presented different chemical composition. *In vitro* experiments demonstrated that *T. vulgaris, O. vulgare, S. montana* essential oils inhibited the growth of the fungus while the other oils showed low activity on the growth of the fungus. The antifungal activity of oils was determined as fungistatic rather than fungicidal. *Thymus* spp, *O. vulgare* and *S. montana* are rich in phenolic compounds (thymol, *p*-cymene and carvacrol) and these can explain their higher antifungal activity compared with other oils.

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Original scientific paper

PROPOLIS PROFILING OF SAMPLES FROM SOUTHWESTERN ROMANIA (LUGOJ REGION): NEW DATA

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ABSTRACT

Propolis represents an important component of modern apitherapy, due to its multiple biological effects: antimicrobial, anti-inflammatory, local anesthetic, antioxidant, immunomodulating, chemoprotectant, hepatoprotective. These effects are attributed to plant metabolites like flavonoids, phenolic acids, terpenes, tannins, polysaccharides, mostly present in the bud exudates of various trees. Data on the propolis profile from different European regions are currently emerging (Mediterranean, Sicily, Greece); they are obtained through GC-MS analysis of selected compound classes. The aims of our study were: i) to point out specific constituents found in Romanian propolis (performing GC-MS analysis of hexane extracts), and ii) to obtain UV spectra of propolis extracts in different solvents – providing thus a tool for the standardization and comparison to other Romanian propolis types. Among the identified compounds we cite : triterpenes (13,14- Epoxyursan-3-ol acetate, Olean-12-ene-3,15,16,21,22,28-hexol), sesquiterpenes (1,4-Methanoazulen-3-ol, decahydro-1,5,5,8a-tetramethyl-[1S-(1α, 3β, 3aβ, 4α, 8aβ)), steroids (ethyl iso-allocholate), carotenoids (3,3',4,4'-Tetrahydrospirilloxanthin), cyclohexenone derivatives (3-Cyclohexen-2-on-1-carboxylic acid, 1-methyl-3-(tetrahydrofuran-2-on-4-yl) methyl, methyl ester)). The above constituents can be considered marker compounds for propolis of SW Romanian origin. The comparison with UV spectra obtained for other Romanian propolis [1] places the currently analyzed samples in a high-flavonoid propolis group.

***Keywords**: propolis, triterpenes, GC-MS*

INTRODUCTION

Propolis represents an important bee product used for centuries in therapy due to its therapeutic effects: antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, immunostimulatory, local anesthetic, chemoprotectant, hepatoprotective, hypoglycemic [2-7].

Due to its multiple uses in medicine many studies were developed for propolis composition, functions and mechanism of action. The chemical composition of propolis varies depending on its origin and influences its therapeutic action. Bud exudates from poplar, birch, pine,

alder, willow, beech, horse chestnut represent the main source for bee glue [8]. Bees mix it with wax and their own substances forming a resinous complex used to protect their hive [9]. The composition of propolis depends on the geographic and climatic conditions of the place of collection, being influenced by the local flora. The differences in propolis composition make difficult the standardization and the quality control of this natural product [10].

The European propolis chemistry has been well studied but there are still surprises concerning the plant origin and chemical composition [11]. In the temperate zone, the resinous exudates of the buds of poplar trees represents the main source of bee glue [12] and it was possible to observe similarities between the chemical composition of propolis and that of buds from *Populus* sp. suggesting propolis origin [13]. There is the assumption that the chemistry of propolis could influence its biological properties [14, 15], so propolis of different origin could present different therapeutic actions.

The principal components of propolis are flavonoids, aromatic acids, diterpenic acids, phenolic compounds, aldehydes, amino acids, waxy acids, sugars. New compounds are listed as first discovered in the analysis of various types of propolis from different regions [2, 15, 16,].

Recent studies tried to establish the propolis compounds that are responsible for the biological activity and their mechanism of action. Due to its complex composition it is still difficult to point out all the compounds and the differences of therapeutically action of different origin propolis.

MATERIAL AND METHODS

Propolis sample was harvested from an apiary in the area of Lugoj, Timiş county. The solvents used for the extracts were spectroscopic purity ethanol analytical purity anhydrous glycerin and chromatography purity hexane. UV absorbtion spectra were realized with a spectrophotometer Spectronic-300 with double optical path . Fused quartz cuvettes of 1 cm were used.

The ethanolic extract was obtained by dissolving propolis in ethanol, stirring periodically and storage in dark place for two weeks. For the glycerin extract the sample was heated during the two weeks. Hexane extract spectra were recorded after dilution with hexane while ethanolic and glycerin extracts spectra were recorded after dilution with ethanol. Recording of spectra was performed with scanning speed of 30 nm/min.

The GC-MS analysis was performed with a Bruker GC-MS (GC 450 / 320 MS, auto sampler HP 8400), non polar column VF 5 MS, 50m, 0,22 mm i.d., 0,25 μm film thickness. The temperature was programmed starting with 60° C increasing to 220 °C, at a rate of 10° C/min. Helium was used as a carrier gas, flow rate 2,01 ml/min. The split ratio was 1:10, the interface temperature 220 °C and the ionization voltage 70 eV.

The identification was accomplished by comparing the obtained spectra with those from NIST MS 2.0 data library.

RESULTS AND DISCUSSION

The chemical composition of a propolis sample from the southwestern Romania was analyzed by CG-MS and the UV spectra of propolis extract in different solvents were recorded. Data on propolis chemical composition from different European regions are currently emerging (Mediterranean, Sicily, Greece). New compounds have been identified and it is believed that they represent marker compounds for the propolis collected from those regions [17]. Propolis from Greece and Sicily has a specific diterpenic profile. Flavonoids pinocembrin, pinobanksin, chrysin, galangin were the main compounds identified in European propolis [11,17,18]. Propolis from Brazil is rich is phenolic compounds and diterpenes that are responsible for its therapeutic actions [19].

There is a great interest in the standardization of propolis and the need to characterize the content of it to indicate the compounds responsible for the therapeutic activity. The phenolic compounds and the terpenes found in propolis seem to be responsible for its antibacterial action.

GC-MS is the most used technique for determination of propolis composition [5, 15, 20]. Our study identified by GC-MS in propolis sample the following compounds: triterpenes (13,14- Epoxyursan-3-ol acetate Fig. 2(b), Olean-12-ene-3,15,16,21,22,28-hexol Fig. 2(d)), sesquiterpenes (1,4-Methanoazulen-3-ol, decahydro-1,5,5,8a-tetramethyl-[1S-(1α, 3β, 3aβ, 4α, 8aβ) Fig. 2(a)) steroids (ethyl iso-allocholate Fig. 2(e)), carotenoids (3,3’,4,4’-Tetrahydrospirilloxanthin Fig. 2(f)), cyclohexenone derivatives (3-Cyclohexen-2-on-1-carboxylic acid, 1-methyl-3-(tetrahydrofuran-2-on-4-yl) methyl, methyl ester) Fig. 2(c)). Triterpenes, sesquiterpenes and steroids were also identified in propolis by other studies [5, 8, 15, 16, 21]. Ethyl iso-allocholate (Fig. 2(e)) is a bioactive compound with antimicrobial, diuretic, anti-inflammatory and antiasthma activity isolated in extracts from other plants [22].

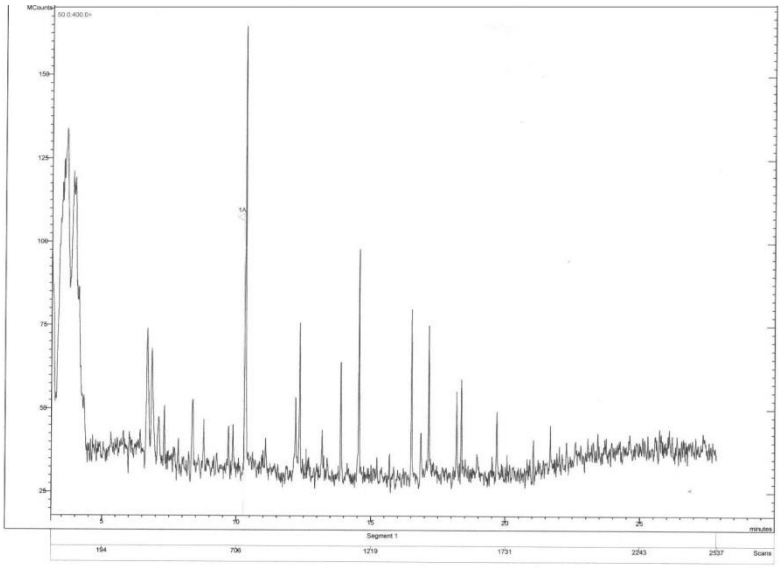

Fig.1. Gas chromatogram of hexanic extract of propolis

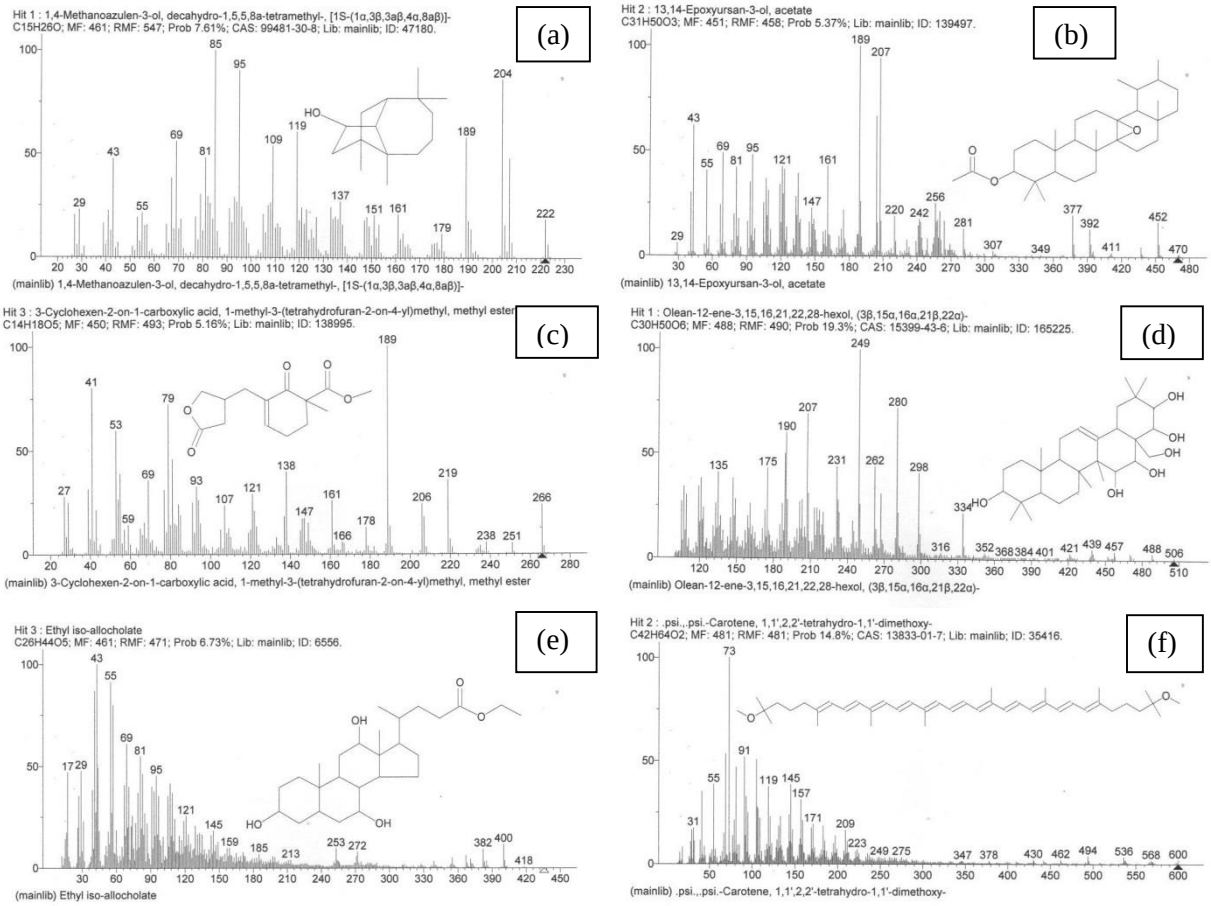

Fig. 2. Mass spectra of isolated compounds

There were compounds in the sample that could not be identified because of the lack of data library spectra.

Flavonoids are main constituents of propolis and their proportion varies according to the type of propolis. The UV spectra obtained (Fig. 3 (a-d)) shows λ_{max} at 295 nm and comparing the results with those from literature we can place the currently analyzed sample in a high-flavonoid propolis group (5%-8% flavonoid content). According to Mărghitaș et al. propolis with this flavonoid content has a high radical scavenging activity (more than 18%) [1]. There are differences between ethanolic, glycerinic extract and hexane extract. This could mean that these solvents could extract different compounds from propolis.

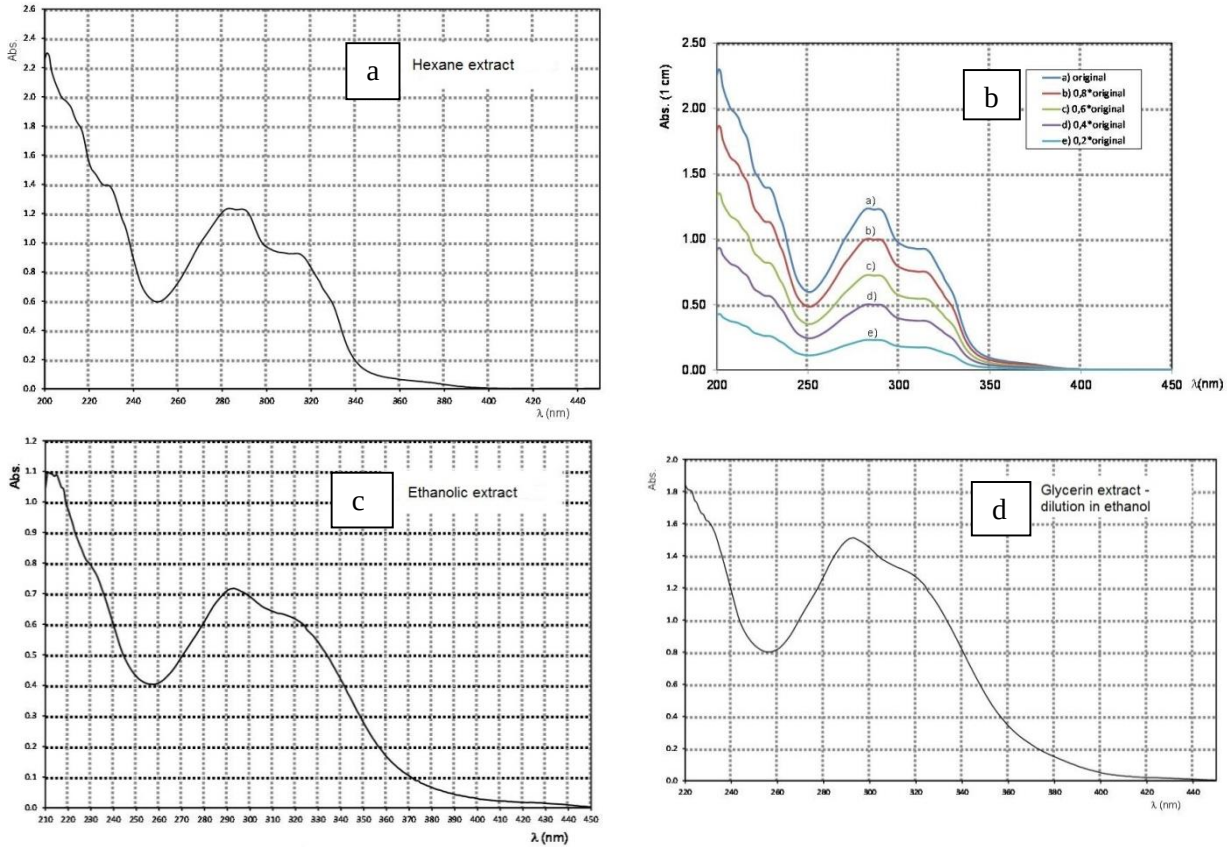

Fig.3 UV spectra of propolis extracts in (a) hexane, (c) ethanol, (d) glycerin diluted with ethanol and UV spectrum of successive dilutions in hexane (b)

CONCLUSION

The constituents identified can be considered marker compounds for propolis of SW Romanian origin. The analyzed sample could be classified in a high-flavonoid propolis group with antioxidant activity. Further studies are required to establish the marker compounds for this type of propolis and the plant sources.

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Original scientific paper

DETERMINATION OF FUMONISINS AND BEAUVERICIN IN ANIS SEED BY LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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ABSTRACT

The purpose of this study was to measure the potential levels of fumonisin B1 (FB1), fumonisin B2 (FB2) and beauvericin (BEA) contamination in anise seed. Mycotoxins were isolated by liquid extraction with a mixture of acetonitrile/methanol/water (25/25/50,v/v). Chromatographic separation was achieved using reverse phase chromatography on Zorbax Eclipse C18 column (Agilent, USA). The mobile phase A was methanol containing 0.1% formic acid and mobile phase B was 0.1% formic acid. Elution was performed in gradient conditions (starting from 50% B to 5% B in 25 min) at a flow rate 0.6 ml/min. FBs and BEA were detected by tandem mass spectrometry (LC-MS/MS) with triple quadrupole mass spectrometer (Agilent 6410 Triple Quadruple Mass Spectrometer, USA) in a positive electrospray ionisation mode using multiple reaction monitoring (MRM). For FB1 the two MRM transitions monitored for precursor ion 722.5 (*m/z*) were the primary product ion 334.4 (*m/z*) and the secondary product ion 352.3 (m/z) and for FB2 the precursor ion was 706.4 (*m/z*), and the product ions 336.3 and 318 (*m/z*). For BEA the precursor ion was 784.4 (*m/z*) and product ions were 262 and 244 (*m/z*). The mean recovery for fumonisin B1 and B2, at two spiking levels, ranged from 77% to 105% (%RSD 3.8 to 8.5) and for beauvericin the recovery at two spiking levels ranged from 85% to 116% (%RSD 4.2 to 9.6). The limit of detection was lower than 0,5 μgkg^{-1} for all analytes. A validated method was applied successfully on samples of anise seed taken at two localities. Beauvericin was found in concentrations ranged from 2 μgkg^{-1} to 18 μgkg^{-1}, while fumonisins were not detected in any of the samples.

***Key words**: anise, liquid extraction, mycotoxins*

INTRODUCTION

Mycotoxins are secondary metabolites produced by a wide range of fungi known to contaminate a variety of food and agricultural commodities worldwide. Their occurrence in food, beverages and feed has been recognized as potential threat to humans and animals.

They are mainly produced by fungi in the *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. Over 400 mycotoxins are known and the foodborne toxins of most interest are aflatoxins, trichothecenes, fumonisins, ochratoxin A, zearalenone and Alternaria toxins [1].

The fumonisins are a group of mycotoxins produced primarily by *Fusarium verticillioides* and *Fusarium proliferatum*. There are at least 28 different forms of fumonisins, most designated as A, B, C, and P-series. Fumonisin B_1 is the most common and economically important form, followed by B_2 and B_3.

Beauvericin is a cyclohexadepsipeptide mycotoxin which has insecticidal properties and which can induce apoptosis in mammalian cells. Beauvericin is produced by some entomo- and phytopathogenic *Fusarium* species (*Fusarium proliferatum*, *F. semitectum*, and *F. subglutinans*) and occurs naturally on corn and corn-based foods and feeds infected by *Fusarium* spp. [2]. Reports from the literature indicate that these toxins can also be found in a variety of botanicals [3, 4]. Numerous medicinal plants, tea and other botanicals enter markets around the world as food supplements. In the last years, there is a progressive increase of interest in food supplements as they are now consumed more and more. Some are used daily by consumers for various reasons. The presence of mycotoxins in food supplements, leads to human health problems. Anise - *Pimpinella anisum*, is a flowering plant in the family *Apiaceae* native to the eastern Mediterranean region and Southwest Asia. Its flavor has similarities with some other spices, such as star anise, fennel, and liquorice. It is sold as a spice, and the seeds are used as a breath freshener. Anise has a history of use as a spice and fragrance. It has been cultivated in Egypt for at least 4,000 years. In ancient Greek history, writings explain how anise helps breathing, relieves pain, provokes urine, and eases thirst. The fragrance is used in food, soap, creams, and perfumes. Anise is often added to licorice candy or used as a "licorice" flavor substitute. Anise is used widely as a flavouring in all food categories including alcohols, liqueurs, dairy products, gelatines, puddings, meats, and candies.

To determination of FB1 and FB2 in food and feed samples, many liquid chromatography methods with fluorescence detection (HPLC/FLD) [5-7] and liquid chromatography–mass spectrometry (LC –MS and LC–MS/MS) [8-10] have been developed. However, HPLC/ FLD methods require a derivatization step, usually with OPA reagent. LC–MS/MS require a shorter pre-treatment sample preparation with higher selectivity and sensitivity for the analysis of fumonisins than HPLC/FLD methods.

The aim of this study was to develop a multi-component analytical method, based on LC-MS/MS for the simultaneous determination of fumonisins (B1, B2 and B3) and beauvericin (BEA) in anise samples, one of the most common spice and fragrance.

MATERIAL AND METHODS

Beauvericin (BEA), Fumonisin B1and B2 (**≥98%,** HPLC**)**, and stock solution of Fumonisin B_1-$^{13}C_{34}$ (in acetonitrile) were obtained from Sigma Aldrich, (Germany). Referent material (Product No. P64/F428) purchased from R-Biopharm Rhône Ltd, (Glasgow, Scotland). Acetonitrile and methanol, all LC grade, were supplied from Merck (Darmstadt, Germany). Formic acid (98/100%, laboratory reagent grade) was from Fischer Scientific (Loughborough, UK). Pure water was obtained from Purelab® ELGA water purification system (Vivendi Water Systems Ltd UK). Glassmicrofiber filters (GF/A) were from Whatman, Cat. No. 6880-2504 (Maidstone, UK). Econofilters regenerated cellulose (0.45

μm) were from Agilent, Germany. A stock standard solution for fumonisins was prepared as micture at 1000 μg ml^{-1} (FB1) 30 μg ml^{-1} (FB2) in acetonitrile : water (50:50, v:v) and stored at -20 °C.

Chromatographic analyses were performed in an Agilent 1200 HPLC system equipped with a G1379B degasser, a G1312B binary pump, a G1367D autosampler and a G1316B column oven (Agilent Technologies, USA), using an Zorbax Eclipse C18 column (100 mm x 4.6 mm), with 1.8 μm particle size, from Agilent, USA. The analytical separation was performed using water as mobile phase A, and methanol as mobile phase B, both containing 0.1% formic acid. Elution was performed in gradient conditions (starting from 50% B to 5% B in 25 min) at a flow rate 0.6 ml/min.

The mass analysis was carried out with an Agilent 6410 Triple Quadrupole mass spectrometer equipped with multi source (Agilent Technologies, Palo Alto, CA, USA). Data acquisition and quantification was conducted using Agilent MassHunter Workstation software B.01.04 (2008). The following ionization conditions were used: electrospray ionisation (ESI) positive ion mode, drying gas (nitrogen) temperature 350°C, drying gas flow rate 5 L/min, nebulizer pressure 50 psi and capillary voltage 2000 V. Detection was performed using multiple reaction monitoring mode (MRM) with the following transitions: the precursor ion for FB1 was 722.5 (*m/z*) producing the primary product ion 334.4 (*m/z*) and the secondary product ion 352.3 (*m/z*) and for FB2 the precursor ion was 706.4 (*m/z*), and the product ions 336.3 and 318 (*m/z*). For BEA the precursor ion was 784.4 (*m/z*) and product ions were 262 and 244 (*m/z*). Fragmentor voltage and collision energy for FB1 (FB2), were 140 and 40 V(35V), respectively. Fragmentor voltage and collision energy for BEA, were 100 and 25 V, respectively. Dwell time was 100 ms. Internal standard method was used for quantification.

Two samples of anise seeds were obtained from Institute for Medical Plants »Dr Josif Pančić«, Belgrade, Serbia for development and validation of analitical method. Ground samples (10 g) were extracted with 100 ml acetonitrile : methanol: water mixture (25:25:50, v:v:v) by blending, using commercial blender with glass blender jars (Vicam) for 2 minutes. Sampe extracts were filtrated trough Whatman No. 4 filter paper and portion of extract was centrifuged for 5 minutes at 12000 rpm. Aliquot of supernatant was filtrated through CA filter 0.45 μm (Whatman). A 100 μl filtrate was transferred to insert and a 20 μl isotope label (^{13}C34) FB1 was added and stirred vigorously for 30 seconds, and analyzed by LC-MS/MS [11, 12, 13].

RESULTS AND DISCUSSION

Optimization of the chromatographic separation for FB1, FB2 and BEA

Optimization of the ESI-MS conditions was performed by direct injection of standard solutions of each mycotoxin in acetonitrile/water. Identification of precursor ions was performed in the full scan mode by recording from *m/z* (mass to ratio) 100 to 800 in PI mode, showing as predominant ion the protonated molecule [M+H]+. Further identification of the most abundant product ions, and selection of the optimum collision energies for each mycotoxin, was carried out in the product-ion scan mode. Two ion transitions were selected according to the highest sensitivity and the optimal selectivity for the target compounds, for quantification was preferred the one with best signal intensity (*Q*) and for confirmation was used the second transition (*q*) and the ratio of abundances among both ion transitions (*Q*/*q*).

Cone voltages were selected according to the sensitivity of the precursor ions and collision energies were chosen to give the maximum intensity of the fragment ions obtained. Table 1 lists the mass spectrometer parameters as precursor and product ions as well as the optimized cone voltages and collision energies used. For FB2 and FB3, different retention time was obtained but with the same transitions, the precursor ion was *m*/*z* 706, and the product ions *m*/*z* 336.3 and 318. Fumonisin B3 (FB3) was qualitatively determined in CRM, since analytical standard was not purchased.

Table 1. Summary of MRM transitions and MS operating parameters selected for analysis of the fumonisins and beauvericin

Mycotoxins	**Formula**	**M (g/mol)**		**Precursor Ion**		**Product Ion**	**Frag (*V*)**	**CE**
BEA	$C_{45}H_{57}N_3O_9$	783.95	Q	784.4	→	262.0	100	25
			q	784.4	→	244.0	100	30
^{13}C34-FB1	$^{13}C_{34}H_{59}NO_{15}$	755.58	Q	756.4	→	374.2	160	40
			q	756.4	→	356.2	160	45
FB1	$C_{34}H_{59}NO_{15}$	721.8	Q	722.5	→	334.4	140	40
			q	722.5	→	352.3	140	40
FB2	$C_{34}H_{59}N0_{14}$	705.8	Q	706.4	→	336.3	140	35
			q	706.4	→	318.0	140	35
FB3	$C_{34}H_{59}NO_{14}$	705.8	Q	706.4	→	336.3	140	35
			q	706.4	→	318.0	140	35

One significant drawback in ESI/MS quantitative analysis is matrix effect. Coeluting undetected matrix components may reduce or enhance the ion intensity of the analytes and effect the reproducibility and accuracy of the assay. The suppression and enhancement effect depends on the interface used, the sample pretreatment procedure, the matrix nature and the analyte considered. In our case we used label isotope standard for elimination matrix effect.

Method validation

Calibration curves were linear in the studied working range with a correlation coefficients greater than 0.99. (Table 2). Average recoveries of FB1 and FB2 obtained by adding different spiking levels to analyte-free anise seed samples are presented in Table 2. and they varied from 77% FB2 to 105 % FB1 with a relative standard deviation 3.8% for FB1 and 4.5% for FB2. Figure 1 present chromatogram of fortified sample with FB1 and FB2. The precision of the method in terms of repeatability (r) (intra-day precision) and reproducibility (R) (inter-day precision) was evaluated calculating the relative standard deviation (%RSD) of reference material analyzed in triplicate on different days (Table 2). The limits of detection (LOD), defined as the lowest concentration that the analytical process can reliably differentiate from background levels, was estimated for those concentrations that provide a signal to noise ratio of 3:1. These values of the LODs are 0.25 μg kg^{-1} for the FB1, 0.5 μg kg^{-1} for FB2 and 0.2 μg kg^{-1} for BEA. LOQs estimated as those concentrations of analyte which yield a signal-to-noise ratio of at least 10:1, ranging from 1 μg kg^{-1} for FB1and BEA, and 2 μg kg^{-1} for FB2 (Table 2.). Limits of quantifications (LOQs) obtained were well below the maximum levels for FBs set by the European legislation for foodstuffs, while no regulatory limits were set for herbal tea and spices plants.

Table 2. Method performance data: R^2, LODs and LOQs, recoveries and precision

Compound	Range (ng ml^{-1})	R^2	LODs (µg kg^{-1})	LOQs (µg kg^{-1})	Rec. (%)	Precision	
						Intra-day	Inter-day
FB1	25-500	0.9985	0.25	1	105	3.8	7.3
FB2	7.5-150	0.9978	0.5	2	77	4.5	8.5
BEA	10-500	0.9981	0.2	1	100.5	4.2	9.6

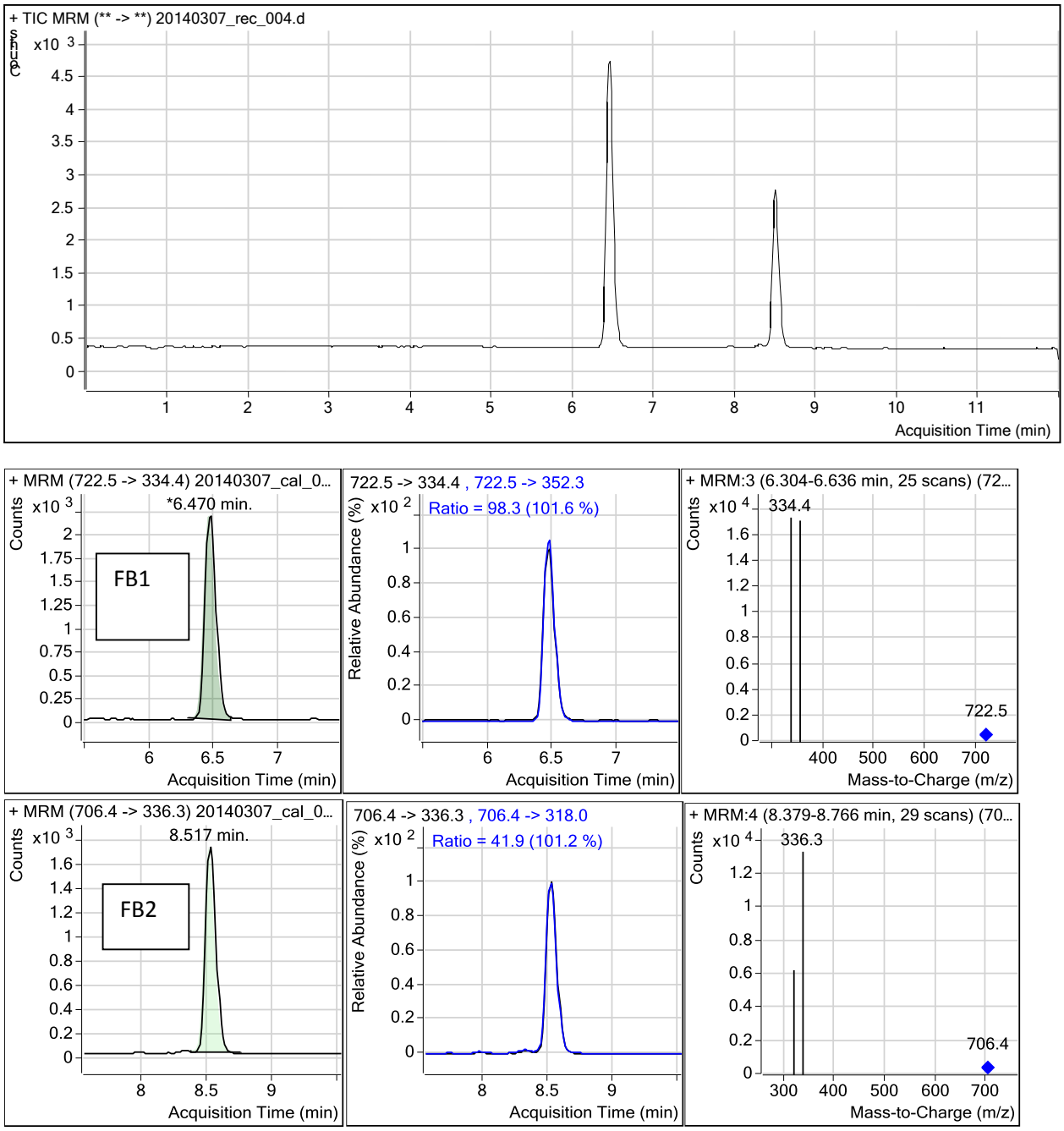

Figure 1. Chromatogram of fortified sample with FB1 (500 µg/kg) and FB2 (150 µg/kg)

Application to real samples

The applicability of the proposed method was evaluated by analyzing of anise seed (two samples) taken at two localities (Radinci 1 and Radinci 2). Those samples were contaminated by *Fusarium solani, F. tricincctum, F. sambucinum, F. equiseti, F. sporotrichoides, F. semitectum, F. verticilioides* and *F. oxysporum*. Fumonisins were not detected in any of the samples, but beauvericin was found in concentrations ranged from 2 μgkg^{-1} to 18 μgkg^{-1} (Fig. 2).

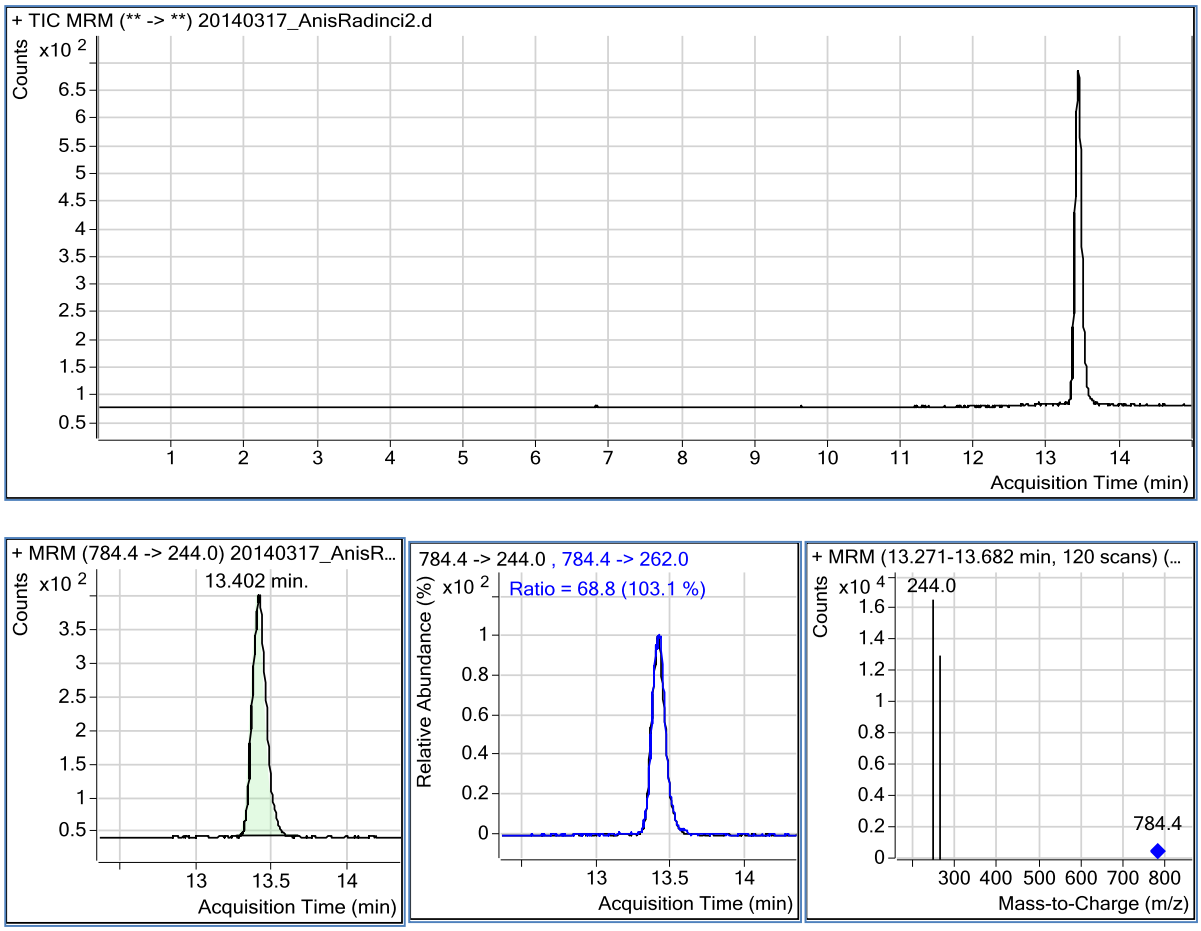

Figure 2. LC-MS/MS chromatogram of anise seed samples and MS spectra of beauvericin

CONCLUSION

Fast, sensitive and reliable LC-MS/MS method has been developed for determination of multiple

mycotoxins (fumonisins and beauvericin) in anise seeds. Fumonisins and beauvericin were analyzed in two samples taken at two localities. The most dominant fungi recorded were *Fusarium verticillioides* and *Fusarium oxysporum*, already well known producers of fumonisins. FB1 and FB2 were not detected in any of the samples, but beauvericin was found in all analyzed samples. As there is a long tradition of using medicinal plants in our

country, the content of FB1 and FB2 should be monitored in all the products infected by *Fusarium* sp.

ACKNOWLEDGEMENT

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Original scientific paper

HOW DO LYCOPENE AND ANTIOXIDATIVE ACTIVITY VARY IN TWO TOMATO GENOTYPES UNDER DEFFICIT IRRIGATION TREATMENTS

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ABSTRACT

One of the main characteristics of tomato fruit ripening is a significant accumulation of carotenoids, mainly lycopene. Despite the detailed knowledge of the carotenoid biosynthetic pathway, the regulation of synthesis by phytohormones, especially ABA, is poorly known. More attention needs to be given to the regulation of carotenoid accumulation, because its biosynthesis is not only of agricultural importance but also of scientific interest in terms of the chemical, biological, and genetic regulation.

The aim of the paper was to investigate the lycopene content and antioxidative activity in fruits of the wild type 'Ailsa Craig' and its ABA deficient mutant *flacca*, under different irrigation treatments. Plants were grown in greenhouse under optimal irrigation (FI), partial root drying (PRD) and regulated deficit irrigation (RDI) treatments. Lycopene content in final size fruits were measured as well as antioxidative activity.

Our results showed that concentration of lycopene was similar in *flacca* (125 mg/kg) and wild type, 75 mg/kg, but antioxidative activity was 2.5 times lower in *flacca* (38.6 μmoL TU/g) than in wild type, indicating positive effect of ABA on antioxidant potential of tomato plants.

***Key words**: Lycopersicon esculentum, wild type, flacca, antioxidant activity*

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the world's most important vegetables with 4.4 million ha production and 115 million t tomato yield worldwide in 2004 [1]. It is an excellent source of many nutrients and secondary metabolites, which are important for human health [2]. Apart from contributing nutritive elements, color and flavour to the diet, tomatoes are also a valuable source of antioxidants, or chemo-protective compounds, and may thus be termed a "functional food" [3]. The antioxidant potential of tomato is derived from a mixture of lycopene, ascorbic acid, phenolics, flavonoids and vitamin E [4]. Lycopene, constituting 80-90% of total carotenoid content present in tomato, have been shown to act as powerful

antioxidants in humans [5]. Tomato diet containing moderate amounts of lycopene has been associated with the prevention chronic diseases, such as cardiovascular disease [6] and cancers [7].

It is well known that the ripening process in tomato fruit is accompanied by an increase in the carotene content, especially lycopene and β-carotene, and decrease in the xanthophylls level [8]. The characteristic foliar carotenoids found in green fruit, i.e. lutein, neoxanthin, and violaxanthin, decreased as the ripening process progressed, and ripe fruit accumulated saturated carotenes such as two isomers of ζ-carotene, 15-cis-phytofluene, and especially 15-cis-phytoene and its epoxide.

The total antioxidant activity of tomato fruits varies greatly, depending on tomato genotypes, agricultural practices, and stages of maturation, but particularly on lycopene content and storage conditions [9, 10]. The lycopene content and antioxidant activity of tomatoes varies significantly among cultivars [5]. It is found that lycopene content ranged from 4.3 to 116.7 mg kg^{-1} on the fresh weight basis and founded in high concentration in small sized tomato fruit, e.g. cherry [4, 11].

Deficit irrigation practices, plant nutrient interactions, temperature and sunlight conditions have been identified as the conditions that enhance lycopene production in tomato fruit. [12] Moisture stress, for example, reduced lycopene (as well as β-carotene) content in some tomato varieties but increased it in others. On the other side, water deficit increases both total carotenoids and lycopene content in tomato fruit [13]. Antioxidants are also very important for plant response interaction to deficit irrigation, because they act as oxygen scavenging system able to detoxify the various forms of activated oxygen generated during water deficit period [14]. Regarding to PRD, there is no available data concerning benefit of different irrigation techniques on fruit quality [15, 16], especially in case of lycopene and antioxidant activity, but it is well known that PRD increase ABA concentration [17] more than RDI.

ABA is a plant hormone important for plant response to stress conditions, especially drought, but fruits of *flacca* ripen with delay compared with wild type fruits, even in optimally watered conditions. It is interesting to see how much the synthesis of carotenoids differs in these two genotypes and which is the role of ABA in ripening process. Since some xanthophylls in green fruit metabolize into ABA [18], and concentration of ABA in fruit pericarp increase in the mature green fruit [10], it is possible that ABA is involved in regulation of fruit ripening, probably by increasing fruit sensitivity to ethylene [19].

The study with different tomato genotypes (wild type and *flacca*) would indicate that the applied irrigation techniques contribute most to the increase of lycopene and total antioxidant activity of tomato fruits and also production of food with greater human health benefits.

MATERIAL AND METHODS

Tomato plants (*Lycopersicon esculentum* Mill.), 'Ailsa Craig' and it's ABA mutant, *flacca* were grown from seed and at the fifth leaf stage repotted into pots (one plant per pot) filled with 11 kg of commercial compost (Potground H, Klasmann-Deilmann, Germany) and grown in a chamber (photoperiod 14h; light intensity at plant level 300 $\mu molm^{-2}s^{-1}$, day/night temperature 25/18°C and relative humidity 70%) at the Faculty of Agriculture, University of

Belgrade. Pots (height 65 cm, diameter 20 cm, volume 20 dm^3) were specially designed for PRD experiments in such a way that they were vertically separated into two equally sized compartments. Root of each fifth leaf old plants were divided into approximate halves and repotted into these two hydraulically separated pot compartments. Compartments were classified as PRD-L (left side) and PRD-R (right side). Ten days after repotting, 15 plants per genotypes were subjected to optimal irrigation treatment, in which the whole root system was irrigated daily to reach field capacity around 35 %. In partial root-zone drying (PRD), the amount of water in one half of the root system of each plant was kept to 35% (wet side) while the other half was allowed to dry (dry side). The irrigation from wet to dry side was shifted when volumetric soil water content of the dry side had decreased to 15%-20%, and so alternating until the end of the experiment. In regulated deficit irrigation (RDI) water was evenly applied to the whole root system to reach 15-20% soil water content. The volumetric soil water content of both compartments of each pot was measured daily using TDR probes (time domain reflectometer, TRASE, Soil Moisture Equipment Corp., USA) at 20 cm depth. In total PRD and RDI, plants received about 30 % and 40 % less of the water that was applied for irrigation of the FI plants, respectively.

Since 52 % of the total antioxidants (48 % lycopene, 43 % ascorbic acid, 53 % phenolics) is located in the epidermis of the fruit, it should not be discarded during consumption [20]. Therefore, whole ripe tomato fruits were homogenized in a blender. The extraction of lycopene from fruit is carried out with a mixture of hexane : methanol : acetone (2:1:1) containing *butylated hydroxy toluene* (BHT). The suspension was centrifuged for 15 minutes at 8000 speed/minute at a temperature of 4 °C (2-16K, Sigma, Germany). The upper hexane layer was removed and absorbance at 505 nm of a 1:10 dilution of the extract was measured (SPECTRO UV-VIS RS, 1166, Lambomed, Inc. USA), using hexane as a blank test. Based on the absorption of lycopene concentration is calculated by the extinction coefficient of 3400. Results are expressed as the content of lycopene in mg per kg of fruit fresh weight [5].

Ripe tomato fruits (1 g) were homogenized with 10 ml of 80 % ethanol. The suspension was centrifuged for 10 minutes, 10000 speed/minute at room temperature. The upper layer was separated, and obtained ethanol extract was used for the antioxidant activity analysis. The antioxidant activity was determined by the method [21]. The resulting solution was diluted with a 5 mM phosphate buffer, pH 7.4. The absorbance was measured at 734 nm (SPECTRO UV-VIS RS, 1166, Lambomed, Inc. USA) for two minutes after the initial mixing - in of the vortex, with PBS as a blank test.

Statistical analysis

Two-way analysis of variance (ANOVA) was carried out to determine values of least significant difference (LSD) and degree of significance between treatments (FI, PRD and RDI) and genotype (wild type and *flacca*) at significance level $p \leq 0.01$.

RESULTS AND DISCUSSION

Our results showed that in FI treatment the concentrations of lycopene were not significantly different between *flacca* (125 mg/kg) and the wild type (WT). In PRD and RDI WT lycopene concentrations were 79.3 mg/kg and 92 mg/kg, respectively, similar to those in FI (Table 1).

Table 1. Differences among treatments and genotypes for lycopene and antioxidative activity (AA) (two-way ANOVA test).

	Lycopene		AA	
Treatment	WT	*flacca*	WT	*flacca*
FI	75.0	125.6	109.8	38.6
PRD	79.3	20.0	97.1	108.1
RDI	92.0	77.0	89.2	79.0
Treatment affect	n.s.		$p \leq 0.01$	
Genotype affect	n.s.		$p \leq 0.01$	

In *flacca* the concentration of lycopene in PRD and RDI was 20 mg/kg and 77 mg/kg, respectively. Similar results of PRD influence on lycopene content were reported by [15]. However, the content of lycopene is more dependent on the stage of maturation, than genotypes [22].

In FI, 2.5 times higher antioxidative activity was in WT (109.8 μmoL TU/g) than in *flacca* (38.6 μmoL TU/g). In WT PRD and RDI decrease antioxidative activity ($p<0.001$), while in *flacca* antioxidative activity 2 times significant increasing in RDI (79 μmoL TU/g) and 2.8 times in PRD (108.1 μmoL TU/g) than in FI (Table 1).

In literature there are no much data regarding the effect of PRD on antioxidant activity in different plant organs (fruits, tubers, seeds) of agricultural crops. Available data on the effects of PRD irrigation showed an increase in activity of several antioxidant enzymes in PRD and RDI irrigated olives [23]. It has been suggested that tomato plants enhance some of antioxidant enzymes activities to protect themselves, and they could be correlated with plant-water deficit adaptation [24]. Both PRD and RDI treatments possibly induced oxidative stress, which resulted in up-regulated some of antioxidant enzymes activities under water deficits in olive [25].

CONCLUSION

Results of the experiment showed that under PRD or RDI, WT plants used about 30-40 % less water than under FI, but at the same time there were no significant reductions of lycopene. Results of total antioxidant activity revealed differences between genotypes and applied irrigation system. Thus, the PRD irrigation system in *flacca* showed a significant positive effect of increasing the total fruits antioxidant activity compared with FI, while in the

wild type was not observed significant differences between the irrigation treatments. The increase of the antioxidant activity under PRD and RDI is very desirable characteristic that could be beneficial from the aspects of health-promoting value of tomato fruits. Further research of both techniques and application to much more tomato cultivars will allow assessment of potential practical impacts of these techniques for tomato production in the areas with restricting water resources. The future investigations will be focused on dynamic of synthesis carotenoids during fruit development in wild type and *flacca*.

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Original scientific paper

QUANTITATIVE ANALYSIS OF GLYCYRRHIZIC ACID OF GLYCYRRHIZA L. TAXA FROM THE CZECH REPUBLIC AND KYRGYZSTAN

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ABSTRACT

Licorice (*Glycyrrhiza* L., *Fabaceae*) is a perennial medicinal plant. Roots and rhizomes are frequently used in pharmacy and food technology. Since 1995, genetic resources of the genus *Glycyrrhiza* L. have been evaluated at MENDELU, respectively Faculty of Horticulture in Lednice. The genus *Glycyrrhiza* L. consist of about 30 species and few of them (*Glycyrrhiza glabra* L., *Glycyrrhiza echinata* L., *Glycyrrhiza pallidiflora* Maxim, *Glycyrrhiza macedonica* Bois. Et Orph., *Glycyrrhiza foetida* Desf. and *Glycyrrhiza uralensis* Fisch.) are evaluated. The second place of origin of *Glycyrrhiza* genus wild plants is Kyrgyzstan, means plant material from Sukuluksky area and area near Bishkek. The quantitative analysis of glycyrrhizic acid of *Glycyrrhiza* L. taxa was carried. The content of glycyrrhizic acid (glycyrrhizin) was measured by the HPLC. The content of glycyrrhizin ranged between 34.26% – 1.07%. The highest content (34.26%) of glycyrrhizic acid among all analysed taxa was determined in *Glycyrrhiza uralensis* Fish. (wild plants from Sukuluksky area, Kyrgyzstan) and lowest content (1.07%) was determined in *Glycyrrhiza pallidiflora* Maxim (cultivated plants in Lednice, Faculty of Horticulture, Mendel University in Brno, Czech Republic).

***Key words**: Glycyrrhiza L., glycyrrhizin, HPLC, Czech Republic, Kyrgyzstan*

INTRODUCTION

Licorice (*Glycyrrhiza* L.) species are perennial herbs native to the Mediterranean region, central to southern Russia and Asia Minor to Iran, now widely cultivated through Europe, the Middle East and Asia. The genus *Glycyrrhiza* L. consist of about 30 species [1]. The *Glycyrrhiza* L. genus was subdivided into two parts [2] representing true (*Euglycyrrhiza* Bois.) and pseudo (*Pseudoglycyrrhiza* Regel. Krug.) licorice species. *Euglycyrrhiza* group (*G. glabra, G. uralensis, G. inflata, G. korshinskyi, G. aspera*) produce glycyrrhizin and the others, *G. echinata*, *G. macedonica*, *G. lepidota* and *G. pallidiflora*, in group *Pseudoglycyrrhiza* Regel. Krug. produce macedonoside C as a major saponin [2, 3]. *Glycyrrhiza glabra* includes three varieties: Persian and Turkish licorices are assigned to *G. glabra* var. *violacea*, Russian licorice is *G. glabra* var. *gladulifera* and Spanish and Italian licorices are *G. glabra* var. *typical* [1]. Species *Glycyrrhiza glabra* L., *Glycyrrhiza uralensis* Fisch. *and Glycyrrhiza aspera* Pall. are occurring in Kyrgyzstan, especially in the Chujsky

region, respectively Sukuluksky area [4]. In the Czech Republic, licorice isreamin of earlier cultures. This old cultural plant was widely cultivated since the 16th century in South Moravia, where they have been extensive culture until the mid19th century [5]. The item (42A4400001) of the genetic resources comes from these wild plants. Licorice, the roots and rhizomes of some *Glycyrrhiza* species (*Fabaceae*) has been used in human being for at least 4000 years [1]. A drug was used in the orient for its sweetening power, as well as for its medicinal benefits, and recommended by the Greeks to treat ulcers. This *radix dulcis* was prescribed by Arab physicians to treat cough and to relieve the side effects of laxatives. The dried root and stolones of licorice, whole or cut, and peeled or not (Eur. Ph. 3rd Ed.) currently have many uses, chiefly in pharmacy and food technology [6]. Licorice is an essential ingredient of the Chinese five-spice blend, which is used in sauces and barbecues. It is also used in soft drinks, ice cream, candy, smoothies, drinks, and beer. In the Unites States and Europe, licorice is used in cough syrups, confectionares, and lozenges. It also masks bitterness in medicines. Licorice can be a noncaloric sweetener but must be used at very low amounts in food so it does not impart a licorice taste. The root has about 20% to 30% of water-soluble extractive and 4% of a glycoside (glycyrrhizin), which is fifty times sweeter than sugar. It has the sweet and slightly astringent-like flavor of anise. "Block juice" is the black brittle concentrated extract from the root that is free of insolubles. It of sugar, starches, and gums in addition to 12% to 20% glycyrrhizin. Licorice contains vitamin C, niacin, sodium, potassium, magnesium, and calcium[7]. Licorice root is a traditional medicine used mainly for the treatment of peptic ulcer, hepatitis C, and pulmonary and skin diseases, although clinical and experimental studies suggest that it has several other useful pharmacological properties such as antiinflammatory, antiviral, antimicrobial, antioxidative, anticancer activities, immunomodulatory, hepatoprotective and cardioprotective effects. A large number of components have been isolated from licorice, including triterpene saponins, flavonoids, isoflavonoids and chalcones, with glycyrrhizic acid normally being considered to be the main biologically active component [8]. Coumarins and the triterpenoid saponin glycyrrhizin present in samples of some *Glycyrrhiza* species have been also shown to have free radical scavenging activity [9]. The roots and stolons of *Glycyrrhiza glabra*, and *Glycyrrhiza uralensis* contain large amounts of glycyrrhizin, an oleanane-type triterpene saponin, which is considered as the main active constituent and a well-recognized natural sweetener [10]. Recently glycyrrhizin has been shown to have anti-tumor activity, highly active in inhibiting replication of HIV-1 and SARS-associated virus and exhibits a number of pharmacological effects [11]. The licorice root (*Glycyrrhiza radix*) is an indispensable ingredient of traditional *Kampo* medicines in Japan. Although *G. uralensis* and *G. glabra* (but not *G. inflata*) are both listed in the Japanese Pharmacopeia. According to the standards of the japanese Pharmacopeia XV, medicinal licorice must be used in licorice-containing *Kampo* medicines, and the minimum content of glycyrrhizin in these medicines should be 2.5% according to the standards of the Japanese Pharmacopeia [12]. In the Czech Pharmacopoeia [13] is mentioned part with title *Liquiritiae radix*–there are dried unpeeled or peeled, whole or cut root and stolons of species *Glycyrrhiza glabra* L. and/or *Glycyrrhiza inflata* Bat. and/or *Glycyrrhiza uralensis* Fisch. Roots and stolons must contain at least 4.0% of glycyrrhizic acid ($C_{42}H_{62}O_{16}$) counted on the dried drug. The content of glycyrrhizinic acid (glycyrrhizin) ranged between 4.30–23.20% in *Glycyrrhiza uralensis.* The highest content of glycyrrhizic acid (7.7%–18.6%) is in the fruit ripening period. Glycyrrhizic acid content depends on soil and climatic conditions, especially the amount of rainfall, the physical and chemical properties of the soil and groundwater levels. The content of glycyrrhizin is lower in the roots and rhizomes of licorice, when grown on dry soils. Different content of glycyrrhizin was also caused by different kinds of roots. When vertical roots were harvested the content of glycyrrhizin ranged from 8.6% to 18.5%. In the roots was content of

glycyrrhizin in the range of 9.00% to 18.7% [14]. The evaluation results of glycyrrhizic acid content are as follows: *Glycyrrhiza glabra* from 2.68% to 11.53%, in *Glycyrrhiza uralensis* 3.98%–12.06%, in *Glycyrrhiza echinata* 0.30%–8.05% and 3.54%–8.06% in *Glycyrrhiza pallidiflora*[15]. Results of evaluation roots of *Glycyrrhiza glabra* and *Glycyrrhiza uralensis* ranged from 8.00% to 24.00% glycyrrhizic acid [16]. The roots of *Glycyrrhiza uralensis* evaluated in 2009 contained 3.98%–12.06% glycyrrhizic acid [17]. The glycyrrhizin content of *Glycyrrhiza glabra* in 1-year-old roots rapidly increased from October to November, whereas the isoliquiritigenin glycoside content increased up to October. In 3-year-old plants, although the isoliquiritigenin glycoside content rapidly increased from June to July, the glycyrrhizin content did not show any significant increase from May to August. The glycyrrhizin content increased during the senescence of the aerial parts as well as during the early stage of shoot elongation. These results indicated that the biosynthesis of glycyrrhizin is differently regulated from that of isoliquiritigenin glycoside in the thickening root of *G. glabra* [18].

MATERIAL AND METHODS

Climatic conditions

Genetic resources of licorices were progressively integrated to the collection in the years 1995-2009. The altitude in FH MENDELU Lednice is 164 m, the average annual temperature

is 9 °C and average precipitation is 516.6 mm. Climatic conditions of Sukuluksky region are as follows-altitude 530-2,800 m, the average annual temperature is 8.5-9.8 °C, average precipitation is 350-400 mm. Bishkek is situated at 760 m altitude, the average annual temperature is 11.3 °C, average precipitation is 452 mm per year.

Plant material

Material comes from Czech Republic-cultivated plants and from Kyrgyzstan-wild plants. The harvesting of *Glycyrrhiza* samples was done during October 2011 – July 2012. The samples of roots and rhizomes material of *Glycyrrhiza* taxa were collected to a maximum depth of 0.25m. All samples were washed and dried by use not more than 40 °C. Plant material was analysed in laboratories at Mendel University in Brno, Faculty of Horticulture. List of all analysed samples represents **Table 1**.

Table 1 List of plant species

sample No.	species	origin/remark */**	date of harvesting
1	*Glycyrrhiza uralensis L.*	CZ/ *42A4400015	16.04. 2012
2	*Glycyrrhiza echinata*	CZ/ 42A4400008	16.04. 2012
3	*Glycyrrhiza macedonica*	CZ/ 42A4400013	16.04. 2012
4	*Glycyrrhiza pallidiflora*	CZ/ 42A4400005	16.04. 2012

5	*Glycyrrhiza pallidiflora*	CZ/ 42A4400009	16.04. 2012
6	*Glycyrrhiza glabra*	CZ/ 42A4400011	16.04. 2012
7	*Glycyrrhiza foetida*	CZ/ 42A4400010	16.04. 2012
8	*Glycyrrhiza glabra*	CZ/ 42A4400001	16.04. 2012
9	*Glycyrrhiza uralensis*	CZ/42A4400015	19.07. 2012
10	*Glycyrrhiza echinata*	CZ/ 42A4400008	19.07. 2012
11	*Glycyrrhiza macedonica*	CZ/42A4400013	19.07. 2012
12	*Glycyrrhiza pallidiflora*	CZ/42A4400005	19.07. 2012
13	*Glycyrrhiza pallidiflora*	CZ/ 42A4400009	19.07. 2012
14	*Glycyrrhiza glabra*	CZ/ 42A4400011	19.07. 2012
15	*Glycyrrhiza foetida*	CZ/ 42A4400010	19.07. 2012
16	*Glycyrrhiza glabra*	CZ/ 42A4400001	19.07. 2012
17	*Glycyrrhiza glabra*	KGZ/ Sokulukskij rajon 5	15.10. 2011
18	*Glycyrrhiza glabra*	KGZ/ Sokulukskij rajon 6	15.10. 2011
19	*Glycyrrhiza glabra*	KGZ/ Sokulukskij rajon 7 **Kaška Suu at the bottom	15.10. 2011
20	*Glycyrrhiza glabra*	KGZ/ Sokulukskij rajon 8 **Kaška Suu at the top	15.10. 2011
21	*Glycyrrhiza glabra*	KGZ/ near Bishkek 1	07.04. 2012
22	*Glycyrrhiza uralensis*	KGZ/ Sokulukskij rajon 3	31.03. 2012

* item of ECN genetic resources,**Kaška Suu - located on the northern slopes of the Kyrgyz ridge 35 km from the capital Bishkek, at an altitude of 2,000 m above sea level

Rhizomes and roots of Glycyrrhiza samples 50-150 mm long were dried in an oven at 40 °C for at 35-42 hours. The samples were ground in universal grinding mill (maximum grain size 3 mm). Content of glycyrrhizin was performed immediately after preparation of samples.

Content of glycyrrhizin

The glycyrrhizin of *Glycyrrhiza* L. taxa was measured by the high performance liquid chromatography (HPLC) according to Czech Pharmacopeia 2009 (*Liqiuriteae radix*).

The analyses were performed by RP-HPLC in a LCO-101 analytical column. The acetic acide in combination of acetonitrile and water (6:30:64) was used as the mobile phase in a gradient system, and the eluate was monitored with UV-VIS detector SMARTLINE 2600 with absorption at 254 nm, (ECOM Ltd., Praha). The content of glycyrrhizin was expressed in percentage. The results were recalculated on the dried drug.

RESULTS AND DISCUSSION

Results for all analysed samples presents **Table 2**. The highest content of glycyrrhizic acid showed the sample 22 *Glycyrrhiza uralensis* from Kyrgyzstan, Sukulukskij region 4 (34.26%).

Table 2 Results of glycyrrhizic acid content

sample	species	glycyrrhizic acid [%]
1	*Glycyrrhiza uralensis*	7.35
2	*Glycyrrhiza echinata*	3.58
3	*Glycyrrhiza macedonica*	1.45
4	*Glycyrrhiza pallidiflora*	1.07
5	*Glycyrrhiza pallidiflora*	8.66
6	*Glycyrrhiza glabra*	7.46
7	*Glycyrrhiza foetida*	12.81
8	*Glycyrrhiza glabra*	13.38
9	*Glycyrrhiza uralensis*	4.70
10	*Glycyrrhiza echinata*	7.27
11	*Glycyrrhiza macedonica*	3.88
12	*Glycyrrhiza pallidiflora*	5.52
13	*Glycyrrhiza pallidiflora*	4.95
14	*Glycyrrhiza glabra*	4.78
15	*Glycyrrhiza foetida*	2.49
16	*Glycyrrhiza glabra*	5.21
17	*Glycyrrhiza glabra*	3.86

18	*Glycyrrhiza glabra*	3.56
19	*Glycyrrhiza glabra*	3.51
20	*Glycyrrhiza glabra*	4.41
21	*Glycyrrhiza glabra*	17.46
22	*Glycyrrhiza uralensis*	34.26

The comparing of content of glycyrrhizic acid in samples from two different countries you can see at **Fig. 1**

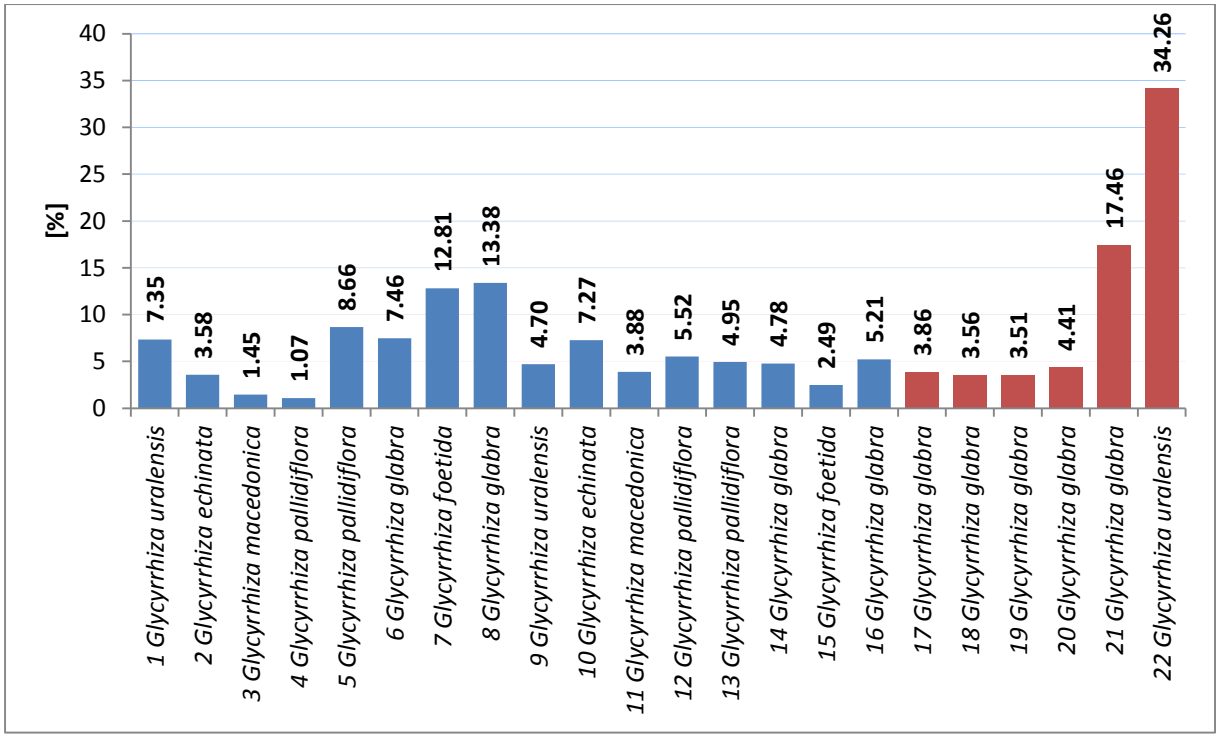

Figure 1 Glycyrrhizic acid content, difference in content and origin

The results above can be considered as a screening of content of glycyrrhizic acid in the kinds of licorice. Various species belonging to sections *Euglycyrrhiza* and *Pseudoglycyrrhiza* were evaluated. According to literature data [2, 3] by *G. echinata*, *G. foetida*, *G. macedonica* species does not contain any glycyrrhizic acid. The reason for the different results may be the fact that some seeds were obtained from botanical gardens and identification of taxa could be wrong. Glycyrrhizic acid content in roots and rhizomes is affected by altitude, precipitation, soil quality and other soil and climatic factors in locations of collection and cultivation. According to the Czech Pharmacopoeia requirements (minimum is 4.00% glycyrrhizin) comply with all evaluated samples of *G. glabra* and *G. uralensis* except *G. glabra* (sample number 17, 18, 19) harvested in Sukulukskij rajon on October. All evaluated samples (*G. glabra* and *G. uralensis* would) correspond to conditions of the Japanese Pharmacopoeia (minimum of glycyrrhizin is 2.50%). Quite exceptional content was found in the sample

number 22 *G. uralensis* (34.26%). This content does not match any literature data, this evaluation must be repeated. This is a necessary condition for obtaining exact results.

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Original scientific paper

DETERMINATION OF MACRO NUTRIENT CONTENT IN SOME HERBAL DRUGS FROM THE BLACK SEA PROVINCES IN TURKEY

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ABSTRACT

Levels of macro nutrients (N, P, K, Ca, Mg and S) and protein content in six useful herbal drugs were determined using standard analytical methods. Chamomile (*Matricaria chamomilla* L.), St. John's wort (*Hypericum perforatum* L), Nettle (*Urtica dioica* L), Laurel (*Laurus nobilis* L), Yarrow (*Achillie millefolium* L.) and Linden (*Tilia cordata* L.) were used as plant material in this study. The samples of herbal drug were collected from the local markets at Black Sea provinces in Turkey and subjected to nutrient analysis. The results showed that N, P, K, Ca, Mg and S contents in the samples of these selected plants were 1.41-3.78%; 0.11-0.54%; 0.63-2.85%; 0.89-4.64%; 0.15-0.65% and 0.15-0.47%, respectively. Also, crude protein was found ranging from 8.82-23.61% in the samples. Accumulation of macro nutrient and protein content varied from plant to plant. Elemental analysis of the samples indicated that the nettle leaves contained appreciable levels of nutrient. Nutrient status of nettle was higher than those of other medicinal plants. Nutrient status of nettle was found to be as follows: crude protein 23.61%, N 3.78%, P 0.54%, K 2.85%, Ca 4.64%, Mg 0.65% and S 0.47%. These results suggest that the herbal drugs accumulate the elements at different concentrations and they are good source of important macro elements and can be consumed to supplement our daily nutrient needs.

***Key words**: Essential elements, herbal plant, nettle, spice, St. john's wort*

INTRODUCTION

Medicinal plants are one of the most important resources of medicines. Minerals elements in medicinal plants are an important factor for their pharmacological actions (1). Mineral elements play an important role in structural components of tissues and function in cellular and basal metabolism and water and acid-base balance in the living organism (2). Besides, their deficiency causes diseases, whereas the excess presence may cause toxicity for human health (1). Medicinal plants also play an important and vital role in traditional medicine and

are widely consumed as home remedies. According to the World Health Organization (WHO), about 65–80% of the world's population in developing countries, due to the poverty and lack of access to modern medicine, depend essentially on plants for their primary health care (3). Although these plants are mainly used for medicinal purposes, their food values should be evaluated. Recently, mineral nutrient content of herbal and medicinal plants has been investigated by many researchers (2, 4, 5, 6). However, a few plants have been studied for the assessment of their nutrient status. The aim of this study was to determine macro element contents of some herbal drugs consumed for medicinal purposes in Turkey.

MATERIAL AND METHODS

The herbal drugs were collected from local markets of Ordu, Samsun Giresun, cities of the Black Sea in Turkey. Chamomile (*Matricaria chamomilla* L.), St. John's wort (*Hypericum perforatum* L), Nettle (*Urtica dioica* L), Laurel (*Laurus nobilis* L), Yarrow (*Achillie millefolium* L.) and Linden (*Tilia cordata* L.) were used as plant material in this study. The plant samples were dried, powdered before processing for analysis. The dried and ground analytical samples were analyzed for total nitrogen by the Kjeldahl method (7). The crude protein content (NX6.25) of the samples was estimated by the macro-Kjeldahl method (8). The plant samples were analyzed for P, K, Mg, Ca and S by ICP-AES. 200 mg of the plant sample was put in a burning cup and 5 ml HNO_3 acid (65%) and 2 ml H_2O_2 30% were added. The samples were incinerated in a microwave at 200 °C and cooled at room temperature for 45 minutes and filtrated using filter paper. The filtrated extracts were collected by high-deionised water in 20 ml bottles and were kept at 4 °C in laboratory for ICP-AES analysis. Each sample was analyzed in triplicate. Merck standards (R1and R2 groups) were used as analytical reagent grade chemicals. Standard solutions of P, K, Ca, Mg and S were prepared in 1% HNO_3 immediately before the analysis by serial dilution of 1000 mg L^{-1} stock solution. Peach Leaves (Standard Reference Material, 1547) and Corn Bran (Standard Reference Material, 8433) were used as reference materials (13). The ICP-AES was used to determine P, K, Ca, Mg and S in the extracts. …….

RESULTS AND DISCUSSION

The macro element compositions in selected medicinal plants are presented in Table 1. The present results revealed that the plant samples comprised considerable amount of N, P, K, Mg, Ca, and S.

Table 1. Macro elements and protein content in some herbal drugs

	N	P	K	Ca	Mg	S	Protein
	----------	----------	----------	%	----------	----------	----------
Chamomile (Matricaria chamomilla L.)	1.85	0.39	2.27	1.14	0.28	0.21	11.56
St. john's wort (*Hypericum perforatum* L)	1.92	0.34	1.23	0.75	0.22	0.22	12.03
Nettle (Urtica dioica L)	3.78	0.54	2.85	4.64	0.64	0.47	23.61
Laurel (Laurus nobilis L)	1.48	0.11	0.63	1.21	0.15	0.15	9.24

Yarrow (Achillie millefolium L.)	1.41	0.27	1.68	0.89	0.24	0.18	8.82
Linden (Tilia cordata L.)	1.96	0.30	1.70	1.40	0.38	0.17	12.26

Nitrogen levels varied from 1.41 to 3.78% in the herbal drugs. Laurel and yarrow had the lowest N concentration whereas nettle had the highest. The greatest variability was observed in N concentration of plants. Phosphorus levels ranged from 0.11 to 0.54% in plant samples. Nettle leaves had the highest P concentration, whereas the lowest P levels were recorded in some leaves, such as laurel and yarrow. The mean P levels of St. john's wort, nettle and chamomile in of Poland medicinal samples by Pytlakowska, et al. (5) were found to be 2780, 5731 and 4270 mg kg^{-1}, respectively. Queralt et al., (9) also reported that P level of chamomile was 3500 mg kg^{-1}.

Potassium levels varied between 0.63 to 2.85% in the herbal drug samples. Laurel had the lowest K concentration whereas nettle had the highest. The mean K levels of St. john's wort, nettle and chamomile in of Poland medicinal samples by Pytlakowska, et al. (5) were found to be 1048, 2782 and 2085 mg kg^{-1}, respectively. Queralt et al., (9) also reported that K level of Chamomile was 23467 mg kg^{-1}. Magnesium levels ranged from 0.15 to0.65% in the samples and higher concentrations were found especially in nettle, lower concentrations in laurel. The mean Mg levels of St.john's wort, nettle and chamomile in of Poland medicinal samples by Pytlakowska, et al. (5) were found to be 134, 552 and 279 mg kg^{-1}, respectively. Queralt et al., (9) also reported that Mg level of Chamomile was 2642mg kg^{-1}. Calcium levels were variable among the herbal drugs, ranging from 0.89 to 4.64%. St. john's wort had the lowest Ca concentration whereas nettle had the highest. The mean Ca levels of St. john's wort, nettle and chamomile in of Poland medicinal samples by Pytlakowska, et al. (5) were found to be 133, 1426 and 169 mg kg^{-1}, respectively. Queralt et al., (9) also reported that Ca level of Chamomile was 9279 mg kg^{-1}. Sulfur levels ranged from 0.15 to 0.47% in samples. Laurel had the lowest S concentration whereas nettle had the highest. Queralt et al., (9) reported that S level of chamomile was 4091 mg kg^{-1}.

The results indicated that the herbal plants contain large amounts of nutrients and are rich in N, P, K, Ca, Mg and S as previously reported (1, 4, 5, 6, 9). Also, crude protein was found ranging from 8.82 to 23.61% in the samples. Nettle leaves had the highest crude protein content, whereas the lowest levels were recorded in some leaves, such as laurel and yarrow. Accumulation of macro nutrient and protein content varied from plant to plant. Elemental analysis of the samples indicated the nettle leaves contained appreciable levels of nutrient. Nutrient status of nettle was higher than those of other herbal drugs. The nutrient levels obtained in plant are in the same range of those reported by other authors for herbal drugs from several countries, our data agree with those published in other regions, such as Sheded, et al. (10), Pytlakowska, et al. (5) and Gjorgieva et al.(11), Ajasa et al., (12).

CONCLUSIONS

Medicinal and aromatic plants may be a good source for mineral elements in human foods. On average, the increasing order of the macronutrients among the investigated herbal drugs is N > K >Ca> P > Mg > S. Elemental analysis of the herbal drugs showed that nutrient content of nettle is relatively high than the other plant samples. The results of present study revealed

that the herbal drugs contain the elements at different concentrations and they are good source of important macro elements and can be consumed to supplement our daily nutrient needs.

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Original scientific paper

ESSENTIAL OIL OF COMMON JUNIPER (*JUNIPERUS COMMUNIS* L.) IN ALBANIA

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ABSTRACT

Genus *Juniperus* has about 60 species. The most wide-spread species is common juniper (*Juniperus communis* L.). The main raw material for the production of historical typical Slovak alcohol beverage „*Spišská borovička*" is common juniper fruits. Nowadays berries are mainly imported from Albania, where the plants are growing typically on the rocks, on barren grounds, in fields, grass fields, clear-cut areas, in open forests and in other places. In 2013 the fruits were collected from 16 localities in Albania, naturally dried, extracted essential oils and analysed by GC/MS. The content of essential oil varies in the range of 1.2 % to 3.8 % and from 34 to 47 substances was identified. The Albanian plants have more geographic types, which were identified on base of the essential oil composition. The first has the dominant compounds β-myrcene (44.5 ± 3.04 %) and α-pinene (19.6 ± 3.35 %). The second type is characterised by the contents: α-pinene (25.1 ± 1.78 %), β-pinene (13.4 ± 4.41 %) and β-myrcene (21.2 ± 4.79 %) and the third: α-pinene (31.6 ± 1.81 %), β-pinene (13.6 ± 1.78 %) and β-myrcene (18.5 ± 5.60 %). The last has very high content of α-pinene (37.7 ± 1.92 %), β-pinene (12.4 ± 2.22 %) and β-myrcene (18.6 ± 3.65 %). This biodiversity monitoring of Albanian juniper plant population contributes for increasing efficiency and enhancement of spirit distillate production.

***Key words**: common juniper, essential oil, GC/MS, Juniperus communis L.,*

INTRODUCTION

Common juniper, *Juniperus communis* L., is a shrub or tree species belonging to the cypress family (*Cupressaceae)*. It has wide ecological amplitude. Juniper occurs abundantly in dry sunny hillsides, as well as the subalpine level. It extended almost the entire territory in Slovakia. The human activity affected its spreading. Juniper habitats are scattered to concentrate along with other light-requiring species of plants, mostly shrubs that occur within communities of grasslands and scrubland vegetation. It expands on the extensive use of pasture land or land that is no longer used by grazing. Juniper is therefore an indicator of succession after the disappearance of grazing. The Natura 2000 project - coherent European

network of protected areas, in Slovakia 66 habitats of European importance, include the code Kr2 which note "juniper populations", quantifying of social value of 15.93 euros per 1 m^2 (1).

Juniper berries are used also in phytotherapy, as an infusions, alcoholic extracts and essential oils (2). Significant application is in the manufacture of alcoholic beverages. Essential oil is used for production typical Slovak alcoholic drink „*Spišská borovička*" (PRELIKA, Co., Presov, Slovakia). The aim of the contribution is evaluated the habitats with the juniper populations in Albania for them chemotype biodiversity.

MATERIAL AND METHODS

Plant material. The fruits of common juniper were collected from different individuals on 16 localities in Albania in 2013:

Isolation of the essential oil. Ten grams of each sample of juniper was grounded in a blender and then subjected to hydro distillation for 2 h according to the standard procedure described in the European Pharmacopoeia (2004). The oils were solubilised in *n*-hexane and stored under N_2 at +4 °C in the dark until were analysed. The plant materials gave yellow reddish oils.

Gas chromatography and components identification. GC/MS analyses were carried out on a Varian 450-GC connected with a Varian 220-MS. The separation was achieved using a FactorFour™: Capillary Column VF 5ms (30 m × 0.25 mm i.d., 0.25 μm film thickness). Injector type 1177 was heated on temperature 220 °C. Injection mode split less (1 μL of a 1:1,000 *n*-hexane solution). Helium was used as a carrier gas at a constant column flow rate of 1.2 ml/min. Column temperature was programmed: initial temperature 50 °C for 10 minutes, then to 100 °C at 3 °C/min; isothermal for 5 minutes and then continued to 150 °C at 10 °C/min. Total time for analysis of one sample took 46.67 minutes. Identification of components were made by comparison of their mass spectra with those stored in NIST 02 (software library) or with mass spectra from the literature and a home-made library, as well as on comparison of their retention indices with the standards.

RESULTS AND DISCUSSION

In 2013 the fruits were collected from 16 localities in Albania, naturally dried, extracted essential oils and analysed by GC/MS. The content of essential oil varies in the range of 1.2 % to 3.8 % and from 34 to 47 substances was identified. The Albanian plants have more geographic types, which were identified on base of the essential oil composition (Table 1). The first has the dominant compounds β-myrcene (44.5 ± 3.04 %) and α-pinene (19.6 ± 3.35 %). The second type is characterised by the contents: α-pinene (25.1 ± 1.78 %), β-pinene (13.4 ± 4.41 %) and β-myrcene (21.2 ± 4.79 %) and the third: α-pinene (31.6 ± 1.81 %), β-pinene (13.6 ± 1.78 %) and β-myrcene (18.5 ± 5.60 %). The last has very high content of α-pinene (37.7 ± 1.92 %), β-pinene (12.4 ± 2.22 %) and β-myrcene (18.6 ± 3.65 %). This biodiversity monitoring of Albanian juniper plant population contributes for increasing efficiency and enhancement of spirit distillate production.

The dominant compounds of juniper essential oil are: α-pinen, β-pinen and myrcen. On the basis of their content and the ratio can be divided into four samples groups:

- *The first group*: The highest content is myrcene and its average is 44,5 % and the contents of α-pinene is 19.57 % and β-pinene is very low (1.5 %).
- *The second group:* The content of α-pinene is 25.04 %, myrcene 21.21 % and these results are nearly same. Quantity of β-pinene is 13.41 % and in this group was detected the the highest content of caryophylene (9.08 %).
- *The third and fourth group* are similar. The myrcene content is 18.5 % (the 3rd group) and 18.56 % (the 4th group). Quantity of β-pinene is 13.56 % (the 3rd group) and 12.43 % (the 4th group). The very important is the differences of the α-pinene characteristics, which are 31.56 % (the 3rd group) and 37.68 % (the 4th group).

This plant species extended almost the entire territory in Slovakia. In the comparison with the Albanian research, the juniper fruits were collected from three localities in Northeast Slovakia in the October 2012. The essential oil content varies in the range from 0.20 to 0.42 %. The 34 to 47 chemical components were identified. Dominant compounds were α-pinene, the content ranged from 25.81% to 43.35% and β-pinene, which amount ranging from 13.29% to 20.64%.

The results of the juniper berry essential oil content and its composition from both countries present the significant variation, which is depending on the age of the plant and concrete localities.

Gonny et al. (3) identified 22.1 % amount of α-pinene and only 1.5 % of β-pinene in the fruits of *Juniperus communis* L.. On the other hand he determined the highest amount of limonene (49.3 %). In the analyses of the Slovak samples, the limonen amount was 0.77 % in Vyrava and 5.63 % in Kišovce.

Chatzopoulou et al. (4) analysed the essential oil from the juniper grown in Greece. The essential oil was extracted after 1, 2, 3, 4, 5 and 6 hours of hydro distillation. 26.04 % of α-pinene was identified after 2 hours of hydrodistillation and β-pinene was not noted. The amount of limonene was 1.37 % and highest amount determined in β-caryophyllene 6.97 %, sabinene 9.23 % and germacrene D 12.89 %. After different time period they identified different amount of some components, for example α-pinene after one hour distillation was identified 27.78 % and after six hours only 9.78 %.

High amount (10.9 %) of germacrene D was measured by Marongiu et al. (5) in the juniper fruits cultivated in Sardegna, Italy. In the same sample were identified 44.0 % of limonene, 2.6 % of α-pinene and 0.8 % of β-pinene.

Gonny et al. (3) and Chatzopoulou et al. (4) determined myrcene (6.30 % and 9.21 %). In our samples was not identified.

El-Chorab et al. (6) performed two types of analyses. Its two column chromatographic fractions (eluted with hexane and ethyl ether) were analysed by gas chromatography/mass spectrometry. The major compounds in the dichloromethane extract were α-pinene 23.73 %. A fraction eluted with hexane contained 44.24 % α-pinene. These results could present the importance of the extraction solution.

Marongiu et al. (5) recorded in three different samples of juniper fruits 22.93 – 60.07 % of α-pinene, 1.50 – 5.60 % of β-pinene, 0.68 – 15.72 % of myrcene and 0.68 – 9.96 % of p-cymene.

Table 1. Qualitative and quantitative characteristics of the common juniper population – essential oil in Albania.

	Number of samples	α-pinen	sabinen	β-pinen	β-myrcen	limonen	tepinen -4-ol	bornyl acetate	β-caryophylen
group No.1	8	19.57 ± 2.74	< 1	1.50 ± 0.43	44.50 ± 2.46	5.12 ± 1.02	< 1	< 1	4.25 ± 0.84
group No.2	24	25.04 ± 0.75	5.58 ± 0.89	13.42 ± 1.86	21.21 ± 2.02	4.46 ± 0.72	< 1	< 1	9.08 ± 1.38
group No.3	16	31.56 ± 0.95	4.50 ± 0.82	13.56 ± 0.95	18.50 ± 2.97	4.25 ± 0.71	< 1	< 1	5.87 ± 1.31
group No.4	16	37.69 ± 1.02	4.06 ± 0.71	12.44 ± 1.18	18.56 ± 1.93	3.87 ± 0.77	< 1	< 1	3.75 ± 1,20

CONCLUSION

Common Juniper, *Juniperus communis* L. is a shrub or tree species belonging to the cypress family (*Cupressaceae*) with wide ecological amplitude. The fruits were collected from 16 localities in Albania in 2013, naturally dried, extracted essential oils and analysed by GC/MS. The content of essential oil varies in the range of 1.2 % to 3.8 % and from 34 to 47 substances was identified. The Albanian plants have more geographic types, which were identified on base of the essential oil composition. The first has the dominant compounds β-myrcene (44.5 ± 3.04 %) and α-pinene (19.6 ± 3.35 %).

The obtained results suggest that the content and composition of essential oil of juniper berries (*Juniperus communis* L.) varies depending on the age of the plant and localities. For the determination of essential oil components has a significant effect distillation length as well as the method of analysis. It is necessary to assess the more detailed study of environmental factors in studied localities.

ACKNOWLEDGEMENTS

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Original scientific paper

ESSENTIAL MACRO NUTRIENT PROFILES OF SELECTED MEDICINAL AND AROMATIC PLANTS FROM THE FAMILY OF LAMIACEAE

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ABSTRACT

Determination of nutrient contents of medicinal and aromatic plants has recently generated considerable attention in all over the world. In accordance with this, concentrations of essential macro nutrients (N, P, K, Ca, Mg and S) in selected medicinal and aromatic plants from the *Lamiaceae* family were monitored in the present study. Turkish oregano (*Origanum onites*), rosemary (*Rosemarinus officinalis*), lavender (*Lavandula stoechas*), sage (*Salvia officinalis*), basil (*Ocimum basilicum*) and lemon balm (*Melissa officinalis*) were used as plant material. The plant samples were obtainedat local spice shops from three provinces situatedat the Black Sea coastal area of Turkey and subjected to chemical analysis to quantify macro nutrient levels. The results indicated the presenceof variable amounts of macro nutrients in selected medicinal and aromatic plant samples. The results showedthat N, P, K, Ca, Mg and S contents, on a dry weight basis,in the samples were within the ranges of 1.04-3.37%, 0.07-0.34%, 1.21-2.72%, 0.83-3.01%, 0.23-0.77% and 0.14-0.37%, respectively. On average, the increasing order of the macronutrients among the investigated plant samples is K > N >Ca> Mg > S > P. The samples of basil and lemon balm comprised the highest levels of macro nutrients, while the lavender samples contained the lowest. In conclusion, these findings suggest that medicinal and aromatic plants may have a potential of serving as supplementary sources of essential macronutrients.

Key Words: *Herbs, macro elements, oregano, sage, spice plants*

INTRODUCTION

Turkey, a bridge between Europe and Asia, is situated at the junction of three major floristic regions (Mediterranean, Irano-Turanian, and Euro-Siberian) and has a wide range of climatic and ecological conditions [1]. Turkey is known to be one of the most biologically diverse countries in the world; its unique diversity is manifested by the presence of over 11 000 plant species, 30% of which are endemic [2]. Medicinal and aromatic plants from the families of

Lamiaceae, *Umbellifera*e, *Composita*e, etc.represent an important component of this biodiversity endowment of the country. The flora of Turkey is estimated to contain over 3000 aromatic plants with high percentage of endemism [1, 3]. *Lamiaceae* family is represented in Turkey by 46 genera and 571 species of which more than 40% are endemic [1].

Since ancient times, medicinal and aromatic plants play an important role in traditional medicine and are widely consumed as home remedies particularly in rural areas. In Turkey, as elsewhere in the world, the demand for medicinal and aromatic plants has started to increase in the past recent years. Mineral element composition of medicinal and aromatic plants has gainedan interest in the last few decades [4, 5, 6, 7]. Nutrient elements, alongside their nourishing effect, may have preventive and curative roles in human health, but at elevated levels, they can be dangerous and toxic [8, 9, 10].

Eight common spices (coriander, cumin, anise, nigella, mustard, carum, black pepper, and fenugreek) were studied for their mineral compositions [11]. The results indicated that all these eight spices are generally rich in Ca, Mg, and P contents. Adnan et al. (2010) studied the nutrient contents of five medicinal plant species (*Bupleurum falcatum*, *Valeriana officinalis*, *Forsskalea tenacissima*, *Lavandula angustifolia* and *Otostegia limbata*) collected in humid and sub-humid regions from Northwest Pakistan [12]. The results revealed that, the increasing order of the macronutrients among the investigated plant samples of both regions is Ca> K > Mg >Na. It was observed that the concentration of Ca, Na, K, and Mg were found more in sub-humid region species compared to humid region species.

In Turkey, most of medicinal and aromatic plants used in traditional public health are composed of plants collected from natural habitat. The first hand source providing plant drugs are spice shops (Aktar in Turkish) which are of considerable importance in traditional folk medicine in Anatolia for ages. Mineral composition of medicinal and aromatic plants may vary greatly depending on a number of factors; used plant parts, climate and soil parameters, management practices and post-harvest conditions [13, 14, 15, 16]. Some works have been done on micronutrient and heavy metal contents of medicinal plants from Turkey, butlittle attention has been paid towards their macro nutrient contents [17, 18, 19]. The objective of the present study was to monitor macronutrient profile of selected medicinal and aromatic plants from the family of *Lamiaceae* that is an important component of biodiversity endowment of Turkey.

MATERIAL AND METHODS

In the present study, six medicinal and aromatic plant species commonly used in Turkey were subjected to chemical analysis for their macronutrients content. The dried plant samples were obtained from spice wholesalers and local spice shops in three provinces (Samsun, Ordu and Giresun provinces) located at the Black Sea coastal area of Turkey in September 2013. Some characteristics of the six plant species monitored for macronutrient contents are given in Table 1. The plant samples of 100 g in the plastic bags were kept at normal room temperature until to be analyzed. Before chemical analysis, all the plant samples were cleaned and washed with deionised water and air dried. The plant samples were then dried at 70 °C for 48 hours in an oven and finely ground. Laboratory procedures for the preparation of plant samples and determinationof macronutrients were used as outlined by Şekeroğlu et al. [15].

Table 1. Some characteristics of selected medicinal plants from the family of Lamiaceae

Plant scientific name	Common name	Turkish name	Part used	Usage purpose
Origanum onites	Turkish Oregano	Kekik	Herb	Spice, herbal tea
Rosemarinus officinalis	Rosemary	Biberiye	Leaves	Spice, herbal tea
Lavandula stoechas	Lavender	Karabaşotu	Herb	Traditional medicine, herbal tea
Salvia officinalis	Sage	Adaçayı	Herb	Herbal tea
Ocimum basilicum	Basil	Fesleğen	Leaves	Traditional medicine, spice
Melissa officinalis	Lemon Balm	Oğul otu	Herb	Traditional medicine, herbal tea, spice

For macronutrient analysis, 0.2 g of the ground samples were put into a burning cup and then 5 ml HNO_3 65% and 2 ml H_2O_2 30% were added. After that, the samples were incinerated in a microwave at 200 °C and cooled at room temperature for 45 minutes and filtrated using filter paper. The filtrated extracts were collected by high-deionised water in 20 ml bottles and were kept at 4 °C in laboratory for ICP-AES analysis. Each sample was analyzed in triplicate. Merck standards (R1and R2 groups) were used as analytical reagent grade chemicals. Standard solutions of P, K, Ca, Mg and S were prepared in 1% HNO_3 immediately before the analysis by serial dilution of 1000 mg L^{-1} stock solution. Peach Leaves (Standard Reference Material, 1547) and Corn Bran (Standard Reference Material, 8433) were used as reference materials [20]. The ICP-AES was used to determine P, K, Ca, Mg and S in the extracts. Nitrogen analysis was carried out using the Kjeldahl method [21].

RESULTS AND DISCUSSION

The mean concentrations of various macronutrients, on dry weight basis, in selected medicinal and aromatic plant samples are given in Table 2. The present study revealed that all the macronutrients (N, K, P, Ca, Mg and S) were accumulated to greater or lesser extents by all investigated medicinal and aromatic plantspeciesform the family of *Lamiaceae*.Potassium, nitrogen and calcium were predominant macronutrients in medicinal and aromatic plants. On average, the increasing order of the macronutrients among the investigated plant samples is K > N > Ca > Mg > S > P. The samples of basil and lemon balm comprised the highest levels of macro nutrients while the lavender samples contained the lowest.

Table 2. Macro nutrientcontents, on dry weight basis, of selected medicinal and aromatic plants form the family of *Lamiaceae.*

Plants	N	P	K	Ca	Mg	S
	----------	----------	----------	% ----------	----------	----------
Turkish Oregano (*Origanumonites*)	1.31	0.09	1.21	2.36	0.37	0.24
Rosemary (*Rosemarinus officinalis*)	1.04	0.07	1.46	1.61	0.29	0.17

Lavender (*Lavandulastoechas*)	1.63	0.18	1.69	0.83	0.23	0.14
Sage (*Salvia officinalis*)	1.90	0.14	2.03	1.35	0.24	0.31
Basil (*Ocimumbasilicum*)	3.37	0.34	2.72	3.01	0.77	0.36
Lemon balm (*Melissa officinalis*)	2.33	0.26	2.55	2.25	0.62	0.37

Nitrogen levels in the plant samples analyzed were noticeably variable. The basil samples had the highest nitrogen content (3.37%), whereas the lowest nitrogen value was obtained in basil leaves (1.04%). The phosphorus levels ranged from 0.09 to 0.34%, with the highest and the lowest mean values found in Turkish oregano and basil samples, respectively. In the medicinal and aromatic plant samples, K content was found in the range of 1.21-2.55%. The highest mean level of K was recorded in lemon balm, while the lowest was measured in Turkish oregano.

All plant samples contained noticeably variable levels of Ca; the highest Ca content was 3.01% in basil leaves and the lowest concentration was 0.83% in lavender herb. The Mg concentrations in the plant samples varied from 0.24% to 0.77%, with sage herb containing the lowest and basil leaves having the highest. The variability in sulfur concentrations of the plant samples was lower than those observed in the other macronutrients. The sulfur levels ranged from 0.14 to 0.37%, with the highest and the lowest mean values found in lemon balm and lavender samples, respectively.

The data obtained here in this study show that selected medicinal and aromatic plants, with respect to their mineral content, have a good level of nutritional potential. It was reported that highest mineral concentrations were measured between 16.31-37.25 Ca, 3.09-6.83 Mg and 0.39-1.84 P % in some medicinal and aromatic plants grown in Morocco [22]. It is interesting that Ca, Mg and P contents in thyme, lavender and rosemary reported in the former study is much greater than those obtained in our study. Lasisi et al. [23] found Ca, Mg, K and P contents ranging from 2350to 30520 mg/kg, 65.20 to 5470 mg/kg, 4375 to 25300 mg/kg and 100 to 3500 mg/kg in eight herbal plants of the South-Western Nigeria.In another study, it was found that important species of *Artemisia* found in Pakistan accumulated significant amount of K, Ca and Mg [24]. Similarly, a study with 24 different medicinal plants from Pakistan, used in folk medicine, revealed that investigated medicinal plants were good source of K, Ca, Mg [25]. An elemental study on medicinal plants from Macedonia [26] showed that they contained large amounts of nutrients and were rich in Mg, Ca and K. According to Subramanian et al. [27], medicinal plants collected from local market in India were rich in some essential minerals, especially Fe and Mg. It is obvious that the abundance of K, Ca and Mg in the medicinal plants analyzed in the present study was in good agreement with previous findings available in the literature.

CONCLUSION

In the present study, selected medicinal and aromatic plants (Turkish oregano, sage, rosemary, lavender, basiland lemon balm) commonly used in Turkey were subjected to chemical analysis to quantify macronutrient levels. The plants analyzed in the study have

been traditionally used in folk medicine and in Turkish cuisine in Anatolia for years.The results revealed that these plants were rich in essential macronutrients and could play a meaningful role in human nutrition. On average, the increasing order of the macronutrients among the investigated plant samples is K > N >Ca> Mg > S > P. The samples of basil and lemon balm comprised the highest levels of macro nutrients while the lavender samples contained the lowest. In conclusion, these findings suggest that medicinal and aromatic plants may have a potential of serving as supplementary sources of macronutrients.

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Original scientific paper

CORRELATION BETWEEN ANTIOXIDATIVE POTENTIAL OF PURE CAPSAICIN AND CAPSICUM OLEORESINS

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ABSTRACT

Capsaicin is a biological active compound which is isolated from the fruit of hot pepper species. It has been known for its analgesic, antireumatic, antiseptic, antidiabetic and several other pharmacological properties. Its antioxidative potential is also a subject of many experiments conducted, in the past few years. The aim of this study is to examine the antioxidant potential of capsaicin and capsicum oleoresins produced from *Capsicum* sp. cultivated in R. of Macedonia.

This experiment comprises four different genotypes of *Capsicum annuum* L., which were used for obtaining ethanolic oleoresins. Their antioxidant potential was measured and compared to the antioxidative potential of the pure capsaicin standards. As a method for measuring the total antioxidant capacity was used FRAP (Ferric reducing antioxidant potential) method. This is a simple photometric method for estimation of *in vitro* antioxidative potential which is expressed as μmol/L Fe^{2+}.

As expected from the previous findings of capsaicin, results from this study are also showing that it posses antioxidative potential that is not so high. But, there is a good correlation between antioxidant potential of capsaicin and capsicum oleoresins in addition of the capsaicin content measured in oleoresins.

The results show that antioxidative potential of hot peppers does not come only from the vitamins and phenolic compounds in them, but alkaloids (capsaicinoids) are also included.

***Key words**: capsaicin, antioxidants, hot peppers, fruit, FRAP*.

INTRODUCTION

Capsaicinoids refers to a group of pungent compounds found in chili peppers. Pepper fruits (*Capsicum annuum* L.) are important vegetables used as vegetable foods, spices or dry fruits intended for isolation of capsaicin [1]. Peppers are a good source of vitamins C and E [2, 3] as well as some of the carotenoids as compounds with well-known antioxidant properties [4-6]. Hot cultivars are rich in capsaicinoids, alkaloids with pharmacological properties, giving the specific taste to pepper fruit [3, 7]. Interest in their biological activity is increasing. Previous studies have indicated that red pepper and capsaicinoid decrease blood cholesterol concentration, [8, 9] possibly mediated by inhibition of intestinal cholesterol absorption [10]. Capsaicinoid has also been shown to be effective in weight reduction mediated by enhancing β-oxidation of fatty acids *in vivo* and increasing adrenergic activity and energy expenditure [11]. Accumulated evidence has also demonstrated that capsaicinoid has potential beneficial effect on the human cardiovascular system [12-14]. It has also been reported that capsaicinoid possesses antitumor activity [15]. Capsaicinoids have been shown to possess antioxidant activity with an ability to prevent excessive formation of reactive oxygen species (ROS) [16].

There are few reports on the antioxidant activity of capsaicin [17]. This activity may be the result of the presence of groups in the phenolic ring (a methoxy group in ortho position to OH) of capsaicinoids and ferulic acid ester, which influenced the antioxidant properties. This is in agreement with Henderson et al. [18], who showed that the amide group present in capsaicin does not play a major role in its antioxidant activity under free radical oxidation conditions, that the antioxidant behavior for capsaicin was due primarily to the phenolic moiety in the molecule, and that the main product of capsaicin oxidation is its dimmer dicapsaicin. A comparison of the results of antioxidant activities of pure capsaicin and *Capsicum* extracts obtained in the present studies showed that the antioxidant activity of oleoresins is dependent of the concentration of capsaicin. We have determined the antioxidant activity of the capsaicinoid fraction isolated from hot peppers and the antioxidant activity of the ethanolic oleoresins. The primary objective of this study was to determine which genotype has higher antioxidant potential and how it correlates with antioxidant potential of the pure capsaicin.

MATERIAL AND METHODS

Plant Material: Fruits of three hot pepper cultivars (genotypes Vezena, Feferona, Bombona), and one mild genotype Sivrija, (as a control), were taken from the field experiment conducted in 2012-2013. Fruits were harvested at the stage of full ripeness (red) [19]. After the fruits have been washed and the seeds removed, fresh pepper fruits were cut and dried at a room temperature, in a dark and dry place for about two weeks. They were dried to constant weight, and the percent of water in them was counted. Dried fruits of the peppers were grounded and kept in an exsiccator.

Method of extraction: For the extraction of oleoresin we have used the maceration method [20]. Process of extraction with vacuum filtration was made using 0.2 g of grounded peppers in 25 ml of extraction solvent. According to the literature a few organic polar and non polar solvents can be used for capsaicin extraction. We have tried to use acetone and ethanol, but acetone has shown as an inappropriate solvent for spectrophotometric measurements on wavelength of 280 nm. Therefore, in the focus of this experiment were taken only the ethanolic extracts. Maceration process was performed in volumetric flasks for 5 hours, on temperature of 50ºC. Separation of extracts from the powder was conducted with vacuum

filtration using a Buchner funnel and a water vacuum pump. Final extracts for this experiment were diluted 2:25 with the same solvent.

Method for quantification of capsaicin: Concentrations of capsaicin in the standard solutions and in ethanolic extracts were measured by one of the cheapest method for quantification of capsaicin, the UV/VIS spectrometric method [21, 22]. Measurements of the concentration of capsaicin in the extracts were evaluated through their absorbencies measured on wavelength of 280 nm.

Method for measuring the antioxidant potential of capsaicin. As a method for measuring the total antioxidant capacity (TAC) of the pure capsaicin or *Capsicum* extracts was used the *in vitro* spectroscopic method FRAP (Ferric Reducing Antioxidant Power) assay [23]. The method is based on the reduction of the Fe^{3+} ions to Fe^{2+} ions, when they are captured in TPTZ (2,4,6-Tri(2-pyridinyl)-1,3,5-triazine), under the influence of the antioxidant (reductance) in the system. The reaction in this assay is running under acidic conditions. $FeSO_4$ has been used as a standard solution in this measuring, so the results are expressed as µmol/L Fe^{2+}. Detection of the final coloring of the samples on which this assay was applied was measured on wavelength of 595nm.

RESULTS AND DISSCUSION

The capsaicin content in the ethanolic oleoresins was determined on the basis of standard solutions of capsaicin (Sigma-Aldrich Chemie, Steinheim, Germany) [24, 25]. The results for concentration of capsaicin in the samples were calculated using the linearity curve and linearity equation y= 9.484.x + 0.016, obtained from the standard solutions of capsaicin (Figure 1).

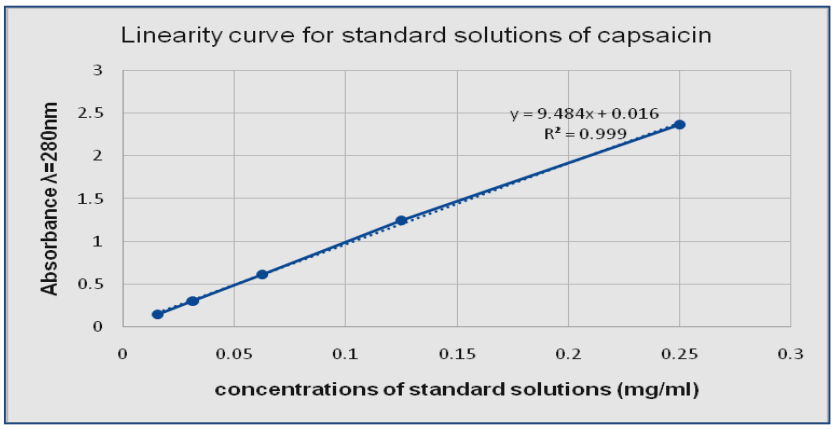

Figure 1. Linearity curve for standard solutions of capsaicin

Table 1 is presenting the results of capsaicin concentration (λ=280 nm) and TAC values of the standard solutions of capsaicin, measured by spectrophotometer on wavelength of 595 nm.

Table 1 Capsaicin concentration and TAC of the standard solutions of capsaicin

Standard solutions	Concentration of capsaicin (mg/ml)	Absorbance in FRAP assay	TAC (Fe^{2+} µmol/L)

St. solution 1	0.25	1.175	1395
St. solution 2	0.125	0.726	763
St. solution 3	0.062	0.457	383
St. solution 4	0.031	0.332	207
St. solution 5	0.016	0.265	113

Table 2 Capsaicin concentration and TAC of the ethanolic oleoresins

Extracts	**Concentration of capsaicin (mg/ml)**	**Absorbance in FRAP assay**	**TAC (Fe^{2+} µmol/L)**
Vezena	0.014	0.227	59
Feferona	0.019	0.228	61
Bombona	0.052	0.249	90
Control (Sivrija)	0.018	0.198	18

Table 2 is giving the results of Capsaicin concentration and TAC in the ethanolic oleoresins. Antioxidant potential of the extracts was measured and compared to the total antioxidant potential of standard solutions of pure capsaicin in Diagram 1 (Antioxidant potential of capsicum oleoresins vs. capsaicin standard solutions). These measurements were made using water as negative and ascorbic acid as positive control, against ethanol as sample blank.

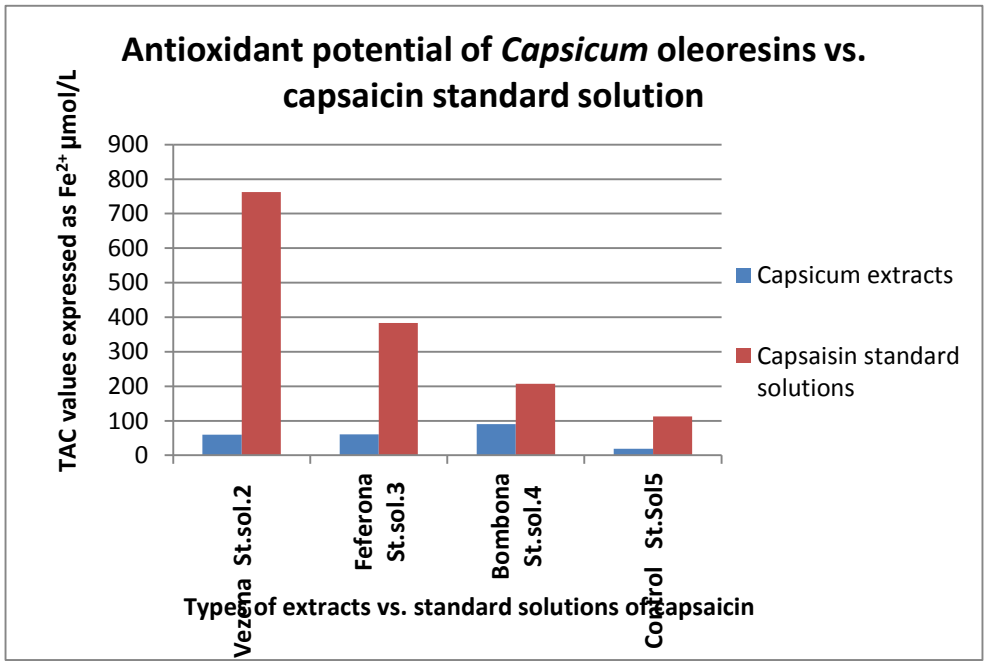

Diagram 1 Antioxidant potential of Capsicum oleoresins vs. capsaicin standard solutions

Genotype Bombona has higher concentration of capsaicin, so we can see, higher antioxidant potential, from the examined genotypes of hot peppers. The total antioxidant capacity of the oleoresins obtained from genotypes Vezena and Feferona, (capsaicin concentrations are 0.014 and 0.019 mg/ml) are lower than TAC values for pure capsaicin solution with concentration of 0.016 mg/ml. The control, as a mild genotype, has obviously lower antioxidant potential, than hot pepper genotypes.

As expected, results showed a good correlation between capsaicin concentration in the extracts and their antioxidant potential. Beside the high concentration of vitamin C and E as well as carotenoids, which poses high antioxidative potential, capsaicin also showed that it is an important metabolite in the *Capsicum* species. It is important, not only because of its analgesic activity, but also as a biological active compound with antioxidative effects. The extract with the highest concentration of capsaicin showed the highest antioxidative ability, and on the opposite site, the extract from the control, which was a mild genotype of pepper, showed the lowest antioxidative capacity. This confirms that a high percent of the total antioxidative capacity of the hot peppers is due to the presence of capsaicin in this extracts. Compared to the TAC values of pure capsaicin the total antioxidant potential of oleoresins is little lower but it has a good correlation with their capsaicin concentration.

CONCLUSION

As a conclusion: On the basis of the data in this study and in the literature, there is a likelihood that pepper fruits may provide the types of nutritional and health benefits associated with the consumption of fresh pepper fruits in general [26-28]. From the results obtained by FRAP (ferric reducing antioxidant power) method, oleoresin obtained from hot peppers genotype that has higher concentration of capsaicin exhibits higher total antioxidant potential than other genotypes, especially from the mild control. This can mean that hot peppers can be used as a potential source of antioxidants. This in vitro method for measuring the antioxidant potential is not a specific test for showing the in vivo effects of the antioxidant, because of the known ADME (absorption, distribution, metabolism, elimination) effects on the capsaicin in the human organism. Although the bioactive forms of the capsaicin might not be those found in the plants, the pepper fruits are widely used as a source of antioxidants. Because of their common use as a spice and as a food protector further studies into the activity of this compound of peppers are needed to evaluate their potential antioxidative effect and their health benefits for the human organism.

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Original scientific paper

TOXICOLOGICAL EVALUATION OF *JUNIPERUS* SPECIES FROM FLORA OF THE R. MACEDONIA

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ABSTRACT

This study evaluated the acute toxicity of methanol extracts of needles of several *Juniperus* species from flora of the Republic of Macedonia. Some are used in folk medicine for treatment of infections, skin diseases and other different ailments. An evaluation of the toxicity of extracts of these plants is crucial to support the therapeutic claims. The present work is the first one to investigate the *Juniperus* species toxicity. Plant samples of four *Juniperus* species were collected from 4 localities in Republic of Macedonia in 2010/11. Evaluation of the toxic potential of *J. foetidissima, J. excelsa, J. oxycedrus* and *J. communis* was carried out using Brine shrimp (*Artemia salina)* lethality assay. The results are expressed as medium lethal concentration (LC_{50}) in relation to positive ($K_2Cr_2O_7$) and negative controls (artificial sea water). LC_{50} values with 95% confidence intervals were determined by the Finney's probit analysis. Assays were performed in triplicates with observations up to 60 h. The analysis showed different LC_{50} values for the examined *Juniperus* species, tested at six concentrations: 0.01, 0.1, 1, 3, 5 and 10 mg/mL, for each extract. Toxicological bioactivity of *Juniperus* methanol extracts was in the following order: *Juniperus oxycedrus* > *Juniperus excelsa* > *Juniperus foetidissima* > *Juniperus communis.* According to the Meyer's toxicity index, *Juniperus* methanol extracts could be classified as non-toxic and toxic, which possibly determines their use in traditional medicine. Moreover, the used method could serve as a predictive tool for oral acute toxicity in plant extracts.

Keywords*: herbal extracts, acute toxicity, Juniperus species, Artemia salina, LC50.*

INTRODUCTION

According to WHO, curative plants are the best source to obtain various drugs (medicines) and a possible way for curing illnesses caused by multidrug resistant bacteria [1]. According to the World Health Organization's poll, it is announced that 70-80% of the population

worldwide relies on unconventional medicine, mainly from plant sources, in the primary health protection [2].

Republic of Macedonia presents the treasure of the ecosystems, habitats, communities and unique plant localities that placed it on the top of the list of countries with extraordinary biodiversity in Europe. In the flora of the Republic of Macedonia, land plants include about 3200 species divided into 147 families [3]. Many of them are used in the traditional medicine, in the form of various preparations, for treating many illnesses. However, part of them are toxic and the community has no knowledge about it, but also there is no scientific literature to support this assumption. The toxicity of the plants may originate from different contaminants or from plant chemical compounds that are synthetised from the plant. Various assays were involved for researching the toxicity of the herbal extracts, based on different biological models such as *in vivo* assays on laboratory mice, as well as species of *Artemia salina, Artemia franciscana* [4] and *Artemia urmiana* [5], species of *Thamnocephalus platyurus* [6] etc. The results for toxicity on *Artemia salina* show strong correlations with the data obtained for acute oral toxicity of plant preparations on rodents and humans [7 , 8, 9]. The analysis of the toxicity with the species of *Artemia salina* (Brine shrimp) was proposed and developed by Michael et al., since 1956 [10]. This assay was later modified by Meyer [11] and McLaughlin [12]. The larvae of *Artemia salina* are used as a biological model to confirm the toxic potential of the bioactive compounds in the plant extracts [12]. According to Meyer, the criterion for toxicity of the plant extracts is modelled from the gained values for the parameter of the average lethal concentration - LC_{50}, according to which, extracts with LC_{50} < 1000 μg/mL were considered as toxic, while extracts with LC_{50} > 1000 μg/mL were considered as non toxic [11]. Clarkson et al. modelled the criterion for the toxicity of the plant extracts in accordance with the following levels of LC_{50}: extracts with LC_{50} above 1000 μg/mL are non-toxic, LC_{50} of 500 - 1000 μg/mL are low toxic, extracts with LC_{50} of 100 - 500 μg/mL are medium toxic, while extracts with LC_{50} of 0 - 100 μg/mL are highly toxic [13]. Yet, there is no officially proposed criterion from WHO, FDA and other authorities, how to reach compliance for interpretation of the gained results.

The method for evaluating the toxicity with *Artemia salina* has a broad range of advantages compared to *in vivo* methods for toxicity with experimental animal models like mice and rats. Since it does not include higher animals, the data for acute toxicity obtained with the *Artemia salina* method are collected after 60 hours, accentuating that low quantities are enough to conduct the bioassays [14]. The *Artemia* species are very sensitive to toxic substances in their early developmental phases, defining it as a proper choice for this kind of assay [15, 16]. This method, beside its application in toxicity testing of various plant extracts [12, 14], wields a wider spectrum of applicability such as identifying fungal toxins [17], toxicity testing of heavy metals [18], identifying cyanobacterial toxins [19], testing of pesticides [20], cytotoxicity testing of dental materials [21], toxicity testing of nano-particles [22], testing of extracts prepared from natural sea organisms [23] etc. Additionally, this method is used for research and identification of the cytotoxic activity of the plant agents that are utilized for treatment of human cancerogenous cell lines, for example lung cancer, (A-549) and the colon carcinoma (HT-29) [23]. The examined herbal extracts often show strong toxic potential, which limits their safe application [14, 24, 25, 26, 27, 28]. According to these findings, the purpose of this paper is the toxic evaluation of the herbal extracts from *Juniperus* species of the flora of Republic of Macedonia, that will contribute to their safe application in the herbal medicine. Evaluating the toxic potential using a Brine shrimp (*Artemia salina)* lethality assay, these extracts would gain a scientific confirmation for their safe application.

MATERIAL AND METHODS

Plant material

The *Juniperus* plant material was harvested from various locations in the Republic of Macedonia during 2010/11 (Table 1).

Table 1. Plant samples of *Juniperus* from R. Macedonia

	Species	**Location**	**Year**	**Sample abbreviation**
1.	*J. communis*	Commercial sample		Jc-c
2.	*J. excelsa*	Chalakli, Dojran	2011	Je-D/11
3.	*J. excelsa*	Udovo	2011	Je-U/11
4.	*J. excelsa*	Velestovo, Ohrid	2010	Je-O/10
5.	*J. foetidissima*	Chalakli, Dojran	2011	Jf-D/11
6.	*J. foetidissima*	Udovo	2011	Jf-U/11
7.	*J. foetidissima*	Veles	2011	Jf-V/11
8.	*J. foetidissima*	Velestovo, Ohrid	2011	Jf-O/11
9.	*J. oxycedrus*	Velestovo, Ohrid	2010	Jo-O/10

Bothanical identification was conducted at the Institute of Pharmacognosy, a voucher of the samples was deposited in the herbarium at the Faculty of Pharmacy-Skopje. Plant samples were appropriately dried and stored until analysis.

Hatching of nauplii (larvae) *Artemia salina*

The hatching of larvae of *Artemia salina* is done in accordance to Meyer's procedure [11]. In a plastic bottle, 0.5 L of artificial sea water was prepared in laboratory conditions (NaCl – 38 g/L), with adjusment of pH=9. The larvae of *Artemia salina* are hatched during 48 hours, under regulated temperature (25 ^{o}C), air flow (using an air pump) and continuous light source. The bulb provides direct light and heat (about 25 °C) during the embriogenesis [11, 12]. The hatched brine shrimps are attracted with light, 10 shrimps are collected using a micropipetor and simultaneously placed in laboratory flasks containing plant extracts, positive and negative control.

Preparation of the plant extracts

30 g of the plant material was dissolved in 300 mL methanol. The extraction was performed in ultrasonic bath (Selecta p.) during 1 h, followed by filtration of the prepared extract. The filtrate was evaporated until dry in rotary evaporator. The liofilized extract was reconstituted with DMSO (0.05 %) in concentrations of: 10 mg/mL, 5 mg/mL, 3 mg/mL, 1 mg/mL, 0.1 mg/mL and 0.01 mg/mL.

Assays for lethality with *Artemia salina*

Ten brine shrimps are collected with micropipetor (1-10µl) and placed in laboratory flasks, each containing 5 mL total volume of a plant extract with proper concentration. The experiment is performed in 60 h time frame, with periodical monitoring of the mortality of *Artemia salina* in time intervals after 6 h, 10 h, 24 h, 30 h, 36 h, 48 h, 54 h and 60 h. During the assay the brine shrimps are not fed, because they are still using reserves from their own yolk [21]. The experiment is performed simultaneously with a positive and a negative control. Potassium dichromate ($K_2Cr_2O_7$) is used as a positive control, while the artificial sea water is a negative control. The larvae that are motionless for more than 10 seconds are counted for dead [23].

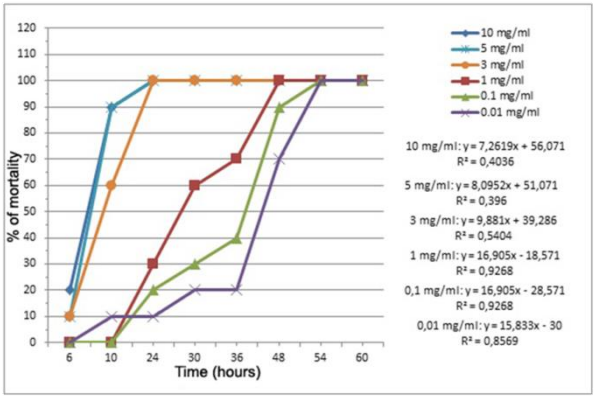
Determining LC_{50}

The medium lethal concentrations (LC_{50}) are confirmed in 95% interval of confidence determined during 24 h with probit analysis according to Finney [29], using a commercial statistical software IBM SPSS 20.0. The values for LC_{50} are calculated with regression analysis of the lethal percentage in relation to the logarithmic value of the concentration of the extract.

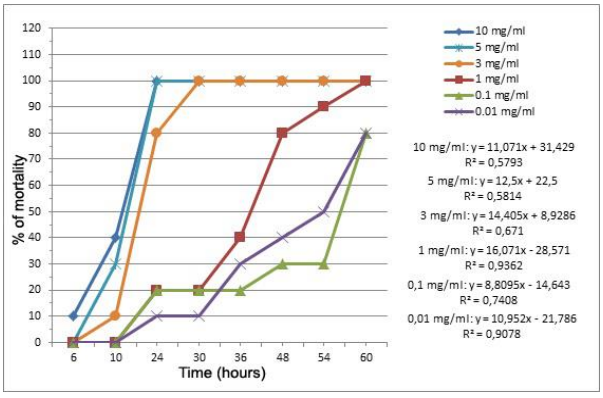
RESULTS AND DISCUSSION

The species of *Juniperus* (Cupressaceae) are usually applied in the alternative medicine for the treatment of different illnesses: rheumatism, inflammatory illnesses, bronchitis, urticaria, dysentery, bleeding, scabies, fungi, hemorrhoides etc. [30, 31]. The flora of Republic of Macedonia abounds many species of *Juniperus*, from which the local community prepares various concoctions for different applications. However, the lack of knowledge for the toxic potential of these plants obstructs their safe application.

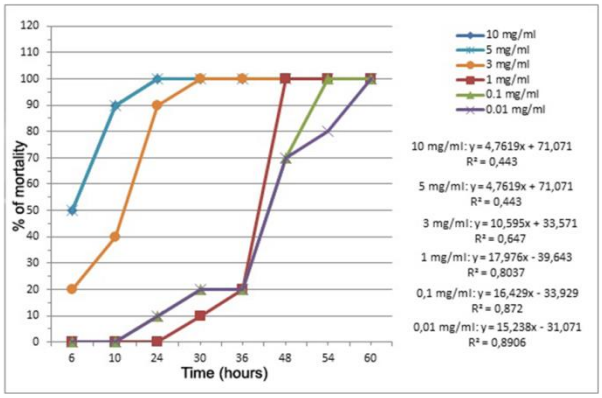
The degree of the toxicity of the plant extracts is determined according to the percentage of lethality of *Artemia salina* in presence of different concentrations of methanol extracts of *Juniperus* species, and in relation to the calculated LC_{50} values, within 24 h time interval. The trend of mortality is presented in Figure 1 as percentage (%) of lethality of *Artemia salina* versus time of observation for four samples: Jf-D/11, Jo-O/10, Jc-c (commercial sample) and Je-O/10.



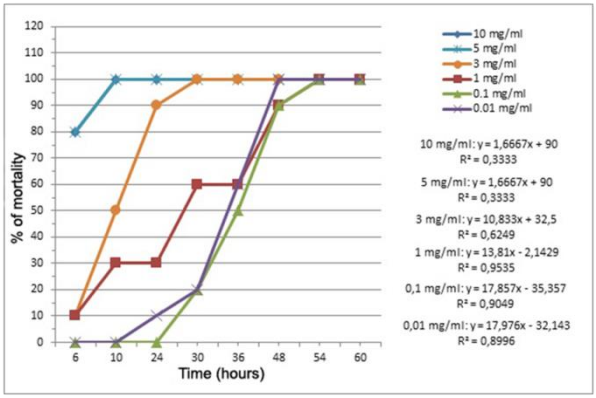
a. Lethality of *A. salina* - *J. oxycedrus* (Velestovo)



b. Lethality of *A. salina* - *J. excelsa* (Velestovo) vs time



c. Lethality of *A. salina* - *J. communis* (commercial sample) vs time



d. Lethality of *A. salina* - *J. foetidissima* (Chalakli) vs time

Figure1. Percentage of lethality of *A. salina* vs time: a. *J. oxycedrus* (Velestovo), b. *J. excelsa* (Velestovo), c. *J. communis* (commercial sample) and d. *J. foetidissima* (Chalakli)

Methanol extracts of the *Juniperus* species show positive results, which indicates that these samples are biologically active. The graphic representation of the % of lethality of *A.salina* vs time for extracts concentrations of 10 mg/mL, 5 mg/mL, 3 mg/mL, 1 mg/mL, 0.1 mg/mL and 0.01 mg/mL indicates linear correlation, suggesting a directly proportional relation between the concentrations of the extracts and the lethality degree. This is supported by the fact that in all assayed samples, the maximal mortality is registered at a concentration of 10 mg/mL, while concentrations of 0.1 mg/mL and 0.01 mg/mL cause minimal mortality within 24 h, a period considered as most convenient for evaluation of the mortality.

The results for LC_{50} (24 h) for all assayed samples, including the positive control - $K_2Cr_2O_7$, are confirmed in 95% interval of confidence. These results are calculated with probit analysis according to Finney [29], presented in Table 2.

Table 2. Results for LC_{50} (24 h) from *Juniperus* extracts and $K_2Cr_2O_7$ as positive control.

Sample	**Part used**	**Brine shrimp lethality (LC_{50} – 24 h µg/mL)**
Jc-c	needles	798
Je-D/11	needles	513
Je-U/11	needles	449
Je-O/10	needles	581
Jf-D/11	needles	656
Jf-U/11	needles	1716
Jf-V/11	needles	1526
Jf-O/11	needles	1108
Jo-O/10	needles	395
$K_2Cr_2O_7$		41

According to Meyer's coefficient of toxicity, 6 assayed extracts of *Juniperus* out of 9 in total, show toxic potential with LC_{50} < 1000 µg/mL. This LC_{50} range of values applies to Jc-c, Je-D/11, Je-U/11, Je-O/10, Jf-D/11 and Jo-O/10, while Jf-U/11, Jf-V/11, Jf-O/11 are practically non-toxic for *Artemia salina,* with LC_{50} > 1000 µg/mL. According to Clarkson, the extracts with toxic potential can further be classified as low, medium or highly toxic. The gained result from *J. communis* (LC_{50} – 798 µg/mL) is in correlation with the result gained from Krishnaraju et al. (LC_{50} – 690 µg/mL) [28]. None of the extracts was close to LC_{50} = 41 µg/mL of the positive control $K_2Cr_2O_7$, which is confirmed as toxic compound. However, in order to confirm the eventual toxic potential, performing further *in vivo* assays with higher animals is essential, as well as applying more specific and sophisticated bio-assays.

Plants have the ability to produce plenty of secondary metabolities, which enabled the great usage of various plant species during the centuries, for curing different illnesses. It is confirmed that plants possess numerous biological and pharmacological activities along with a potential to serve as chemotherapeutic agents or a starting point for the development of new medicines [32]. For instance, in a study from 2004 which included examination of the species *Phyllanthus engleri* by applying the Brine shrimp (*Artemia salina)* lethality assay, it was calculated LC_{50} of 0.47 µg/mL. Not so long ago, Englerin A has been isolated from this plant, which is a selective anti-cancerogenous compound that is used against cancerogenous cells in kidneys [33]. In this manner, applying the Brine shrimp (*Artemia salina)* lethality assay provides an additional scientific support for the potential of the anti-cancerogenous compounds in plant extracts [34]. Over the last few years, it has been proved that the analysis with brine shrimps is an excellent method for preliminary testing of the toxicity of new curative plants that are used for many applications, but also for a huge number of newly isolated biologically active compounds [35]. The experimental screening of the toxicity of curative plants is of key importance for the confirmation of their safety and efficiency [36].

CONCLUSION

Toxicological bioactivity of *Juniperus* methanol extracts was in the following order: Jo-O/10 > Je-U/11 > Je-D/11 > Je-O/10 > Jf-D/11 > Jc-c > Jf-O/11 > Jf-V/11 > Jf-U/11. The levels of the toxicity of *Juniperus* species are in correlation with the concentrations of the extracts. The biological assay where the *Artemia salina* species are used can determine the toxic potential of *Juniperus* extracts. However, the analysis of the toxicity with brine shrimps cannot explain the mechanism of the toxic effect of the extracts, still presents a valid and predictive factor for the evaluation of the oral acute toxicity, and the bioactivity of the plant extracts.

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Original scientific paper

SEED PROGENY OF PORTUGUESE FENNEL WILD POPULATIONS: MORPHOLOGICAL AND ESSENTIALS OILS VARIABILITY

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ABSTRACT

Previous studies on Portuguese fennels (*Foeniculum vulgare* Mill.) essential oils showed the existence of chemotypes (anethole, anethole / fenchone, anethole / methyl chavicol) that diverge from international standards. Progeny of seeds from forty-nine wild populations of *F. vulgare* accessions were evaluated for diversity through morphochemical traits. The accessions, collected between 2005 and 2009 from wild populations growing in northwest, centre and south of mainland Portugal, were evaluated using a set of standards based on the International Union for the Protection of New Varieties of Plants (UPOV) list of descriptors. The descriptors, modified to fit local specificities, were scored on 20 randomly selected plants per population. The essential oils (EO) were isolated by hydrodistillation from the fruits (seeds) progeny and analysed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectrometry (GC-MS). The percentage of three main compounds from the essential oils was used to determine the relationship between the different essential oil samples by cluster analysis. Cluster analysis for morphological descriptors defined two main groups. Cluster A, including the accessions from north-western Portugal, and Cluster B, including 5 subgroups: B4 grouping accessions from the south of Portugal, and the clusters B31, B32, B21 and B22, grouping accessions originated in central Portugal. The central region of Portugal was the area from which greater genetic variability of fennel wild populations was observed. The populations from northwest and southern areas showed both specific morphological and chemical characteristics: in the south, plants showed smaller umbels, fenchone rich EOs and lower essential oil yield; the fennel wild populations of northwest presented plants with larger leaves and largest umbels, with a high EO yield but the EOs were methyl chavicol (= estragole) and *trans*-anethole rich.

***Keywords**: Foeniculum vulgare; Portugal; evaluation; wild populations, genetic variability*

INTRODUCTION

Foeniculum vulgare Mill. (fennel) is a biennial or perennial plant belonging to the Apiaceae family. Fennel is native to the Mediterranean region, but came to be naturalized at diverse locations. Two subspecies of *Foeniculum vulgare* Mill. (= *F. officinale* All.) occur in Portugal (mainland, Azores and Madeira Archipelagos), *F. vulgare* Mill. subsp. *capillaceum* (= *F. vulgare* Mill. subsp. *vulgare*) and *F. vulgare* Mill. subsp. *piperitum*. The subspecies *capillaceum* includes three varieties: *azoricum* Mill. Thell. (Florence), *dulce* Mill. Thell. (sweet) and *vulgare* Mill. Thell. (bitter) [1, 2, 3].

It is a species with several applications in the food industry and also used in cosmetic and pharmaceutical products. The *vulgare* subspecies is characterized by having as main compounds two phenylpropanoids: estragole and *trans*-anethole and one oxygen-containing monoterpene: fenchone.

The Portuguese Genebank (Banco Português de Germoplasma Vegetal, BPGV) maintains wild medicinal and aromatic plants (MAPs) among which a national wild fennel collection (81 accessions, 2012 inventory). As part of a wider programme of characterization of several MAPs, one of the main BPGV targets is the *ex situ* conservation and evaluation of this genetic resources. For a proper conservation and valorisation of diversity, it is important to use internationally adopted formats and universally understood languages for plant genetic resources data which contributes to describe, conserve, manage and share information about those resources, whether maintained in genebanks or growing in their natural environments such as *in situ* and on farm.

Morphological well-defined descriptors are an indispensable tool for management, maintenance and utilization of plant genetic resources such as the descriptors developed by the International Union for the Protection of New Varieties of Plants (UPOV) and by Bioversity International (formerly the International Plant Genetic Resources Institute, IPGRI). Protocols to enable information sharing such as descriptors lists represent an important tool for a standardized characterization system and they are promoted by Bioversity International throughout the world, contributing to facilitate the international exchange and use of plant resources, aiming at achieving uniformity in data collection, description, storage and retrieval. Characterization of each sample involves a careful description of special characteristics that are inherited, easy to score, and expressed consistently in all environments. The chemical characterization represents a vital additional tool for aromatic and medicinal species evaluation, since it represents an advanced categorization, important to breeders, to governmental entities and to researchers in several scientific domains.

In order to facilitate the assessment of bitter fennel oil quality, the International Standard Organisation [4] defines two essential oil types: *trans*-anethole and phellandrene type. *trans*-Anethole essential oil type is characterized by high *trans*-anethole levels (50-78%), with 10-25% fenchone, 2-11% α-pinene, 1-6% estragole (= methyl chavicol), 1 6% limonene and trace-9% α-phellandrene. The phellandrene type bitter fennel oil contains 15-30% *trans*-anethole, 7-16% fenchone, 2-8% α-pinene, 2-7% estragole (= methyl chavicol), 8-30% limonene and 8-25% α-phellandrene.

According to the Portuguese Pharmacopoeia [5], bitter fennel seeds oil is characterized by 55-75% *trans*-anethole, 12-25% fenchone, 1-10% α-pinene, ≤ 6% estragole (= methyl chavicol) and 1-5% limonene. No relative amount is recorded for α-phellandrene, but it mentions that the α-pinene / limonene is >1%.

Previous studies on Portuguese fennels essential oils [6, 7, 8], have already shown the existence of chemotypes (anethole, anethole/fenchone, anethole/methyl chavicol) that deviate from these international accepted standards [4, 9].

Since 2008, the continuous and thorough evaluation of fennel genetic resources in Portugal is an objective of BPGV, by studying the morphological data and analysing the essential oils. After a preliminary evaluation of nine accessions [8], the study was extended to evaluate the morphological and essentials oils variability on seed progeny from forty-nine wild populations maintained in *ex situ* conditions.

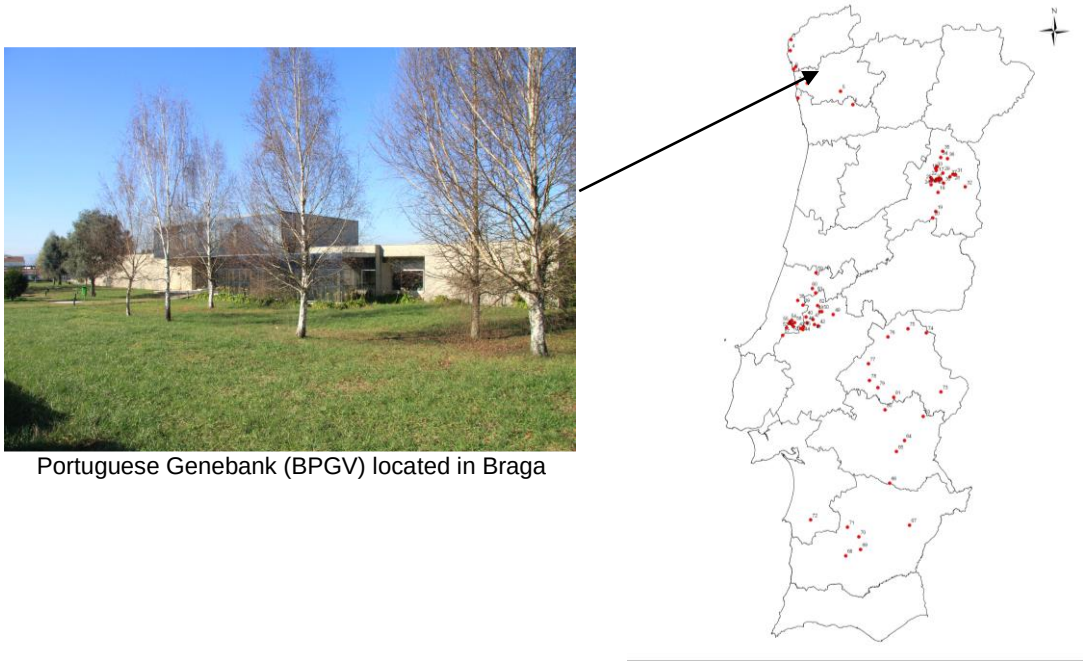
MATERIAL AND METHODS

Plant Material

Forty-nine wild fennel populations collected in Portugal from distinct locations at different altitudes and representing different ecological zones, including nine previously studied [8], maintained under *ex situ* conservation, have been grown in the field as individualized plants to evaluate their morphological characteristics and essential oil compositions (Table 1, Figure 1). Between 2008 and 2012, twenty randomly selected plants of each wild population were evaluated for morphological descriptors and their seeds were also assessed for essential oil composition. Two commercial varieties were also evaluated, bitter (Wild_comm) and sweet (*Doce*) essential oil types, respectively.

Morphological Characters

The set of thirty-three characterization and evaluation descriptors were adapted from the UPOV Test Guidelines for Fennel [10] and from previously defined descriptors developed at BPGV [8]. Eighteen morphological descriptors out of the thirty-three observed *per* plant were selected to this study on the basis of their usefulness and applicability in the analysis of morphological variability by to one-way analysis of variance, Table 2. The descriptors: time to appearance of main umbel, time of appearance of flower buds and time to beginning of flowering, considering the number of days since 1 January, did not show variability among populations. The evaluation was done at Braga, Portugal.



Portuguese Genebank (BPGV) located in Braga

Figure 1. The BPGV wild fennel collection (2012 inventory).

Table 1. Selected passport descriptors of forty-nine wild populations collected in Portugal.

Accession Number	Nº	District	Region
08872-BPGV	1	Viana do Castelo	Northwest
08875-BPGV	2	Porto	Northwest
08877-BPGV	3	Viana do Castelo	Northwest
08879-BPGV	4	Viana do Castelo	Northwest
08880-BPGV	5	Braga	Northwest
08881-BPGV	6	Porto	Northwest
08883-BPGV	7	Braga	Northwest
08884-BPGV	8	Braga	Northwest
08885-BPGV	9	Viana do Castelo	Northwest
09547-BPGV	11	Guarda	Central - mainland
09549-BPGV	13	Guarda	Central - mainland
09557-BPGV	18	Guarda	Central - mainland
09559-BPGV	20	Guarda	Central – mainland
09563-BPGV	24	Guarda	Central – mainland
09564-BPGV	25	Guarda	Central – mainland
09571-BPGV	32	Guarda	Central – mainland
09572-BPGV	33	Guarda	Central – mainland
09574-BPGV	35	Guarda	Central – mainland
09575-BPGV	36	Guarda	Central - mainland
09576-BPGV	37	Guarda	Central - mainland
09578-BPGV	39	Leiria	Central - coastal
09579-BPGV	40	Santarém	Central - coastal
09580-BPGV	41	Santarém	Central - coastal
09581-BPGV	42	Santarém	Central - coastal
09582-BPGV	43	Santarém	Central - coastal
09583-BPGV	44	Santarém	Central - coastal
09584-BPGV	45	Santarém	Central - coastal
09585-BPGV	46	Santarém	Central - coastal
09586-BPGV	47	Santarém	Central - coastal

Accession Number	Nº	District	Region
09587-BPGV	48	Santarém	Central - coastal
09588-BPGV	49	Santarém	Central - coastal
09589-BPGV	50	Santarém	Central - coastal
09590-BPGV	51	Leiria	Central - coastal
09591-BPGV	52	Leiria	Central - coastal
09594-BPGV	55	Leiria	Central - coastal
09596-BPGV	57	Santarém	Central - coastal
09598-BPGV	59	Leiria	Central - coastal
09600-BPGV	61	Leiria	Central - coastal
09601-BPGV	62	Santarém	Central - coastal
09873 -BPGV	63	Évora	South - mainland
09880-BPGV	64	Évora	South - mainland
09884-BPGV	65	Évora	South - mainland
09886-BPGV	66	Évora	South - mainland
09887-BPGV	67	Beja	South - mainland
09901-BPGV	68	Beja	South - mainland
09904-BPGV	69	Beja	South - mainland
09905-BPGV	70	Beja	South - mainland
09908-BPGV	71	Beja	South - mainland
09913-BPGV	72	Setúbal	South - coastal

Table 2. The eighteen descriptors used for the cluster analyses.

Code	Description
x1	Plant height before flowering (cm)
x2	Number of stems per plant
x3	Umbels / plant
x4	Height of main stem at flowering (m)
x5	Leaf length between apex and sheath (cm)
x6	Distance between 1st and 2nd leaf segment pairs (leaflet) (cm)
x7	Size of terminal leaflet (terminal needle-shaped segments) (cm)
x8	Petiole length (cm)
x9	Major leaflet size (cm)
x10	Minor leaflet size (cm)
x11	Sheath length (cm)
x12	Sheath width (cm)
x13	Umbel diameter / plant (cm)
x14	Size of the main peduncle of umbel / plant (cm)
x15	Rays / umbel (number)
x16	Length of Major Rays/umbel (cm)
x17	Length of Minor Rays/umbel (cm)
x18	Average weight of 100 fruits / plant (g)

Isolation, Identification and Quantification of the Essential Oils Composition

The essential oils were isolated and analyzed as detailed in [11].

Statistical Analysis

The interpopulations variability of fennel accessions was evaluated by cluster analysis as described in [8]. The percentage composition of the essential oils was used to complete the characterization of genetic variability of wild populations in *ex situ* conservation.

RESULTS AND DISCUSSION

Cluster analysis of accessions according to their morphological parameters, defined two main clusters, A and B (cophonetic coefficient r = 0.93, Figure 2, Table 3). B is a cluster compound where 5 subclusters were defined: B4 which groups accessions originating from the South of Portugal; the B3.1, B3.2, B2.1 and B2.2 which groups accessions from mainland Portugal central region.

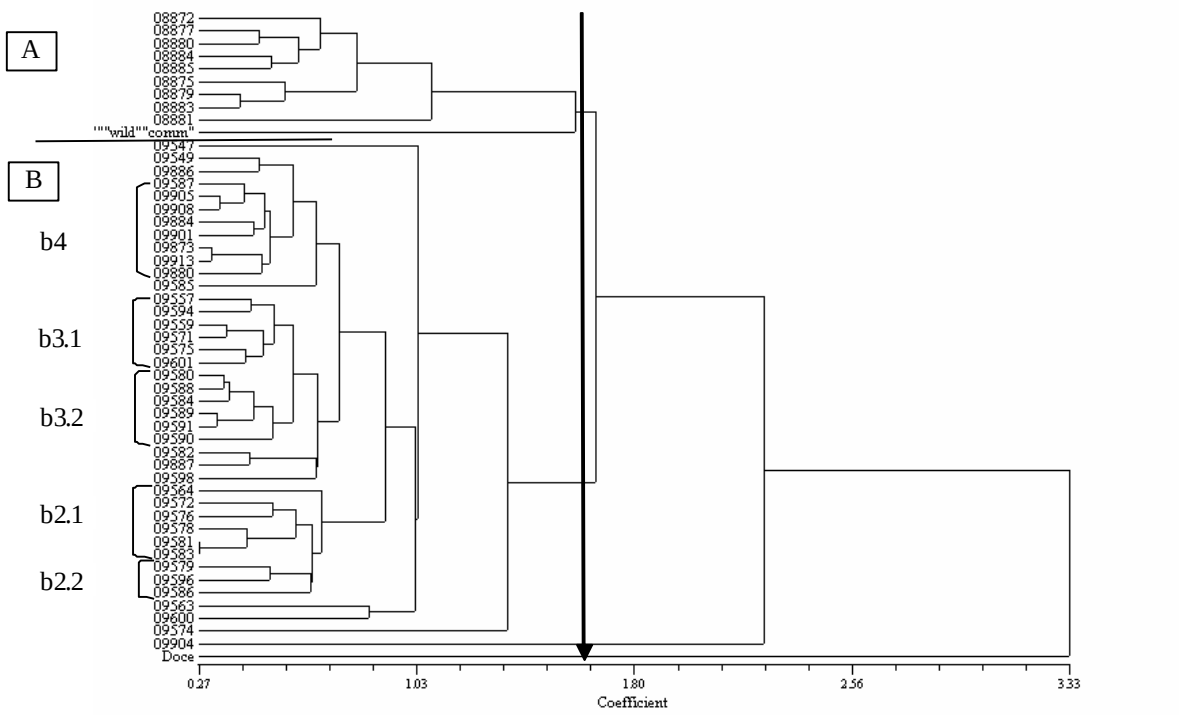


Figure 2. Dendrogram obtained by cluster analysis of the evaluation of eighteen descriptors observed on twenty randomly selected plants of forty-nine seed progeny of Portuguese fennel wild populations.

The analysis was made by comparing the average between cluster/subcluster of each morphological descriptor. To complete the characterization of genetic variability of wild populations the percentage composition of the essential oils and essential oil yield were considered (Table 4).

Cluster A groups accessions with higher expression of morphological descriptors: larger leaves and umbels, greater number of rays of the umbels, umbels with larger peduncle (Table 3). In terms of essential oils' yield, it varies between 2.7 and 3.4% being estragole and *trans*-anethole the two main essential oils components present (Table 4).

Subcluster B4 is formed by accessions showing umbels of smaller diameter, smaller peduncle and minor ray number/umbel ray and lower length of ray. However, the plants show higher "height of main stem at flowering", among those of cluster B accessions. This subcluster is characterised by two main essential oil components, *trans*-anethole and fenchone with yields varying between 0.58 to 1.29 %, (Tables 3, 4).

Subcluster B3.1 describes accessions of cluster B with larger diameter of umbel, greater length umbels peduncle, greater ray number, and greater leaflet size (Table 3). The essential oil yield is higher (3.1 to 4.5 %) and the composition of essential oils is a mix of fenchone and *trans*-anethole (Table 4).

Subcluster B3.2 is composed of accessions with smaller leaves size, the smallest height of main stem at flowering, with umbel's characteristics similar to those of the subcluster B4 but with higher essential oil yield (3.0 to 4.8%) and essential oils' composition similar to B3.1 showing the greatest estragole percentage (Tables 3, 4).

Considering Tables 3 and 4, subcluster B2.1 groups accessions whose umbels have characteristics comparable to those of B3.1 subcluster, although with greater plant height and larger leaves. The essential oil yield is variable (0.49 to 2.94%); the subcluster B2.2 groups accessions characterized by umbels like those of subcluster B3.1. It is also characterized by having the larger sheaths, larger width and length, and longer major leaflet size. The essential oil yield is low and the composition is a mix of the three main essentials oils (estragole, fenchone, and *trans*-anethole).

CONCLUSIONS

The central region of Portugal is the origin area of fennel accessions with greater morphological and chemical inter-variability.

The populations from mainland North-western and Southern areas have specific characteristics, both morphological and chemical: in the South the umbels are smaller while the essential oil contains higher fenchone percentage and essential oil yield is lower. In the Northwest the plants present larger leaves, largest umbels dimension and higher rays number, essential oil yield is significant and the main essential oils components are estragole and *trans*-anethole.

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Table 3. Characterization of forty-nine seed progeny, of Portuguese fennel wild populations, using morphological descriptors. For description of x code see Table 2.

Acc.*	x1 (cm)	x2 (nº)	x3 (nº)	x4 (m)	x5 (cm)	x6 (cm)	x7 (cm)	x8 (cm)	x9 (cm)	x10 (cm)	x11 (cm)	x12 (cm)	x13 (cm)	x14 (cm)	x15 (nº)	x16 (cm)	x17 (cm)	x18 (g)
08872	41.1	7.6	14.0	26.1	203.7	6.4	4.7	0.6	20.7	4.7	10.6	1.7	10.7	10.2	28.7	-	-	0.4
08877	31.5	5.9	20.3	29.5	195.9	7.8	5.8	0.6	25.0	5.8	10.4	2.3	10.6	9.9	26.7	-	-	0.3
08880	46.8	8.1	20.9	31.3	199.5	7.4	7.3	1.2	24.4	7.1	10.1	2.7	11.0	8.4	27.6	-	-	0.3
08884	39.8	9.9	14.3	31.0	245.4	7.6	6.0	0.8	22.9	4.9	11.8	2.3	9.4	7.7	21.2	-	-	0.3

Acc.*	x1 (cm)	x2 (nº)	x3 (nº)	x4 (m)	x5 (cm)	x6 (cm)	x7 (cm)	x8 (cm)	x9 (cm)	x10 (cm)	x11 (cm)	x12 (cm)	x13 (cm)	x14 (cm)	x15 (nº)	x16 (cm)	x17 (cm)	x18 (g)
08885	41.8	7.3	23.3	32.1	230.5	7.1	6.7	1.0	24.3	4.9	12.2	2.4	12.3	9.3	21.1	-	-	0.3
08875	43.3	15.7	23.0	28.7	200.0	7.7	5.1	1.4	21.5	4.9	11.8	2.3	9.8	9.0	20.7	-	-	0.3
08879	51.7	13.0	44.9	31.6	229.5	8.1	6.8	1.6	24.0	5.3	12.7	2.5	10.9	9.1	22.3	-	-	0.3
08883	47.5	12.2	32.1	30.2	239.1	7.7	5.5	1.6	23.7	5.0	11.4	2.5	9.4	9.0	21.5	-	-	0.3
08881	30.0	8.6	13.1	24.7	163.3	3.9	6.2	0.3	19.3	5.1	8.6	2.1	8.1	7.8	15.1	-	-	0.3
Wild-comm	51.5	8.7	72.9	30.5	208.7	8.0	5.1	2.1	22.3	5.1	11.6	2.3	14.3	8.5	28.9	-	-	0.6
09547	123.0	5.0	15.5	22.0	193.5	7.1	3.7	0.5	15.0	6.6	8.1	1.7	7.0	9.9	19.3	3.6	0.5	0.3
09549	103.5	9.0	9.3	22.9	190.5	5.3	3.3	1.0	13.9	2.4	8.8	2.1	6.7	7.3	14.4	3.3	0.7	0.3
09886	157.0	8.7	10.9	20.2	187.6	5.9	3.3	0.9	12.9	1.8	10.5	1.8	6.5	9.2	13.6	3.3	1.1	0.3
09587	172.7	6.1	15.1	22.3	200.0	5.1	3.2	0.7	15.3	1.4	10.8	2.2	6.3	6.0	13.2	3.1	0.7	0.3
09905	163.1	5.7	25.2	23.2	185.8	6.4	2.2	0.9	17.2	1.3	10.2	2.4	6.3	6.9	12.1	3.1	0.9	0.3
09908	145.9	6.6	26.8	21.6	185.7	6.1	2.1	1.0	15.6	1.1	9.9	2.0	5.7	5.7	11.2	2.9	1.0	0.3
09884	172.4	8.1	12.8	23.0	182.3	6.3	3.2	0.8	19.4	1.7	9.7	1.7	6.1	7.1	12.0	3.0	0.9	0.3
09901	163.1	7.6	23.8	19.4	193.9	5.7	3.2	0.9	14.3	2.1	8.8	1.6	5.8	5.3	10.4	2.9	1.0	0.3
09873	164.0	7.3	8.7	22.1	188.7	7.1	3.1	1.0	15.6	1.5	9.2	1.6	6.0	6.4	14.8	3.0	0.8	0.3
09913	153.9	6.0	12.3	21.9	181.9	7.4	1.8	1.0	14.7	0.8	9.1	1.9	5.6	6.3	14.3	2.8	0.7	0.3
09880	174.3	10.5	16.3	20.4	195.0	5.8	3.1	0.9	14.7	1.5	9.3	1.9	6.3	5.8	14.7	3.1	0.8	0.3
09585	124.3	4.4	13.6	23.8	200.0	6.1	2.4	0.4	15.5	1.8	11.7	1.7	6.7	7.6	20.7	3.3	0.9	0.3
09557	78.8	4.4	14.4	17.6	145.3	5.7	3.2	0.6	10.4	2.3	7.3	1.3	7.6	7.6	14.1	3.9	1.2	0.3
09594	86.5	4.3	10.7	19.4	177.8	5.4	2.6	0.5	13.6	1.5	8.4	1.7	8.7	8.2	13.4	4.4	1.2	0.3
09559	100.6	4.9	11.9	19.7	170.0	5.9	3.0	0.9	14.4	2.3	9.2	1.3	7.0	8.6	17.1	3.5	1.1	0.3
09571	88.4	5.2	7.9	21.9	179.7	6.2	3.2	0.9	16.1	2.2	8.0	1.1	7.7	6.9	19.8	3.8	1.0	0.3
09575	100.6	5.3	15.5	17.6	176.4	5.7	2.9	0.5	16.4	2.2	10.0	2.1	8.3	7.9	18.6	4.2	0.8	0.3
09601	119.7	7.3	10.7	15.3	173.2	6.1	2.1	0.6	13.9	1.7	8.9	1.5	8.5	8.2	19.1	4.3	0.9	0.3
09580	118.9	3.6	6.3	18.2	164.8	5.1	1.9	0.3	10.0	1.1	7.6	1.2	6.2	7.2	14.4	3.2	0.8	0.3
09588	107.1	4.5	5.3	19.4	174.7	4.9	3.0	0.8	12.6	1.6	8.1	1.3	5.6	7.3	11.6	2.8	1.1	0.3
09584	121.8	5.5	15.0	19.7	190.2	5.7	2.1	1.0	12.5	1.5	8.3	1.3	6.1	7.2	12.4	3.2	0.8	0.3
09589	108.6	3.9	6.4	19.2	162.9	5.5	2.4	1.1	9.5	1.6	8.3	1.1	5.9	6.0	14.8	3.1	0.8	0.2
09591	90.2	4.4	11.8	17.2	151.5	4.5	2.2	0.6	10.2	1.3	8.7	1.6	6.7	5.6	16.3	3.5	0.9	0.2
09590	90.8	4.5	22.1	15.5	162.0	4.9	1.9	0.9	8.3	1.2	5.8	1.4	7.1	7.2	16.3	3.6	0.9	0.3

Acc.*	x1 (cm)	x2 (nº)	x3 (nº)	x4 (m)	x5 (cm)	x6 (cm)	x7 (cm)	x8 (cm)	x9 (cm)	x10 (cm)	x11 (cm)	x12 (cm)	x13 (cm)	x14 (cm)	x15 (nº)	x16 (cm)	x17 (cm)	x18 (g)
09582	97.3	3.0	5.5	20.4	177.5	6.4	3.9	0.7	11.6	2.5	8.6	1.2	6.5	5.5	9.8	3.3	0.9	0.3
09887	143.9	4.5	6.0	17.7	167.4	6.8	3.3	0.7	12.4	2.1	10.1	1.6	5.8	4.5	10.6	3.0	1.2	0.3
09598	102.4	3.5	1.9	19.1	163.5	4.9	2.1	0.6	11.9	1.4	7.8	1.3	8.2	6.2	12.0	4.1	1.8	0.3
09564	135.2	8.9	19.5	24.8	158.3	6.1	4.4	2.2	19.7	3.3	9.9	2.8	8.6	7.3	20.6	2.3	1.1	0.3
09572	165.5	4.2	22.6	26.1	191.6	7.2	4.4	0.7	19.7	3.0	10.4	2.3	6.6	5.0	18.9	3.3	0.8	0.3
09576	190.7	6.9	11.7	21.7	196.3	6.0	3.9	0.4	17.3	3.0	11.3	2.3	8.4	5.3	17.8	4.2	1.0	0.3
09578	175.6	6.6	24.4	25.8	191.6	7.0	4.2	1.0	19.4	2.7	7.9	2.4	8.3	8.4	16.0	4.1	1.1	0.3
09581	182.4	7.2	11.5	25.7	198.9	7.3	3.7	0.8	18.8	2.5	8.4	2.1	6.5	7.6	15.7	3.3	0.9	0.3
09583	171.8	6.7	7.4	24.6	200.0	7.0	3.8	1.5	18.4	2.3	8.7	2.5	6.9	7.5	15.4	3.6	1.0	0.3
09579	187.1	6.7	26.2	26.1	196.2	6.4	4.7	1.8	18.9	3.1	11.2	2.7	9.3	8.2	19.1	4.7	1.4	0.3
09596	185.7	4.7	27.0	28.5	200.0	6.5	4.0	1.0	22.1	2.6	10.5	3.3	7.6	8.0	16.6	3.8	1.1	0.4
09586	185.1	8.6	25.3	31.0	200.0	8.0	4.5	1.5	22.4	2.5	11.8	2.6	6.4	6.4	14.6	3.2	0.9	0.3
09563	98.4	4.3	19.8	18.3	171.0	5.8	2.7	0.5	16.1	2.3	9.9	1.5	11.3	10.0	19.5	5.6	2.1	0.3
09600	129.0	4.8	26.4	17.2	176.6	4.4	2.9	0.4	14.7	3.1	11.3	2.1	9.5	8.5	21.0	4.8	0.9	0.4
09574	94.5	4.9	2.5	19.3	-	5.2	2.5	0.4	12.7	2.0	9.6	1.6	7.2	8.1	16.7	3.6	0.8	0.3
09904	150.0	5.2	28.5	0.0	150.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	6.9	4.8	15.0	3.2	0.5	0.2
Doce	45.3	10.7	10.9	37.4	62.0	11.6	-	10.3	-	-	-	-	14.8	-	-	-	-	-

*Acc.: Accessions. Commercial varieties: bitter (Wild_comm) and sweet (*Doce*) types

Table 4. Characterization of forty-nine seed progeny, of Portuguese fennel wild populations, using essential oil yield and main components percentage.

Acc.*	α-Pinene	Fenchone	Estragole (= Methyl-chavicol)	*trans*-Anethole	Oil-Yield (%, v/d.w.)
08872	0.3	16.3	28.7	47.5	3.0
08877	0.4	12.3	75.0	4.9	2.8
08880	1.1	12.7	72.2	4.0	2.7
08884	0.8	13.3	47.8	31.5	3.1
08885	1.0	19.8	18.0	58.1	3.0
08875	0.8	15.8	3.0	72.5	3.4
08879	0.8	13.2	35.1	44.2	3.1
08883	0.8	16.6	58.5	17.6	3.1
08881	0.4	16.7	73.5	0.1	2.7
Wild-comm	0.8	24.3	1.4	71.4	4.8
09547	1.3	38.6	34.3	16.8	3.8
09549	0.8	29.5	30.2	31.1	3.8
09886	0.1	26.3	20.9	51.1	1.3
09587	0.3	32.8	15.6	45.9	0.9
09905	0.5	45.0	6.2	38.0	0.6

Acc.*	α-Pinene	Fenchone	Estragole (= Methyl-chavicol)	*trans*-Anethole	Oil-Yield (%, v/d.w.)
09908	0.2	37.0	5.5	55.1	0.8
09884	0.0	18.4	1.8	78.5	0.7
09901	0.1	19.5	1.8	77.1	0.9
09873	0.1	26.2	2.5	69.5	1.1
09913	0.2	34.8	15.3	9.3	1.3
09880	0.4	21.7	2.6	73.4	1.2
09585	5.2	26.1	9.3	42.7	3.8
09557	1.6	18.7	40.5	30.4	3.0
09594	3.1	27.1	31.5	29.7	3.8
09559	0.8	24.8	33.4	29.0	3.1
09571	1.1	26.7	2.3	58.3	4.5
09575	1.6	26.6	30.8	28.9	3.8
09601	3.4	29.1	9.3	44.4	4.1
09580	3.9	20.9	40.5	24.4	3.0
09588	5.2	38.9	14.6	22.8	3.0
09584	4.4	39.4	22.6	16.8	3.8
09589	2.6	20.9	43.7	14.4	4.8
09591	3.9	22.0	7.5	56.5	3.0
09590	2.1	32.0	7.9	49.9	3.0
09582	1.6	33.1	5.4	44.6	3.0
09887	0.1	12.0	9.5	77.9	-
09598	5.9	21.1	22.2	36.8	3.0
09564	0.4	34.7	22.3	39.3	2.5
09572	0.1	20.4	37.8	40.2	0.5
09576	0.2	16.9	2.5	77.7	2.9
09578	0.6	23.4	66.0	7.9	1.6
09581	0.1	18.9	11.3	68.3	0.9
09583	0.2	19.0	37.3	41.5	1.1
09579	0.2	12.5	13.2	71.6	-
09596	0.2	24.8	21.8	51.7	0.7
09586	0.3	28.5	31.1	37.5	0.7
09563	1.2	23.2	3.4	62.0	3.8
09600	1.4	17.7	3.2	69.8	4.0
09574	5.7	24.5	3.2	54.8	4.1
09904	0.2	26.5	22.7	49.0	-
Doce	-	-	-	-	-

*Acc.: Accessions. Commercial varieties: bitter (Wild_comm) and sweet (*Doce*) types

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Original scientific paper

ANTIOXIDANT ACTIVITY, TOTAL POLYPHENOLS CONTENT AND FLAVONOIDS CONTENT OF AQUEOUS EXTRACTS OF SOME ALBANIAN MEDICINAL PLANTS

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ABSTRACT

Antioxidant activity, total polyphenol content and flavonoid content of aqueous extracts of; lemon balm (*Melissa officinalis*), elderberry *(Sambucus nigra),* mint (*Mentha piperita*), chamomile (*Matricaria chamomilla*), elder flower (*Sambucus nigra*), lavender (*Lavandula angustifolia*) were studied.

The antioxidant activity was investigated by the 2,2´-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The highest capacity to scavenge DPPH radicals was found in elder flower (*Sambucus nigra*) extract.

Total phenols content of the aqueous extracts of selected medicinal plants was determined spectrophotometrically following the Folin-Ciocalteau colorimetric method, using acid gallic as a standard. The quantitative estimation of the phytochemical constituents showed that the total polyphenols in lemon balm (*Melissa officinalis*) are found to be much higher than in other herbs.

Determination of the total flavonoids was measured with the aluminum chloride colorimetric assay. The data of the total flavonoids content of the dry herbs were expressed milligrams of (+)catechin equivalents (CE)/g. The highest flavonoid concentration was observed in aqueous extract of elder flower (*Sambucus nigra*).

Key words*; Total phenols, flavonoid, DPPH, antioxidant.*

1. INTRODUCTION

Plants are used by humans in daily life in many different ways, including as food, herbal medicines, and cosmetics [1]. In cosmetics they are used for many beneficial properties, such as sunscreen, anti-aging, antioxidant, anticellulite, and antimicrobial agents [2]. As over-counter drugs botanicals are used in the treatment of acne, inflammatory skin diseases, skin infections, UV-induced skin damage, skin cancer, alopecia, vitiligo, and wounds [3]. Antioxidants such as flavonoids and phenolic acids are supposed to play the main role in

fighting against free radical species that are the main cause of numerous negative skin changes [4, 5]. Antioxidants protect the skin matrix through the inhibition of enzymatic degradation, or to promote collagen synthesis in the skin[6-8].

The increasing interest in the powerful biological activity of plant phenolics and flavonoids outlined the necessity of determining their content in medicinal herbs. In the present study, a comparative evaluation of the polyphenols, flavonoid content and antioxidant capacity were carried out in six albanian medicinal plants: lemon balm (*Melissa officinalis*), elder *(Sambucus nigra)*, mint (*Mentha piperita*), chamomille (*Matricaria chamomilla*), lavender (*Lavandula angustifolia*).

2. MATERIAL AND METHODS

2.1 Reagents and standards

Folin-Ciocalteau 2N (Sigma –Aldrich), sodium bicarbonate methanol, ethanol, gallic acid (Sigma –Aldrich), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ferric chloride, aluminum chloride, ethyl acetate, formic acid, glacial acetic acid, rutin, magnesium powder.

2.2 Samples

Plant used were: lemon balm (Melissa officinalis), elder fruit (Sambucus nigra), mint (Mentha piperita), chamomille (Matricaria chamomilla), elder flower (Sambucus nigra), lavander (Lavandula angustifolia).

2.3 Phytochemical screening

2.3.1 Shinoda reaction

Extracts were mixed with few fragments of magnesium in a test tube and some drops of concentrated hydrochloric acid were added drop wise. The red color appearance indicates the presence of flavonoids in the extracts.

2.3.2 Tannins

1 g of the powdered drugs was extracted with methanol (10 ml) on a water bath at about 60°C for 5 min. The extracts were filtered and then treated with ferric chloride reagent. The blue color appearance indicates the presence of tannins in the extracts.

2.3.3 The TLC analysis of *plants* flavonoids

The TLC analysis of plants flavonoids were done according to Wagner et al. (1984) with some modifications. 1 g of the powdered drugs was extracted with methanol (10 ml) on a water bath at about 60°C for 5 min. The extracts were applied on TLC silica gel F_{254} plates (Merck) as a spots (25µl) for chromatographic separation of the extracts using as mobile phase: Ethyl acetate-formic acid-glacial acetic acid-water (l00:ll:l1:26). As a reference compounds was used rutin (quercetin-3-O-rutinoside) prepared as 0.05% solutions in methanol, and 10 µl was used for chromatography. It was allowed to develop the chromatogram over a path of 15 cm. After completion of the chromatograms the plates were dried in air to remove all acids solvents from silica gel plates before detection. The spots of the plate were initially detected with UV light (254 nm) and then were sprayed with ferric chloride solution. The second plate was sprayed with DPPH (0.2 % w/v) solution for preliminary screening of antioxidant capacity of the extract.

2.4. Bioactive phytochemicals determination

2.4.1 Plant extracts preparation 0.5 g of dried sample of plants was extracted with 50 ml distillated water, on an ultrasonic bath (KQ3200E) for 25 minutes at 40 °C. 5 ml of the extracts was ultracentrifugated for 5 minutes at 14,000 rpm.

2.4.2. Total flavonoids assay
Total flavonoids content was evaluated according to a colorimetric assay with aluminium

chloride, developed by Zhishen, Mengcheng, and Jianming (1999). A 1 mL aliquot of oregano extract (appropriately diluted) or standard solution of catechin (100, 250 and 500 mg/L) was added to a 25 mL volumetric flask containing 10 mL of distilled water, followed by the addition of 0.75 mL of solution of $NaNO_2$ (5%). After 5 min, 1.5 mL of a 5% solution of $AlCl_3$ was added and 6 min later, 5 mL of NaOH (1 mol/L) was added to the mixture. The total volume was made up to 25 mL with distilled water, the solution was mixed and the absorbance was measured at 510 nm (with a UV-VIS Spectrophotometer SPECORD 40) against blank. Catechin was used as the standard for the construction of a calibration curve and the concentrations are expressed as catechin equivalents (mg/g CE). All samples were analyzed in triplicates. Three replicates of each sample were used for statistical analysis and the values were reported as mean.

2.4.3 Total phenolics assay
Total phenolics content in selected medicinal plant species was determined spectrophotometrically according to the Folin-Ciocalteau colorimetric method Vinha et al.(2012 with some modifications) using acid gallic as the standard and expressing the results as acid gallic equivalents (GAE) per gram of dry weight. In brief, 50µl of each extract was mixed with 0.75 ml of Folin–Ciocalteau reagent (1:10), and allowed to stand at 22°C, for 5 min. A 0.7 ml of sodium bicarbonate solution (8%) was added to the mixture. After 60 min at 22°C, absorbance was measured at 725 nm using a UV–Vis spectrophotometer (Specord 40). Total phenolic contents were quantified through a calibration curve obtained from measuring the absorbance of known concentrations of a gallic acid standard (r2 =0.9991) The results were expressed as mg of gallic acid equivalents (GAE)/ml of extract (**Table1**). All samples were analyzed in triplicates and the values were reported as mean.

2.5. DPPH radical scavenging activity (1,1- Diphenyl-2-picryl-hydrazyl radical)
Free radical scavenging ability was measured by the use of DPPH (1,1-diphenyl-2-picrilhydrazyl) stable radical. 100 µl of extract were mixed with 3 ml of DPPH solution. After 16 minutes the absorbance was measured at 525 nm using methanol as blank. All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula.

$$\% \text{ inhibition} = \mathbf{[(Ao-At) / Ao] 100}$$

where $\mathbf{A_0}$ is the absorbance of the control at t = 0 min;

and **At** is the absorbance of the antioxidant at t = 16 min. All samples were analyzed in triplicates and the values were reported as mean.

RESULTS AND DISCUSSION

Phytochemical screening

Table 1 Phytochemical constituents of plants extracts

Plant extracts	Flavonoids	Tannins
Lemon balm (*Melissa officinalis*)	+	+
Mint (*Mentha piperita*)	+	+
Lavender (*Lavandula angustifoglia*)	+	+
Chamomille *Matricaria chamomilla*	+	+
Elder (*Sambuci nigra)*	+	+
Elderberry (*Sambuci nigra)*	+	+

a (+) Presence of phytochemical compounds.

(-) Absence of phytochemical compounds.

We used thin layer chromatography TLC for the preliminary screening of plants flavonoids. The mobile phase used was suitable as a screening system for the TLC investigation of flavonoid glycosides. We used as mobile phase: ethyl acetate-formic acid-glacial acetic acid-water (l00:ll:l1:26. Flavonoids were observed in UV light (254 nm) on plates containing a UV-fluorescent indicator (silica gel F254) and they appeared as dark spots. We used aqueous solution of ferric chloride as a general spray reagent for detection of phenolic compounds. Blu-black spots appeared as the result of reaction of flavonoids with ferric chloride.

We used as reference compounds rutin (R_f 0.36). Several zones of flavonoids appeared in all plants extracts with higher intensity for elderflower extract: two main zones with Rf 0.36 (rutin), Rf 0.44, Rf 0.49, Rf 0.67 and Rf 0.98 that may correspond to phenol carboxylic acids (Wagner et al., 1984), among others.

The marked spots of elderflower extract were scrapped and collected separately along with the adsorbent and eluted with methanol. The resulting filtered solutions were subjected to ultra violet spectral studies. The solutions exhibited major absorption maxima, in the range of the flavonoids.

Total flavonoids

The results of total phenolics, total flavonoids and flavonoids/phenolics ratio are presented in **Table 2.**

The amount of total phenolics varied from 6.02 to 56.08 mg GAE/g dry material. The highest amount of total phenolics was found in lemon balm extract. Other studies suggest that lemon balm is rich in phenolic compounds such as rosmarinic acid (ester of caffeic acid) [16-17]. Rosmarinic acid has a number of interesting biological activities, e.g. antiviral, antibacterial, anti-inflammatory and antioxidant[18-19]. Elder was also rich in polyphenols. However some chemical components such as sugars or ascorbic acid present in the extracts, can interfere (Singleton and Rossi, 1965) with the results.

The total flavonoid content of plant extracts were expressed in term of catechin equivalents (the standard curve equation: $Y = 0.0012X - 0.0056$, $r^2 = 0.9997$).

Total flavonoids was highest in case of extracts of *Sambucus nigra flower*, *Melissa officinalis*, *Lavandula angustifoglia and Mentha piperita,* while lowest percentage inhibition was observed in case of *Matricaria chamomilla and Sambucus nigra* fruit. We find a good correlation between the total flavonoid content in plant extracts analysed with the colometric assay and our preliminary TLC flavonoids analyses.

Table 2. The total phenolic, flavonoid and flavonoid-phenolic ratio of plants.

Plants	**Total phenolics mg GAE/g dry powder]**	**Total flavonoids [mg CE/g dry powder]**	**Flavonoid/ Phenolic**
Lemon balm (*Melissa officinalis*)	**56.08**	34.77	0.62
Mint (*Mentha piperita*)	37.67	25.93	0.69
Lavender (*Lavandula angustifolia*)	39.60	30.88	0.78
Chamomille (Matricaria chamomilla)	6.02	4.99	0.83
Elder (*Sambucus nigra)*	53.28	**47.41**	**0.89**
Elderberry(*Sambucus nigra)*	21.90	10.90	0.51

Values expressed as mean obtained from 3 measurements.

DPPH Free-Radical Scavenging Activity

Phenolics have the ability to neutralise free radicals through transfering of hydrogen [20-21]. Phenolics antioxidant activity depend on both the structural difference and the glycosylation patterns[22].

Table 3. Antioxidant activity of plants extracts

Plant extracts	Percentage inhibition of DPPH radical (%)
Lemon balm (*Melissa officinalis*)	88.28
Mint (*Mentha piperita*)	88.91
Lavender (*Lavandula angustifolia*)	80.97
Chamomille	28.55
Elder (*Sambuci nigra)*	**90.50**
Elderberry (Sambuci nigra)	48.59

Values expressed as mean obtained from 3 measurements.

Our results suggest that percentage inhibition was highest in case of extracts of *Sambucus nigra flower, Melissa officinalis, Mentha piperita and Lavandula angustifolia* while lowest percentage inhibition was observed in case of *Matricaria chamomilla and Sambucus nigra* fruit.

4. CONCLUSION

The studies revealed that *Sambucus nigra flower* has the highest free radical scavenging ability and this might be linked to its higher total flavonoid content. We suggest that elder is an interesting plant for further investigations.

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Additional Paper

PHYTOCHEMISTRY OF THE ESSENTIAL OIL OF *MELLISA OFFICINALIS L.* GROWING WILD IN MOROCCO: PREVENTIVE APPROACH AGAINST NOSOCOMIAL INFECTION

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ABSTRACT

Hydro-distilled essential oil from lemon balm *(Mellisa officinalis* L.) growing wild in Morocco was analyzed by gas chromatography (GC) and (GC–MS) in order to elucidate for the first time the chemical character of this Moroccan balm species. Thirty three components were identified representing 96,2% of the total oil composition. The yield of essential oil was 0.4% and the predominant components were citronellal (14.4%), isogeraniol (6.4%), geraniol acetate (10.2%), nerol acetate (5.1%), caryophyllene (8.1%) and β-Caryophyllene oxide (11%). Antibacterial activity of the oil was tested against four bacterial strains responsible of Nosocomial Infection: *Pseudomonas aeruginosa*; *Klebsiella pneumonia; Staphylococcus aureus* and *Citrobacterkoseri* using disc diffusion method. Results showed that the essential oil from *Mellisa officinalis* exhibited the higher activity against all bacterial strains tested. Therefore, the essential oil extracted from lemon balm can be used to clean the environment of Reanimation Polyvalent and Anesthesia service.

Keywords*: M. officinalis, Essential oils, Antibacterial activity, Nosocomial infection*

INTRODUCTION

Lemon balm (*Melissa officinalis L)* is a perennial herb in the mint family Lamiaceae, native to southern Europe and the Mediterranean region [1]. In Morocco, this plant grows wildly in Sefrou region where it's popularized and applied for tea as a tranquilizer due to its health profit. Reports indicated that lemon balm had many beneficial effects such as anti-bacterial, sedative, spasmolytic, mnemonic improvement, and could reduce excitability, anxiety, stress, gastrointestinal disorders and sleep disturbance [1, 2, 3]. The essential oil of *M.officinalis* is a well-known antibacterial, antifungal and antioxidant agent [4, 5, 6, 7].

The main goal of the present work was to evaluate for the first time the phytochemicals of the essential oil of *M. officinalis* growing wild in Morocco. We determined, moreover, the

antibacterial activity of lemon balm against bacterial responsible of the nosocomial infection contracted at patients in the University Centre Hospital of Fez Morocco. To the best of our knowledge, the antimicrobial activities using lemon balm essential oil belonging to this region has not been carried out before.

MATERIAL AND METHODS

Plant material

Fresh leaves of M. officinalis were collected in the winery from the hills of the Sefrou city "Morocco" in the April 2013 and were dried for 7 to 10 days in the shade at room temperature then stored in cloth bags at 5°C and transferred later to the laboratory for preparation of the plant extracts.

Isolation of the essential oil

200g from the air-dried leaves of the M. officinalis were subjected to hydrodistillation for 3 h with 600 ml distilled water using a Clevenger-type apparatus according to the European Pharmacopoeia [8]. The oil obtained was collected and dried over anhydrous sodium sulphate and stored in a refrigerator at 4-5°C prior to analysis. Yield based on dried weight of the sample was calculated.

Gas Chromatography-Mass Spectrometry (GC-MS):

The analysis of the volatile constituents was run on a Thermo Fischer capillary gas chromatograph directly coupled to the mass spectrometer system (model GC ULTRA S/N 20062969; Polaris QS/N 210729), using an HP-5MS non polar fused silica capillary column (60 m x 0.32 mm, 0.25 μm film thickness). The operating condition of GC oven temperature was maintained as: initial temperature 40°C for 2 min, programmed rate 2°C/min up to final temperature 260°C with isotherm for 10 min; injector temperature, 250°C. The carrier gas was Helium, flow rate 1ml/min. Samples were run in hexane with a dilution ratio of 10:100. The volume of injected specimen was 1μl of diluted oil; splitless injection technique; ionization energy 70eV, in the electronic ionization mode; ion source temperature 200°C, scan mass range of m/z 40-650 and interface line temperature 300°C. The essential oils components were identified by comparing their retention times and retention indexes, as well as their MS spectra with those reported in the literature [9, 10].

Antimicrobial activity assessment

Microorganisms: Pseudomonas aeruginosa; Klebsiella pneumonia; Staphylococcus aureus and Citrobacter koseri. These bacteria were isolated in hospital environment from clinical patients in reanimation service (CHU, Morocco).

Disc-diffusion assay: For the experiments of Susceptibility screening test of the bacterial we are using the agar-disc-diffusion method as mentioned earlier [11]. Each microorganism stock was suspended in Mueller-Hinton (MH) broth and then incubated at 37°C for 18–24h. The overnight cultures were diluted and adjusted in order to get a density of 108c.f.u./ml (0.5 McFarland turbidity standard). They were flood-inoculated onto the surface of MH agar and 6mm diameter, sterile filter discs of Whatman paper N3, were impregnated with 15μg/disc of the essential oil and were delivered into the inoculated agar (MH). The plates were incubated for 18 h at 35°C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the tested microorganisms. The discs antibiogram of: Imipenem (IMI),

Cefaclor (CEF), Oxacilin (OXA), Vancomycin (VAC) are the standard drugs for comparison. The tests were carried out in duplicates. Results were interpreted in terms of a diameter of inhibition zone: resistant (D <6mm), intermediaries (6mm<D<13mm) and sensible (D >13mm).

RESULTS AND DISCUSSION

Essential oil composition

The essential oils obtained from the leaves of M. officinalis from Sefrou region "Morocco" were yellow in colour with a yield of 0.4% V/m and were subjected to GC-MS. The obtained yields are higher than the leaves yielded and studied as usually between 0.06 and 0.39% V/m [8]. The total content in the herb is relatively low increasing its production cost and consequently its commercial price. Thirty three components were identified representing 89,3% of the total oil in leaves composition. Six predominant components followed in the essential oils from Sefrou lemon balm were citronellal(14.4%), isogeraniol (6.4%), geraniol acetate (10.2%), nerol acetate (5.1%), caryophyllene (8.1%) and caryophyllene oxide (11%), representing 55.2% of the total oil. This composition is slightly different to the essential oil of Germany M. officinalis in which the major components were α-Citral (20.13) β-Caryophyllene (17.31) β-Citral (13.58) Citronellal (3.86) [12] and to the essential oil of Turkey M. officinalis in which the major components were citronellal (39%) and citral (33%) [13].

Antibacterial activity

The antibacterial activities of essential oil from M. officinalis was tested by agar disc diffusion method against four bacteria strains (Pseudomonas aeruginosa, Klebsiella pneumonia, Staphylococcus aureus and Citrobacter koseri.) responsible of nosocomial infections in Centre Hospital University of Fez Morocco. The results, given in Table 2, reveal that the essential oil of M. officinalis inhibited the growth of bacteria such as Pseudomonas aeruginosa (16 mm), Klebsiella pneumonia (13 mm) Staphylococcus aureus (20 mm) and Citrobacter koseri. (14 mm) and showed stronger antibacterial activity when compared to standard antibiotics used as controls (Cefaclor, Oxacilin and Vancomycin). This result may be explained by the high content of Citronellyl tiglate, Geraniol acetate, Caryophyllene, Caryophyllene oxide, Isogeraniol and Nerol acetate found in the essential oil. However, it is possible that other minor molecules modulate the activity of the main components.

The biological activity and medicinal value of plants are usually due to their phytochemical profiles, whose composition is totally dependent on geographical and environmental factors. M. officinalis from Morocco is rich in these compounds and shows an interesting antibacterial activity. Lemon balm is used in the Sefrou region in folk medicine for the treatment of headaches, indigestion, colic, nausea, nervousness, anemia, vertigo, syncope, malaise, insomnia, epilepsy, depression, psychosis and hysteria.

Table 1: Chemical composition of the essential oil from leaves of *M. officinalis*.

Compounds	Area (%)	Retention index (RI)
Camphene	2,1	915
α-pinene	0,6	936
cis-p-Meth-2 en-7-ol	3,8	956

2-pinen-4-one	1,75	967
Nerol acetate	**5,1**	980
Citronellal	**14,4**	1021
Nerol	3,5	1036
Patchoulene	1,6	1062
1R-à-Pinene	0,7	1077
Isogeraniol	**6,4**	1089
Geraniol	1,0	1137
Verbenol	0.9	1136
Carane	2,32	1149
Geraniol acetate	**10,2**	1151
Menthol	2,2	1172
Cinerone	0,7	1206
cis-Z-Bisabolene oxide	0,6	1235
Verbenone	0,6	1259
Aromadendrene oxide	1,6	1287
β-Caryophyllene	**8,2**	1309
Aromadendrene oxide	1,8	1333
Andropholide	0.6	1365
Caryophyllene oxide	**11**	1411
cis-Myrtanol	0,9	1446
Germanicol	1,2	1489
Longifolene	0,7	1499
Himachalene	0,7	1515
Himachala-2,4-diene	0,6	1531
Cubenole	0,6	1565
Pimara-7,15-dien-3-one	1,8	1586
Cycloisolengifolene	1,7	1605
Cholest-5-en-7-ol	0,9	1632
Lupan-3-ol acetate	0.5	1665
Total	**89.3**	

Table 2: Zones of growth inhibition (mm) showing antibacterial activity of *Mellisa officinalis* essential oils (diameter of the zone of inhibition includes paper disk diameter, 6 mm), compared to the disk of antibiotic as standard (Mean ± S.D.).

Bacterial species	**Inhibition zone (mm)** Essential oil (15 µl/disc)	**Inhibition zone (mm) Antibiotics**
Klebsiella pneumoniae	**13**	**22 (IMP), 0 (CEF), 0 (OXA), 0 (VAN)**
Pseudomonas aeruginosa	**16**	**27 (IMP), 7 (CEF), 0 (OXA), 12 (VAC)**
Staphylococcus aureus	**20**	**42(IMP), 0 (CEF), 18 (OXA), 15 (VAN)**
Citrobacter koseri	**14**	**12 (IMP), 0 (CEF), 7 (OXA), 11 (VAC)**

Abbreviations: Standard antibiotic disks: Imipenem IMP, Cefaclor CEF, Oxacilin OXA, Vancomycin VAC.

CONCLUSION

The oil was found to have significant antibacterial activity and therefore can be used as a natural antimicrobial agent for the treatment of several infectious diseases caused by these germs, which have developed resistance to antibiotics in Centre Hospital University of Fez Morocco.

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Section III

"MAP Cultivation, Breeding and Biotechnology"

Original scientific paper

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION, AND *IN VITRO* CONSERVATION OF SOME ALBANIAN POPULATIONS OF SAGE (*SALVIA OFFICINALIS* L.)

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ABSTRACT

The effectiveness of the programs of germplasm preservation and exploitation of aromatic-medicinal species requires detailed information on the variation of the species and their distribution inside and among populations. Present work describes the results on the molecular evaluation of populations of *Salvia officinalis* L. from Albanian territory, based on the use of different categories of molecular markers as RAPDs, SSRs, AFLPs, and aimed to compare the level of discrimination offered in each case, and to analyze the respective results. Plant material was collected from 75 populations, and DNA was extracted from fresh leaves based on CTAB method. The results show that two main clusters of similarity (25%) separate the populations of central and coastline southwestern Albania from the populations of the continental south-southeast. From the composition of their essential oils (especially α-β thujone and camphor) analyzed by gas-chromatography was determinate the presence of three different chemotypes inside the sage populations. Llogora population with lower content of ketones (thujone, camphor), but with higher values of cineol and borneol, can be characterized the cineol-borneol chemotype, as a communication bridge between the Greek and Turkish sage. *In vitro* conservation of sage is considered as one of the methods for the preservation of this species considered endangered by the Red Book of Albania. Micropropagation in the base of *in vitro* conservation is carried out using MS/2 nutrient medium supplemented with three phytohormones (auxin NAA, cytokinin BAP, gibberellic acid GA_3 in optimal ratio 1:10:1) during proliferation stage and IBA:GA_3 = 3:1 in rooting stage. The method of minimal growth under low temperature (4°C) is used for short-term conservation of Albanian sage populations.

Keywords*: sage, genetic polymorphism, chemotypes, micropropagation, in vitro conservation*

INTRODUCTION

Mediterranean Albania is rich in different aromatic-medicinal plant species, among them the species of family Lamiaceae. *Salvia officinalis* L. (sage) has been the most exploited during the last decade, endangered also from deforestations, fires, drought, soil erosion, presence of new infrastructure in the forests etc. This spontaneous species is classified as endangered according the "Albanian Red Book" [1]. About 80% of the world production of sage is

harvested from Albanian and Montenegrin areas (exported in USA and Europe) [2, 3]. The sustainable use of medicinal plants can be achieved better through their cultivation. Preliminary genotype selection could be of high importance for developing programs of genetic improvement in order to obtain biotypes with agro-industrial and environmental potential. DNA-based molecular markers have been used in a wide range of plant species either for cultivar identification or in assessment of genetic relationships between individuals and species. A number of researches [4-8] are undertaken in Albania on the evaluation of biodiversity in molecular level at local resources of some aromatic plant species.

Members of family *Lamiaceae* show a plant-to-plant variability of secondary metabolites, due to their gynodioecy, resulting in breeding character, influenced by natural crosspollination. The diversity of environmental conditions also contributes to high crosspollination, increasing further the substantial variability in active ingredient levels and quality. Due to different edaphic and climatic conditions in Albania, different ecotypes with specific biochemical composition of essential oils can be found. According to authors [9-11] variation in the composition of essential oil within a species, including the sage, appears to be the rule rather than the exception and is influenced by 3 factors: (i) individual genetic variability; (ii) variation among plant parts and different stages of development; and (iii) modifications due to the environment.

In 1988, when Chiang Mai Guidelines on the Conservation of Medicinal Plants were drafted [12], new technologies such as micropropagation and *in vitro* preservation had only just appeared on the horizon of possibilities for *ex situ* production and preservation of plant germplasm. Micropropagation represent some advantages [13-15], such as production of "identical plants with mother-plants as an important factor on conservation and propagation of chemotypes with economic values; production of great number of plants during a short time, independently of the season, on the limited space etc. The sage plants obtained through micropropagation had higher concentrations of essential oils comparing to plants cultivated *ex vitro* [9, 16-18].

Objectives of the presented paper are: to identify the polymorphism among different sage populations by RAPD and AFLP markers and to evaluate the genetic relatedness among the populations; to analyze the composition of their essential oils by Gas-Chromatography with aim to determine the chemotypes; and to establish the *in vitro* culture conditions and methods for *in vitro* sage germplasm conservation.

MATERIAL AND METHODS

Plant material: Plant material was collected from populations of *Salvia officinalis* and *S. triloba* from southern and northern Albania, and it was used fresh for DNA extraction based on CTAB method, or dried for essential oils extraction.

Molecular evaluation of populations was based on the use of different molecular markers (RAPDs, SSRs, AFLPs). *RAPDs: Salvia officinalis* ecotypes were taken from eight locations: Malësia e Madhe, Dajt, Laç, Gjirokastër, Fier, Llogora, Delvinë, Kozanë. *S. triloba* ecotypes were collected from Saranda, Vuno and Llogora. DNA was isolated according to the manufacturer's instructions of the Gene Elute Plant Miniprep Kit and with a few modifications, which helped in getting a DNA of higher purity level. Ten RAPD and four

SSR (pairs) primers were used for the PCR amplification. The decamer primers were selected on the basis of their polymorphisms when tested with *S. officinalis* and *S. triloba* ecotypes [4]. RAPD amplification was performed in a volume 10 µl containing 20 ng of template DNA, 0.2 µM of decamer primer, 100 µM of each dNTP, and 1U of Taq DNA polymerase in reaction buffer (1,5mM $MgCl_2$, 10mM Tris-HCl pH=8.3; 50 mM KCl) [4]. The cycling conditions were: 1 min at 95ºC followed by 45 cycles of 10 s at 95ºC; 37ºC/15s; 72ºC/2 min. The samples were kept 5 min. at 72ºC. *SSR analysis* was performed as reported [6]. PCR products were separated in a 1,5% agarose gel (RAPD products) and 3% agarose (SSR products) in 1xTAE buffer (0.4 M Tris acetate, pH=8.3; 0.01 M EDTA)(~5V/cm) and stained with ethidium bromide. *AFLPs* were produced following the Pre-amplification reaction, Cycling conditions and Selective amplification [6]. Products were amplified in polyacrylamide gel, in the sequencer Licor, Global Edition IR2. The information was computed with the soft Automated AFLP Analysis Software SAGA, Version 2.1 and binary matrices were produced. The last were used to create the dendrogram of similarities using the soft NTSYS, based on the Jaccard's coefficient. The dendrogram were built comparing genotypes of the same population, among the eight populations, among populations of close geographical locations, and among the eight genotypes from throughout Albania.

Plant material for essential oils analyses: The plants of natural populations of Gjirokastër, Llogora, Dajt and Shkodër were analyzed. In the framework of the project CERATONIA, in the Biological Research Institute, Tirana, 36 samples of Albanian sage populations, cultivated in the collection at Bari University, Italy were analyzed.

Essential oils isolation: The plant material was air-dried and submitted to distillation (3 h) using a Clevenger apparatus. The essences were collected in toluol (2 ml) [19].

Gas Chromatography: The analyses were carried out on a Shimadzu GC 14B fitted with a flame ionization detector FID using a WCOT fused silica capillary column (30m x 0.32mm i.d., film thickness 1 µm, stationary phase CP-Sil 8 CB) and helium carrier gas. The column temperature was programmed from 60ºC to 220ºC increasing by 4ºC/min. The temperature of injector and detector was kept at 200ºC. Peak areas were determined by an electronic integrator [20]. Gas-Chromatographic analyses were performed in triplicate by injecting 2 µl of the solution in split mode (1% in hexane, split ratio 1/60). The identification of the individual compounds was based on their retention times.

Plant material for micropropagation and in vitro conservation: Objects of these procedures were the plants of natural populations of Gjirokastër, Llogora and Dajt.

In vitro culture: as initial explants were used meristems and buds inoculated in the spring. Nutrient medium MS [21]: **Stage I**-proliferation, MS/2, and in mg l^{-1}: auxin (NAA 0.1), cytokinin (BAP 1.0), gibberellin (GA_3 0.1); **Stage II**-subculture, MS with BAP 1, NAA 0.1; **Stage III**-rooting, IBA 0.3, GA_3 0.1, sucrose 2%, agarose 0.6%.

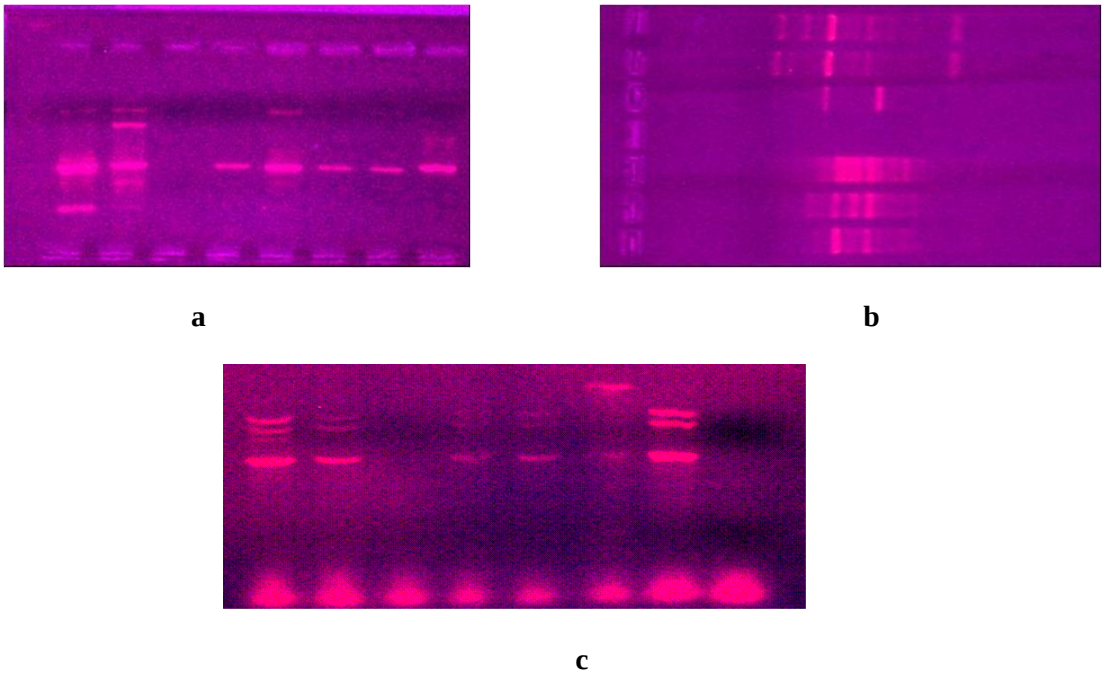
In vitro germplasm conservation: method of minimal growth in temperature 4°C was used [12, 13, 22].

RESULTS AND DISCUSSION

Biodiversity through different molecular markers

Results of RAPDs, SSRs and AFLPs for Salvia species *populations*

RAPD-s and SSR-s were used to detect genetic variability among different ecotypes of *Salvia officinalis* and *S. triloba* grown in Albania. In the RAPD analysis, ten decamer primers were used to amplify all genotypes; four of these showed well-resolved bands.



a **b**

c

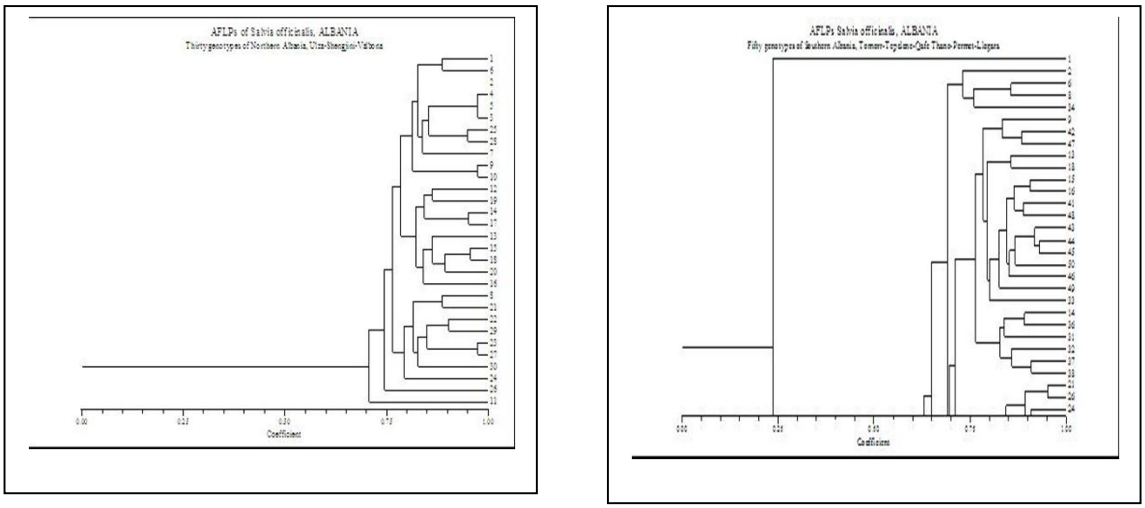
Figure 1. a – RAPDs on 8 *Salvia officinalis* populations; **b** – RAPDs on 3 *S. triloba* populations and **c** – SSrs on 8 *S. officinalis* populations

The highest number of polymorphic bands was obtained by primer SoR5 and the lowest by primer SoR1 and SoR2. The number of bands produced by RAPD varied 1-13 from which 2-7 polymorphic. Amplification with SSR markers was performed using five primers pairs. The primer pair SoS2a/SoS2b produced the maximum number of polymorphic bands, while generally for each primer pair it varied 1-13 (Fig. 1a,b,c).

Results of AFPLs for 8 Salvia officinalis populations of Albania.

AFLPs are the category of molecular markers used to evaluate the intra-populational and inter-populational diversity among 75 sage genotypes of Albania from eight populations collected in geographically distant areas of Albania. A total of 63 fragments were received from which 20 were polymorphic. Genotypes of the same populations shared from 30% to 60% and to 80% similarity; 80 genotypes compared to each other shared at least 70% similarity. The dendrogram of similarity grouped together populations of near geographical locations indicating that the intra-species variability at *S. officinalis* is closely linked to environmental conditions (Figures 2a,b). The AFLPs showed that even though the similarity

among the populations was high, 60%, which is expected in intraspecies level, the eight of them demonstrated to be different as well.



a b

Figure 2. Dendrogram of similarity of different sage populations of Northern and Southern Albania: **a** - according to Jaccard's coefficient; **b** - UPGMA cluster analysis via NTSYS 2.1.

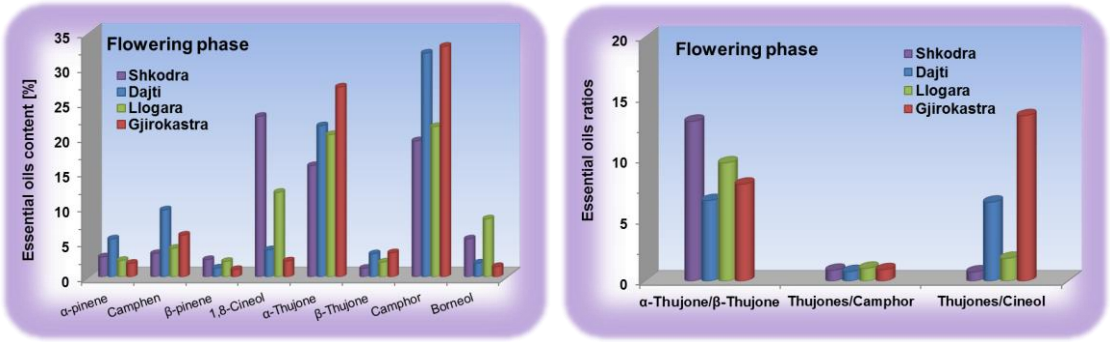
Grouping of the populations according to their geographical locations and based on the fact that the AFPL is very sensitive to the detection of polymorphisms generated from mutations [25], which add or delete restriction sites, and to inversions, insertions, or deletions between two restriction sites, we could conclude that in the case of *S. officinalis,* these categories of morphisms are closely linked to the environmental conditions. The use of AFLPs is the most efficient way to generate a big number of markers connected to a certain gene [26]. The polymophism offered by AFLPs has been important for the populational genetics, phylogenetic analysis, identification of cultivars/accessions studies [27, 28].

Comparison of the essential oils ingredient of sage populations

Results of the essential oils of some Albanian natural sage populations

The essential oils components of the analyzed ecotypes of *Salvia officinalis* are bicycle terpenic ketones: α-thujone, β-thujone and camphor; terpenic oxide: cineol; as well as terpenic alcohol: borneol (Fig. 3a). The essential oils in flowering phase exhibited notable differences. α-thujone content represented higher values in Gjirokastër area and lower in Shkodër: Gjirokastër population 27.15%, Dajti 21.6%, Llogora 20.33% and Shkodër 15.89%. Camphor content was 32.96%, 31.99%, 21.48%, 19.44% respectively in population of Gjirokastër, Dajti, Llogora and Shkodër area demonstrating the same variations as α-thujone content (higher in Gjirokastër and lower in Shkodër area). α- and β-thujone content is higher than camphor content only in Llogora area. The camphor content in other areas is higher than of thujone content. Correlation coefficient between thujone and camphor contents in 4 areas is r = 0.88. Significant differences were observed on essential oils related to cineol and

borneol content too (Fig. 3a). The observed fluctuations on the content of 1.8-cineol have shown the decline from 22.9% in population of Shkodër to 12.1% in Llogora population and up to 3.85% and 2.26% in areas of Dajti and Gjirokastra respectively. Borneol values also represent variation from 28.8% (Llogora), 5.4% (Shkodër) to 1.97% (Dajt) and 1:46% (Gjirokastër).



a b

Figure 3. a - Essential oils content of different sage populations (flowering phase); **b -** essential oils ratio of different Albanian natural sage populations

The differences between ecotypes are even more obvious considering some of the ratios of more characteristic constituents of essential oils (Fig. 3b). Based on these differences can be accepted the presence of three different chemotypes. Essential oils of Llogora area characterized by low ketones content (thujone, camphor), but by high cineol and borneol contents, can be characterized the cineol-borneol chemotype, as a communication bridge between the Greek and Turkish sage. Asllani [2] confirms this conclusion. On the other hand, the population of Shkodër area in terms of components of essential oils is known as *Salvia officinalis* var. *longifolia*.

Results of the essential oils of some Albanian sage populations (Bari collection)

The essential oils of 36 analyzed Albanian ecotypes (presented in genetic collection in Valenzano, Bari) exhibited notable differences especially on thujone and camphor contents (Fig. 4). α-Thujone content represented higher values in % (59.7, 51.8, 48.1 respectively) in some samples (Al 36/60, Al 36/59 and Al 36/57) and lower (0.51, 1.12, 4.52 respectively) in other samples (Al 36/61, Al 36/67 and Al 36/54). Whereas, camphor content represented higher values in % in Al 36/67, Al 36/68 and Al 36/53 (52.1, 51.42 and 46 respectively) and lower in Al 36/59, Al 36/60 and Al 36/57 (4.3, 10.3 and 15 respectively). Comparing the values of two main components can be observed that thujone content was higher than camphor content only in three samples (Al 36/59, Al 36/60 and Al 36/57), almost equal in Al 36/56, whereas in all others camphor content was higher than thujones content, especially on Al 36/61, Al 36/67. Thujones and ketones contents are higher in the Al 36/60, Al 36/59 and Al 36/57 than of the others (Figs. 4, 5a). Differences were also observed on cineol and borneol content too, representing higher values in ecotypes of Al 36/59 (cineol) and Al 36/61 (borneol) (Figs. 4, 5b). While the essential oils of the populations Al 36/61 and Al 36/63

demonstrated low ketone content (thujones and camphor), but high cineol and borneol content.

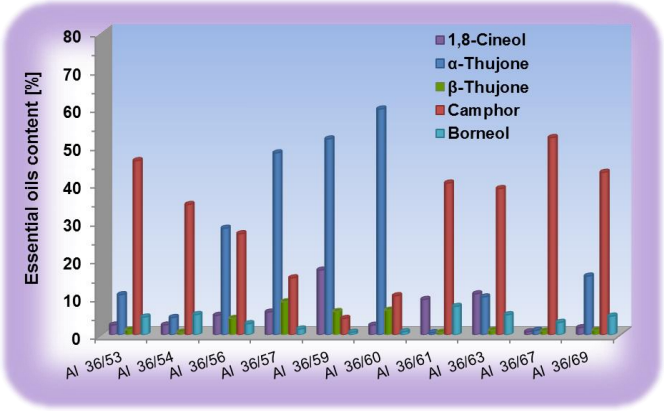
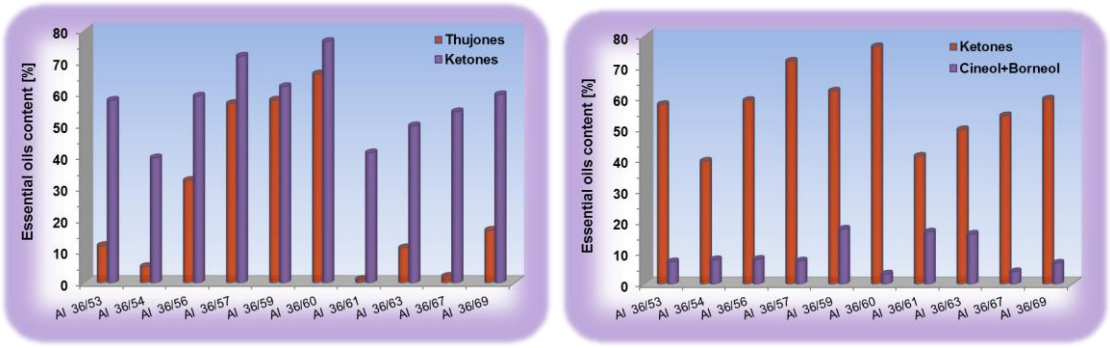


Figure 4. Essential oils content of different Albanian sage populations (Bari collection)



a b

Figure 5. Essential oils content of different Albanian sage populations (Bari collection): **a** - thujones and ketones and **b** - ketones and cineol+borneol.

The presence of three different groups of the analysed ecotypes can be clearly evidenced. In addition two of these populations (Al 36/61 and Al 36/63) can be characterized as cineol-borneol chemiotypes. Our experimental data show a high variation in essential oils yield, but especially in the constitution of Albanian sage populations in areas of Northern, East and South Albania. As shown, this variation is related to both environmental and genetic factors [9-11]. The data obtained from the study of the chemical composition of essential oils of different sage populations are corresponded to the results of genetic variability, and it created a coordinated fuller picture of the genetic and biochemical diversity of these populations. According some studies [23] cluster analyses based on oil composition and RAPD markers corresponded very well to each other, suggesting that there is a strong relationship between the chemical profile and the genetic variability for different cultivars of common sage. Complex chemical and genetic studies carried out simultaneously can provide information for the identification and selection of genotypes of high importance for developing programs of genetic improvement to obtain biotypes with valuable potential.

Results of *in vitro* culture and conservation

Proliferation and bud elongation stage: Optimal medium MS/2, rich in phytohormones BAP, NAA and GA_3 in ratio 10:1:1 promotes morphogenesis, buds formation initiation (Fig. 6a) and influence in the apical dominance. Phytohormones combination is the most significant factor influencing explant dedifferentiation in culture [16]. The differences in buds proliferation initiation appear when the explants were isolated in early spring. Explants derived from Llogora population are characterized by a higher proliferation percentage, followed by Gjirokastër population, and further rank explants from Dajti population. The same ranking is observed about the biometric indicators of plantlets (shoot length, leaves number), which take on the appearance of the bundles (Fig. 6b) due to the presence of BAP in medium in relatively high content.

a b

Figure 6. a - New developed buds 12 days after inoculation; **b -** Plantlets in the bundles.

Subculture stage: The plantlets obtained from first stage (Fig. 6b) undergo the subculture, as divided into separate parts (stem pieces with 1-2 leaves or lateral buds).

a **b**

Figure 7. a - Plantlets of Gjirokastër population in the bundles; **b** – Plantlets of Dajti population with higher shoot length.

Plantlets developing in bundles favor the formation of a large number of new buds. The multiplication coefficient reaches 1:4. This indicator is higher for Llogora population plantlets. The plantlets of Gjirokastër population develop in the bundles (Fig. 7a), whereas Dajti population plantlets are characterized by higher shoot length (Fig. 7b). The high content

ratio BAP/NAA (10:1) induces plant morphogenesis stimulating the formation of new lateral buds in the absence of apical dominance phenomenon.

Rooting stage: Optimal medium is MS with phytohormone ratio IBA:GA_3 (3:1).

a b

Figure 8. a – Sage plantlets in the callogenesis stage; **b** – Rooted sage plantlet

IBA, as "rooting phytohormone" induces plantlets callogenesis in the majority of plantlets (Fig. 8a) and the rhizogenesis in rare cases (Fig. 8b).

In vitro germplasm conservation of sage collections

Method of minimal growth (low temperature 4ºC and reduced light regime), based on the micropropagation is used to conserve different sage populations (Llogora, Gjirokastër and Dajt). Reducing growth temperature close to 0ºC can minimize the growth rate in many crops [14]. Reducing light intensity also affects growth rate by reducing photosynthetic requirements and metabolism. The techniques are based on the reduction of metabolism in order to avoid the growth, but to preserve the possibility of regeneration [14, 22]. This method appeared to be ideal for short to medium-term germplasm preservation in a small area [13]. The sage plantlets were stored *in vitro* during the period of 6 months with the possibility of full regeneration.

CONCLUSION

The sage populations of different areas of Albania exhibited genetic polymorphisms identified based on RAPDs and AFLPs; the AFLPs grouped the populations of *Salvia officinalis* according to their geographical affinity, indicating that the intraspecific variability at this species is closely linked to the environmental conditions. Differences on essential oil components observed among different populations; sage population of Llogora can classify as cineol-borneol chemotype, as a communication bridge between the Greek and Turkish sage. Specific reaction of different populations resulted toward the *in vitro* culture and the micropropagation presented as an opportunity to establish an *in vitro* collection of sage populations according to the minimal growth methods. This method is a necessary activity for geneticists not to lose valuable genetic materials, as holders of interesting features and useful for the improvement of commercial cultivars.

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Original scientific paper

EFFECT OF NITROGEN FERTILIZATION UPON MOUNTAIN SAVORY YIELD AND ESSENTIAL OIL AND ITS STABILITY ESTIMATION

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ABSTRACT

A three-year field experiment was conducted at experimental fields of Bački Petrovac, Institute of Field and Vegetable Crops, Serbia. The effect of six nitrogen doses (0, 40, 80, 120, 160 and 200 kg ha^{-1}) on dry herb yield and essential oil content of mountain savory Serbian population was investigated by using the block design with four replications. The obtained data were processed by ANOVA and AMMI analysis.

Year of investigation, nitrogen doses and interactions had a highly significant influence (F probability <0.01)on both traits, except for interactions on herb yield that showed significant influence (p=0,012). The lowest values of both traits were in 2006 (2812kg $ha^{-1}$and 1.90 ml 100 g^{-1} respectively) whereas between other two years no significant differences were obtained. In three years average, the nitrogen doses increased by 1 kg linearly increased herb yield by 19.2 kg (r=0.98), while essential oil content followed quadratic polynomial regression (r^2=0.99). Maximum oil content was obtained with 160 kg N, and after that decreased.

Due to highly significant influence of interactions, further analyses included the AMMI (Additive Main Effects and Multiplicative Interaction) model. Under three-year environmental conditions, only first IPCA were significant for both traits that may be explained by 87.1% and 96.2% of total interaction variability, respectively. Herb yield was the most stable and over general mean in 2007,while essential oil content was the most stable but below general mean in 2006. Herb yield and simultaneously essential oil content werethe most stable at or over general mean at doses of 80 and 120kg of nitrogen. Herb yield positively correlated with higher nitrogen doses in 2007 and 2008 whereas with lower doses in 2006. Essential oil content positively correlated with lower doses in 2006 and 2007 whereas with higher doses of nitrogen in 2008.

***Keywords**: Satureja montana L., nitrogen fertilization, yield, essential oil content, AMMI*

INTRODUCTION

Mostly wild populations of *Satureja* species have been examined in Serbia. Variability of their morphological and chemical features has been broadly discussed while their cultivation was much less investigated [1,2]. Mountain or winter savory (*Satureja montana* L.)is most frequently used as a culinary herb but it also has marked medicinal benefits. The possibilities of growing of *S. montana* in the Serbian lowland, i.e. its plantation longevity and also the effect of harvest time upon yield and essential oil were investigated [3,4]. Obtained results show that this perennial species may be successfully cultivated at least ten years under given ecological conditions of Serbia. Significant variations of yield and essential oil content by years [5, 6] and essential oil content by wild population [7] were recorded.

This research is aimed at finding out optimal amounts of nitrogen fertilizers for obtaining higher dry herb yield and essential oil content of mountain savory.

Multi-environment trials (MET) are usually conducted in multiple years and locations to adequately represent temporal and spatial variation of environments. Besides plant breeding, MET is also important in agronomy for studying yield stability and predicting yield performance of agronomic management practices across environments[8]. Because of temporal variability, agronomist should locally use multiple year trials to make recommendations to farmers about the superior management practice for each environment. Like the effects of genotypes, the effects of agronomic management practice or treatments can change differentially in relation to environmental changes, producing a treatment x environment interaction (TEI).Statistical models for G x E [9] are equally useful for T x E (treatment x environment). The presence of a large interaction (T x E) limits data interpretation and makes selection of an "optimal" N dose, one that exhibits good performance regardless of the environment but less accurate. However, most of the biotic and abiotic characteristics of an environment cannot be controlled leaving the treatments as the sole source for reducing TEI. This is reason for choosing AMMI model for processing three years data influence of six doses of N fertilizers on yield and essential oil content of winter savory.

MATERIAL AND METHODS

A three-year experiment was conducted on chernozem soil at the experimental field of Bački Petrovac (84 m altitude), Vojvodina province, Serbia. Soil characteristics- pH in H_2O 7.52, pH in KCl 8.13, $CaCO_3$ 2.97%, humus 3.15%, total nitrogen 0.219%, AL-P_2O_5 17.0 mg/100g and K_2O 32.4 mg/100g. Mountain savory Serbian population was investigated by using the block design with four replications. Basic plot area was 10.8 m^2. Planting row distance of 45 cm and distance between plants in row of 30 cm using standard agrotechnical measures were applied [5]. Nitrogen (N) fertilizer (KAN, 27% N) in the doses of 0, 40, 80, 120, 160 and 200 kg N $ha^{-1}$was applied every year. Crop was harvested by hand at flowering stage at the 15 cm above soil surface. Fresh plant mass was dried in solar dryer at 45 ºC. Dried samples (leaves) were distilled after Ph. Jug. V, and quantity of essential oil was determined.

The obtained data were processed by AMMI analysis while ANOVA which is an additive model is effective in partitioning the total sum of squares into main effects off treatment, environment and the TEI, but it does not provide insight into TEI structure and patterns of

treatment responses to changing environmental conditions. To study of underlying the interaction component, more advance techniques such as principal component analysis are required. The AMMI model is a hybrid model involving both additive and multiplicative components of two way data structure. The AMMI model separates the additive variance and then applies Principal Component Analysis (PCA) to the interaction portion to extract a new set of coordinate axis that explains in more detail the interaction pattern. Biplot analysis is very useful for quick visualization and exploration of patterns inherent in the complex TE two way table[9].

RESULTS AND DISCUSSION

According to ANOVA year, treatments had highly significant ($p<0.01$) influence on both herb yield and essential oil content while interaction TE on herb yield had significant ($p<0.05$) and on oil content highly significant influence (Tab. 1).

In total variability of herb yield, the highest part belonged to T (86.4%) while E had only 10% and TEI 3.6%. It means that N doses had more than 8 times bigger variability than years while interaction was very low. Low interaction indicates similar effect of nitrogen in all the years. Partitioning of variability for essential oil content was more even, T (48.1%) was close to E (34.4%), while TEI was higher (17.5%) that indicates different effect of N doses in the years.

Table 1. AMMI analysis of variance including degrees of freedom (df), mean squares (MS) and percent contribution to total sums of squares (%TSS) of six N treatments in 3 year, for herb yield and essential oil content of winter savory

Source of variation	d.f.	Herb yield			Essential oil content		
		MS		%TSS	MS		%TSS
Replication	3	926030			0.0786		
N doses (T)	5	25771453	**	86.4%	0.6947	**	48.1%
Environment (E)	2	7437023	**	10.0%	1.2421	**	34.4%
T*E (TEI)	10	533388	*	3.6%	0.1262	**	17.5%
IPCA1	*6*	*774123*	**	*87.1%*	*0.2024*	**	*96.2%*
Residual PCA	*4*	*172287*			*0.0120*		
Error	51	214909			0.0393		

*Significant at the 0.05 probability level

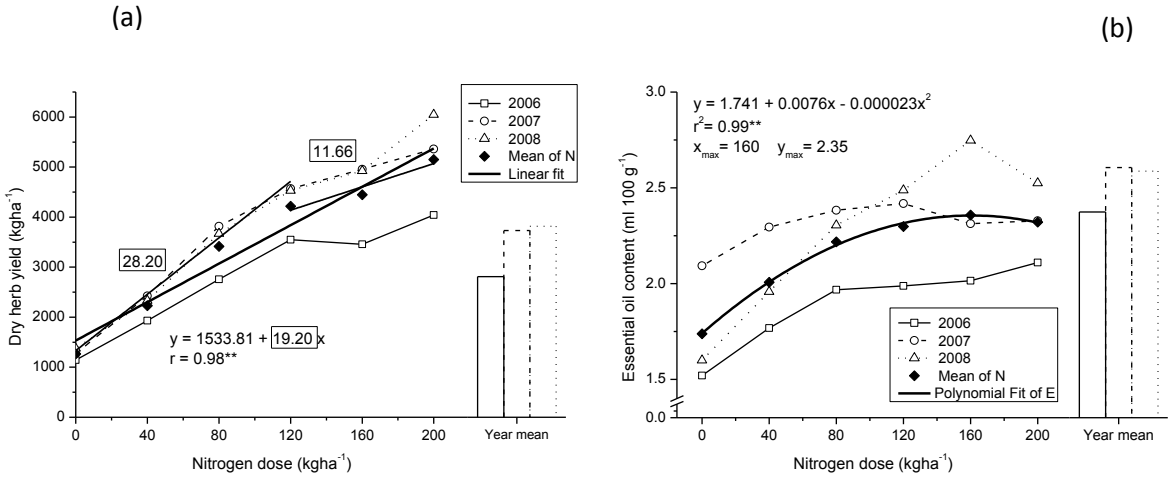
**Significant at the 0.01 probability level

For both traits, the lowest values were in 2006 (2812 kg ha^{-1} and 1.90 ml 100 g^{-1} respectively) while between other two years no significant differences were obtained (Fig.1). In all the years, herb yield regularly increased with increasing nitrogen doses. The lowest slope was in 2006 whereas the highest in 2008. Also, in all the years, a higher slope was obtained with lower doses of N (0 to 120 $kgha^{-1}$) while slope was lower using higher doses. On the average for three years, including all the ranges of N doses (0-200kg),by increasing N for 1 kg herb

yield linearly increased by 19.2 kg (r=0.98) while at lower range doses (0-120 kg) it was 28.2 kg whereas at higher range of N doses (120-200 kg) it was 11.66 kg (Fig. 1a)

Essential oil content also increased with increasing doses of nitrogen, but not linearly to the maximum N doses, like yield. Maximum essential oil content in 2006 (2.00 ml $100g^{-1}$)was obtained with 80 kg of N, in 2007 (2.42 ml $100g^{-1}$) with 120 kg of N and in 2008 (2.75ml $100g^{-1}$) with 160 kg of N. On the average, differences in maximum with increasing N fertilizers essential oil content followed quadratic polynomial regression (r^2=0.99). Calculating maximum oil content of 2.35 ml 100 $g^{-1}$was obtained with 160 kg N, and after that decreased (Fig. 1b).

Figure 1.Three year effect of nitrogen fertilizing on dry herb yield (a)and essential oil content (b) of winter savory



Due to significant influence of interactions for both traits, further analyses by AMMI model were employed. Under the environmental conditions of investigated year, only first IPCA1 were significant for both traits, which explained 87.1 % and 96.2% of total interaction variability, respectively (Tab. 1, Fig. 2).

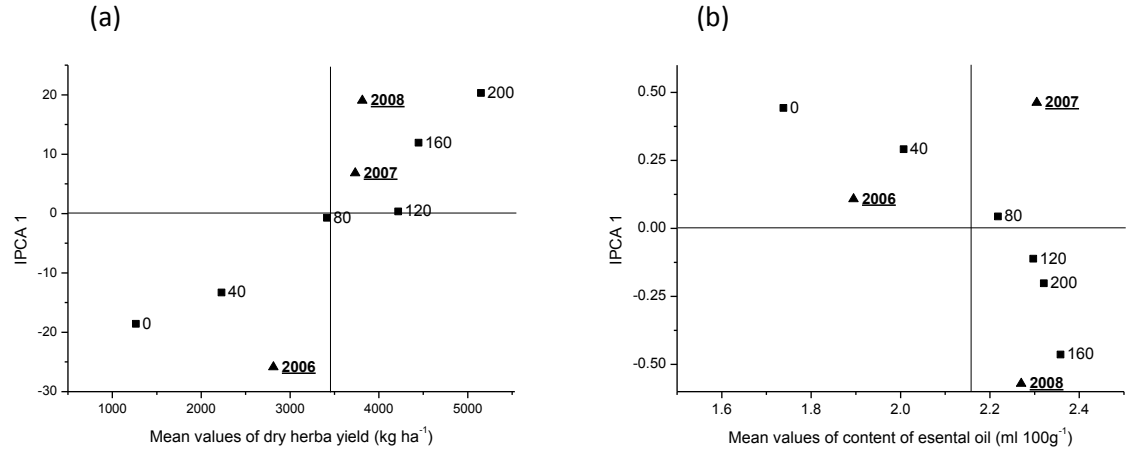
Herb yield over general mean was recorded in 2008 and 2007 in which also the most stable yield was obtained, while in 2006 yield was below and the least stabile. Essential oil content was the most stable in 2006, but below general mean, while in 2008 and 2007 was higher than general mean but less stabile (Fig. 2).

Herb yield and simultaneously essential oil content was the most stable and at or over general mean at doses of 80 and 120 kg of nitrogen. Stability of both traits decreased at lower (0 and 40kg) and at higher (160 and 200kg) doses of nitrogen. At lower doses both herb yield and essential content were below general mean while at higher doses both traits were above general mean. Only differences were found in amount of nitrogen where reached maximum values while for herb yield it was at 200 kg of N and for essential oil content at 160 kgN. It means that nitrogen fertilizing had positive influence on both yield and essential oil content.

Herb yield positively correlated with higher doses in 2007 and 2008, while in 2006 with lower doses of nitrogen. Essential oil content positively correlated with lower doses in 2006 and 2007, while in 2008 with higher doses of nitrogen (Fig. 2).

According to a three year research, the best solution for environmental condition of Vojvodina province and recommendation for farmers is to fertilize Serbian population of winter savory with 120 kg N, since both yield and quality were above overall averages and both traits had the best stability at this N dose.

Figure 2. AMMI1 biplot of herb yield (a) and essential oil content (b) of winter savory



ACKNOWLEDGEMENTS

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Review paper

VALUE CHAIN ANALYSIS OF MEDICINAL PLANTS FROM BERATI REGION IN CENTRAL ALBANIA

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ABSTRACT

Albania as a Mediterranean country with favorable climate conditions provides natural habitats for over 350 medicinal plants. In 2012 there were exported about 7000 t of medicinal plants. They are collected in the whole country and the Berati region is one of the most important for wild medicinal plants. The data provided by the study, showed that during last 5 years, 6137 t medicinal plants from Berati region were collected and exported in various countries like: Germany, United States of America, Greece, Spain, Italy and FYROM. The aim of the study was the analysis of the value chain of medicinal plants from Berati region based on the data provided from direct interviews with all stakeholders of the entire medicinal plants chain. The SWOT analysis showed that medicinal plants value chain was too complex, increasing the price and lacking of transparency among stakeholders. The medicinal plants exporters are often facing with the lack of information as well as with price oscillation in the international market. From the study was noticed the lack of relationship between primary collectors and exporters affecting the price and supply with medicinal plants. An integral relationship among actors is needed for the well functioning of the entire medicinal plants value chain. The medicinal plants cultivation and preservation of natural habitats as well as of genetic material of wild medicinal plants are considered as important steps for sustainability of their trading.

***Key words**: value chain, medicinal plants, benefits, trading*

INTRODUCTION

Albania is considered a rich country in natural resources especially in medicinal plants. As a country with Mediterranean climate, in Albania grow more than 300 medicinal plants [1] with natural and traditional medicine values. Medicinal plants are used as curative plants since ancient times. The traditional utilization of medicinal plants in most of the developing countries is considered very important for the health safety. Rising of awareness of community for the usage of medicinal plants in industrialized countries has led to the production of numerous medicines. Recently the market demand for medicinal plants is

increasing, but some of the medicinal plants are nearly extinct which is associated with genetic diversity loss.

The medicinal plants in Albania play an important role not only in the generation of incomes for local communities, but also for the economic indexes of country. The data of last census showed that 46.5 % of population living in rural areas in Albania depends strongly on natural resources [2].

Our country has a significant discrepancy in the trade balance where the export covers only 48 % [3] of flow goods in the foreign trade. According to INSTAT (2013) for the fourth quarterly were exported 3090 t of medicinal plants with a total value of 926 million ALL. The export data shows that the largest export of medicinal plants by group countries has occurred with EU countries. The most of the Albanian exports (96%) are classified under the code HS 1211 with main destination to Germany, USA, Turkey, France, Italy and FYROM. Under this code are included species like *Salvia officinalis*, *Rosmarinus officinalis* etc.

One of the richest districts with wild medicinal plants is Berati region. The number of households of the rural areas in Berati district is 19470 [2]. A considerable part of the households in the rural areas using the medicinal plants collection as a livelihood source, therefore medicinal plants gathering is playing an important role in the alleviation of poverty in rural areas of Berati region.

The purpose of the study is to analyze the existing value chain of the medicinal plants in the Berati district and based on the findings and the SWOT analysis to give several suggestions for its improvement.

MATERIAL AND METHODS

The Berati region, with an overall area of 1798 square kilometer and a population of 128 000 residents [2], is an important district for medicinal plant collection. This district has a forest area of 44896 ha with an average forest area per capita of 0.319 ha [4].

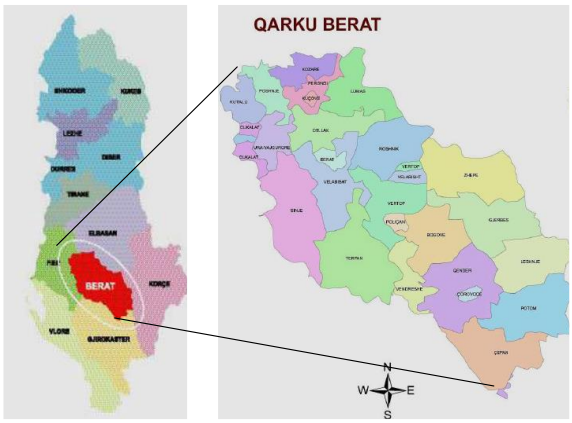

Figure 1. Map of the Berati region

Forest and pasture types in this district are diverse because of local weather patterns and ecological and topographic conditions as well as of anthropogenic influences. Despite

differences in vegetation, habitat types, and human usage, Berati forests and pastures ecosystems are particularly fragile, unstable, and unsustainable because of the interaction of natural factors (steepness, summer droughts, and torrential rains) and social forces (fire, grazing, and overcutting).

The methodology used for providing information comprised face to face interviews with various stakeholders and the desktop review of existing information. The methodological approach is based on a careful analysis of the whole value chain from medicinal plants collector till to the exporter. The value chain includes the entire chain of activities that a product follows [5]. The product in each stage of the value chain is adding its value. The analysis aims to identify all deficiencies in the whole value chain and to offer the proper solutions for improving the current situation.

RESULTS

Quantity of medicinal plants collected from Berati district

In the Berati region the quantity of medicinal plants for the period 2009-2013 based on the data of Forest Enterprise as well as the interviews with local collectors and administrator of *Gjedra shpk* was ranged from 605 to 2000 t per year (Fig 1). The graphic shows that quantity of medicinal plants collected from Berati district is increasing in years. The same positive trend has also the incomes for all stakeholders in the medicinal plants value chain.

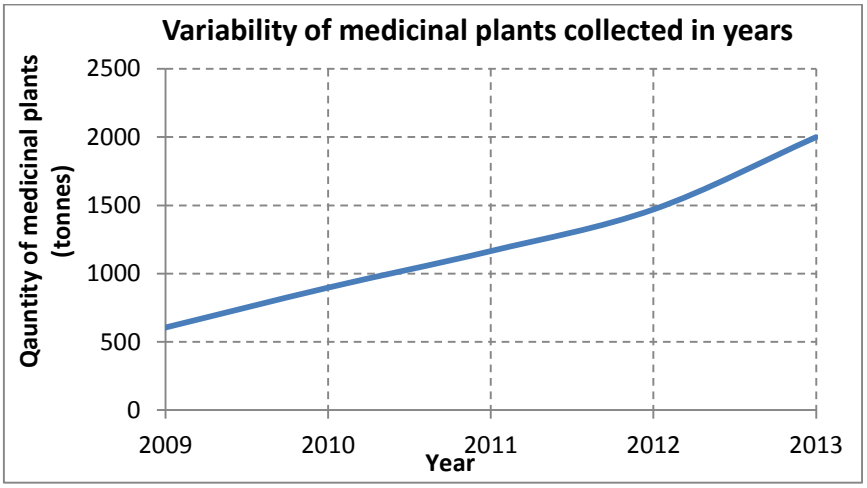

Figure 2. Variability of medicinal plants collected in Berati district

The main species and parts of medicinal plants collected in the Berati district are given in the Table 1.

Table 1.Quantity of medicinal plants collected from Berati region

		Quantity of medicinal plants				
Nr	**Scientific name**	**2009**	**2010**	**2011**	**2012**	**2013**

1	Flores Bellis Perennis	1.5	1.8	2	4	4.5
2	Flores Crataegus	1	2	2.5	2.5	1
3	Flores Papaveris	7	10	6	3	1
4	Flores Malva Silvesstris	5	6	3	2	1.5
5	Flores Crataegus	30	40	60	30	20
6	Flores Camomilla	6	8	3	4	5
7	Folia Farfarea	5	6	8	8	11
8	Folia Rubus Fructicosa	50	80	150	250	300
9	Folia Malva Silvestris	10	12	15	20	22
10	Folia Melissa	20	50	80	100	220
11	Folia Urtica Dioica	10	22	25	32	45
12	Folia Plantago Major	5	6	7	10	12
13	Folia Plantago Loncelata	8	15	17	18	21
14	Folia Olea	5	8	10	15	15
15	Folia Salvia Officinalis	200	300	450	600	850
16	Herba Centaurium	10	15	15	20	25
17	Herba Hypericum	80	100	30	10	20
18	Radix Graminis	10	12	15	18	15
19	Radix Urtica	5	5	8	8	10
20	Radix Taraxasi	2	5	5	8	10
21	Radix Primula	0.5	1	2	2.5	3
22	Cortex Phaseolis	10	12	15	18	20
23	Cortex Junglandis	1	2	2.5	3	3
24	Fructus Sambuci	3	3.5	4	4	5
25	Fructus Malus	50	65	80	100	150
26	Fructus Juniperus Communis	50	80	100	120	150
27	Fructus Juniperus Oxycedrus	20	30	50	60	60

The data from the table show that the largest amount of medicinal plants belongs to *Salvia officinalis*, *Rubus fruticosa* (leaves) and *Juniperus communis* (fruits). The data provided from Gjedra shpk as the only exporter of medicinal plants from Berati region showed that countries where are exported these medicinal plants are: Germany, USA, Greece, Spain, Italy, Hungary, Bullgari and FYROM. Germany is the biggest importer of medicinal plants because the industry of medicine production and cosmetics has a high demand for medicinal plants.

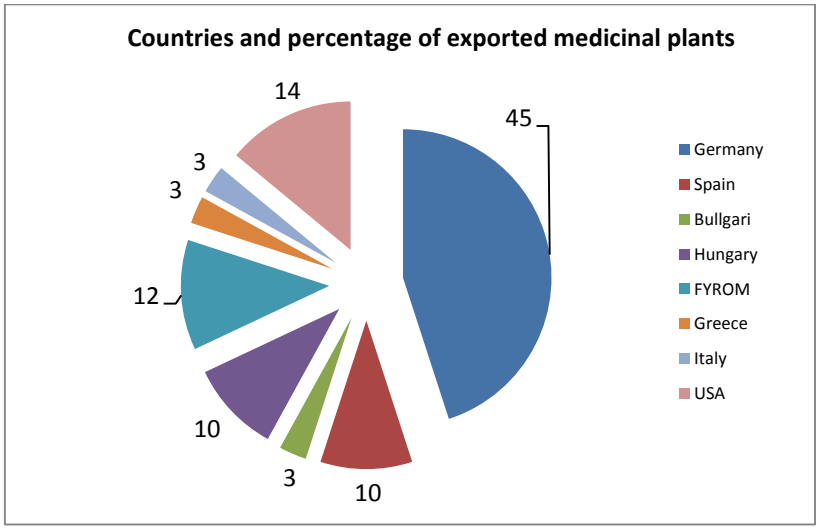

Figure 3. Importer countries of medicinal plants from Berati region

The activity of medicinal plants is considered very important for the livelihood of community in rural areas of Berati region. Based on the interviews with local collectors was noted that 3-4 persons from one family are engaged in the process of medicinal plants collection providing in average about 200 thousand ALL per year for each family.

The value chain of medicinal plants in Berati region

The medicinal plants are collected by farmers in the rural areas and then are delivered to small local traders. At district level are identified 24 local retail traders of medicinal plants that pursue their activity in the whole Berati region. In 90 percent of cases the local traders are buying the medicinal plants in collectors living place and only in 10 percent of cases the farmers are transported the medicinal plants directly to the main storing facilities. The storing facilities are usually old without doing any preliminary processing of medicinal plants. The price of medicinal plants depends on the market price and usually the exporter companies determine the prices of medicinal plants. The price also depends on the quality of medicinal plants after drying. In case of an inappropriate drying process their natural color is vanished affecting also the price of selling. Other important actors in the value chain are the wholesale traders. In Berati region there is only one wholesale company that is also exporter of medicinal plants. This company named *Gjedra* company is operating since 1993 in this activity. The last actors in the value chain of medicinal plants are end-users of medicinal plants or consumers. The main consumers in the Berati region are the local market and pharmacies while in the international trade are pharmaceutical and cosmetic companies. Thus, the scheme of all actors in the entire value chain of medicinal plants from Berati region is:

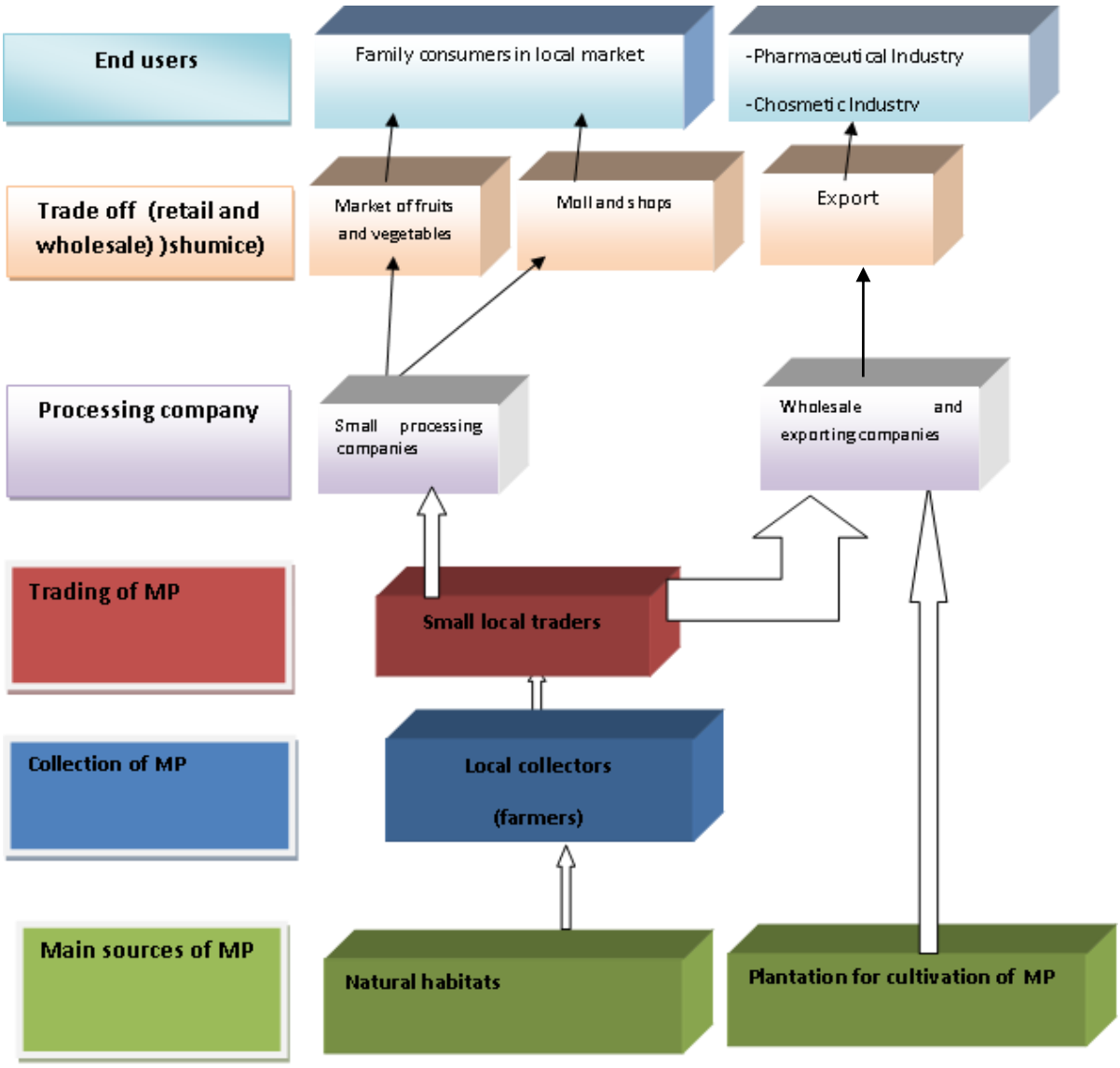

Figure 4. Map of the value chain of medicinal plants in the Berati region

The value map showed that the chain of medicinal plants is organized in 5 levels where in each level one actor is playing in responsible way his role. The relationship among the value chain actors must be characterized by the transparency and trust in information dissemination and price. The actors have a division in their role but are strongly depending on the financial relationship. Thus, the farmers are the main collectors of medicinal plants in forest and pasture areas. Their activity has seasonality duration usually from April to September spending 2 to 6 hours per day for medicinal plants collection. Usually three to four family members are engaged in this process and this activity is combined with livestock grazing, firewood logging etc. The collection process depends strongly from weather conditions and the distance from the village. During interviews they noted the destruction of the habitat of medicinal plants as result of wrong harvesting procedures. They are not organized in any NGO and their work is spontaneous. Despite the collection of medicinal plants they also prepare the medicinal plants for drying in shadow and sun rays, making also the division of leaves from the stem. They represent the biggest community in the value chain and are strongly depending on the prices determined from the local retail traders. During their interviews they emphasized the importance of preliminary contracts signed with biggest traders which will enable continuity in this activity. The local retail traders are another link of

the value chain. They play the role of the dealer in the remote areas buying the medicinal plants directly from farmers. They are an intermediate link in the value chain between farmers and processing or exporter companies. They also carry out other activities for example: qualitative division of the medicinal plants, repacking and transportation. They also depend on the price of medicinal plants from exporter companies. The local retail traders must meet some requirements like: having a trading license, appropriate facilities for medicinal plants storing equipped with weighing etc.

Figure 5. Facilities for medicinal plants storing

The exporter companies have more sophisticated equipments for pre-processing of medicinal plants than local traders. They employ seasonal workers during the summer period enabling to provide incomes for their livelihood. The only exporter company in the Berati region is "***Gjedra Co***".

The retail trading of medicinal plants occurred in the supermarket network, local shops, fruit and vegetable market etc. These products in the local market are sold packaged in the shops (semi - processed) or in bags and bunches (mountain tea, drizzle etc.).

Figure 6. Medicinal plants packaged in the supermarket

SWOT analysis of the medicinal plants market in the Berati region

Based on the information collected from direct interviews with main actors of the value chain of medicinal plants we have conducted the SWOT analysis revealing the strength, weakness, threats and opportunities of this sector in the Berati region.

Strength	Weakeness
• Presence of a huge quantity of medicinal plants in wild state. • Rising of medicinal plants consumption in the international market. • The presence of a numerous stakeholders working with this activity. • The best quality of medicinal plants originated from Albania, has raised the awareness of foreign companies for these products. • Employment of many workers. • Rising of incomes for community in rural areas.	• Few medicinal plants are cultivated. • The lack of contracts avoiding the verbal contracts. • Low quality of raw material because of the mis-treatment based on technical requirements • Non attractive prices for farmers and cultivators. • The lack of information and qualified staff. • The lack of knowledge for cultivation of medicinal plants. • The lack of official data for trading and medicinal plants collection. • The presence of imported medicinal plants of low quality.
Threats	**Opportunities**
• Losing of natural habitats of medicinal plants. • Reduction of local production. • Dishonest competition. • Dependence of medicinal plants export from international market.. • High prices for imported products.	• Improvement of raw material and products quality • Increasing of the quantity of medicinal plants from various sources. • Increasing of assortments originated from medicinal plants. • Increasing of the medicinal plants export. • Rising of attention for medicinal plants sector.

CONCLUSION

The medicinal plants market in Albania plays an important role in the trade flows affecting the trading balance of our country through entering of a considerable foreign currency and employment of people in rural areas alleviating the poverty in poor areas. The medicinal plants market is considered recently consolidated but this sector is suffering the optimal setting up. During the analysis of the medicinal plants in the Berati region were drawn these findings:

- Medicinal plants collectors or famers are not organized in any organizational body and their work is spontaneous. Despite they are the most numerous members of the value chain they represent the weaken link of the value chain of medicinal plants.
- The interviews showed that the majority of the people working with medicinal plants activity were male. The most of the people engaged in this process have an elementary education
- The various level of knowledge of local collectors for medicinal plants indicates that local farmers must be trained from qualified staff before they will engaged in the medicinal plants harvesting.
- The improvement of sanitary and phyto-sanitary standards of medicinal plants storing in conformity with HACCP standards will raise the benefits.
- The most of the medicinal plants exported from Albania are not processed reducing their economic benefits. The processing of medicinal plants inland will add the value and benefits from these products.

- The lack of government subsidy schemes, nonpayment of VAT accounted to 6% and the lack of origin certificate are some of the obstacles that value chain actors are facing during their daily activity.
- The length of the chain of medicinal plants is reducing the incomes for farmers and medicinal plants cultivators as well as reduce the transparency among all stakeholders.
- The bottom-up relationship must be the core of the value chain.

Based on the findings we suggest an improvement of the value chain focusing on: improvement of the process and product, as well as displacement towards a new value chain with less actors and more flexibility.

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Original scientific paper

EVALUATION OF HOW BEESWAX OF ALBANIAN ORIGIN AFFECTS THE SPF OF SUN CREAM

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ABSTRACT

Objective: The objective of this study is to determine whether bees wax affects the SPF obtained by different filters, if the SPF increases or decreases in its presence. To do so first it has to be formulated a stable sun protection cream with bees wax as one of the ingredients.

Material and methods: Bees Wax from albanian farmers. For each sample has been determined characteristics such as identification, melting point, acid value, peroxide value, heavy metals (Pb) based on EP 6.0.

In the next phase have been formulated different sun protection creams. There have been chosen 5 of them as the most stable ones: without a filter only beeswax; with octocrylene, with octocrylene plus beeswax, with PABA; with PABA and beeswax at 2% (p/p) in each formulation. These creams have undergone the analysis to determine the SPF with Labsphere 2000.

Results: The content of Pb in the beeswax of Albanian farmers was about 23µg/g (EP 6.0 max. limit 40µg/g), pH= 5.5 and the melting point 64°C. The cream without a filter has an SPF=2.43; the cream with octocrylene has an SPF of 12.28; the cream with PABA has SPF 8.14; cream with Octocrylene and beeswax has SPF 14,12; the cream with PABA plus Beeswax has an SPF of 10.27.

Conclusion: Our research shows that beeswax can be used as a primary material in cosmetics for skin products up to 2%. It can be used not only as thickeners, emulsifiers and as stiffening agents, especially for Lip balm, cosmetics and medicinal creams, but also as a synergist substance of different filters for sun protection.

***Keywords**: bees wax, formulation, sun cream, SPF*

INTRODUCTION

Natural medicines are often used for many disorders based on tradition or marketing, but not all these uses are supported by reliable or credible scientific research. Yellow Beeswax

obtained by Albanian farmers i**s** light brown, matt, with fainted odour characteristic of honey. Bees Wax mainly consists of myricyl palmitate [$CH_3(CH_2)_{14}COO(CH_2)_{12}CH_3$], and is obtained by melting with hot water the honeycomb of the bee and removing foreign matter. The research intends to determine the characteristics of yellow beeswax of Albania; formulating a stable sun protection cream with and without bees wax; determine how this wax affects the SPF obtained by different chemical filters of these formulations.

MATERIAL AND METHODS

Glass containers 10ml, 25ml, 250ml (class A); analytical Scaltec SBC 22; pipettes 0.5,1 and 2 ml; water bath up to 80-90°C; turboemulsifier Silverson LHR; Magnetic stirrer, (VELP Scientifica) with heater to keep the water temperature 30°±1°C; Thermostat Binder GmbH mod. APT line, seria BD; pH-meter Delta OHM HD 8602; viscosity meter Brookfield spindle 21°; HPLC with UV lamp detector Column C8, mobile phase: methanol: water (650/350); Labsphere 2000; Millipore stratum; Primary ingredients like Bees Wax from albanian farmers, octocrylene, PABA and other ingredients.

We designed 27 formulations of sun protection emulsions. They have undergone the accelerated procedure of stability. Samples of the same emulsion have been kept for the same period one in room temperature, one in thermostat, one in fridge and one have been placed alternatively in Thermostat at 50º-60ºC and then transferred into a fridge at 2º-4ºC. After each period there have been checked regularly the organoleptic characteristics, pH, viscosity of each emulsion.

According to the results of quality control of the above mentioned formulations, there have been chosen 5 of them as the most stable ones (table 1): Formulation B-018 without a filter only beeswax; Formulation B-019 with octocrylene, Formulation B-024 with octocrylene plus beeswax, formulation B-025 with PABA; formulation B-026 with PABA and beeswax. The concentration of bees wax was kept at 2% (p/p) in each formulation.

These creams have undergone the analysis to determine the SPF with Labsphere 2000 using Millipore stratum before and after undergoing UV rays at 2.5 MED.

RESULTS AND DISCUSSION

According to the result of the analysis of bees wax as a primary material to be used in formulations has the odour of honey, acid value at 21 and the melting point 64°C. It becomes a yellow to brown, transparent, consistent liquid. These characteristics make it acceptable to be used in cosmetic products. The ingredients of emulsions for sun protection are illustrated in the table 1.

Table 1. The ingredients of the most stable emulsions

Phase	**Nr**	**Ingredient**	**B-018**	**B-019**	**B-024**	**B-025**	**B-026**
A	**1**	Olivem 1000	5.00	5.00	5.00	5.00	5.00

	2	Finsolv TN	10.00	10.00	10.00	10.00	10.00
	3	Fenoxyparaben	1.00	1.00	1.00	1.00	1.00
	4	PABA	-	-	-	5.00	5.00
	5	Octocrylene	-	6.50	6.50	-	-
	6	Karite butter	5.00	5.00	5.00	5.00	5.00
	7	Abil 350	1.00	1.00	1.00	1.00	1.00
	8	Bees Wax	2.00	-	2.00	-	2.00
B	**1**	Kathon CG	0.05	0.05	0.05	0.05	0.05
	2	EDTA	0.15	0.15	0.15	0.15	0.15
	3	Keltrol	0.30	0.30	0.30	0.30	0.30
	4	Demineralised water ad	100.00	100.00	100.00	100.00	100.00

As far as it concern the formulations that passed the accelerated procedure of stability their organoleptic and rheological characteristics are collected in table 2.

Table 2. Organoleptic & rheological characteristics of five emulsions kept for 3 months under different temperatures.

Characteristic	**B-018**	**B-019**	**B-024**	**B-025**	**B-026**
colour	yellow	slight yellow	yellow	yellowish	yellow
pH	5.38-4.79	4.55-4.92	4.6-4.75	4.95-5.02	4.59-4.64
Viscosity 0.5rpm	45.200-42.200	46.400-30.300	48.200-44.200	39.000-31.800	59.200-5-5.200
centrifuge	stable	stable	stable	stable	stable

Figure 1 gives a sample of the transmittance specter obtained analyzing the sun protection emulsion with Labsphere 2000, while in the table 3 are the medium values of different measurements made for each formulation after radiation 2.5 MED.

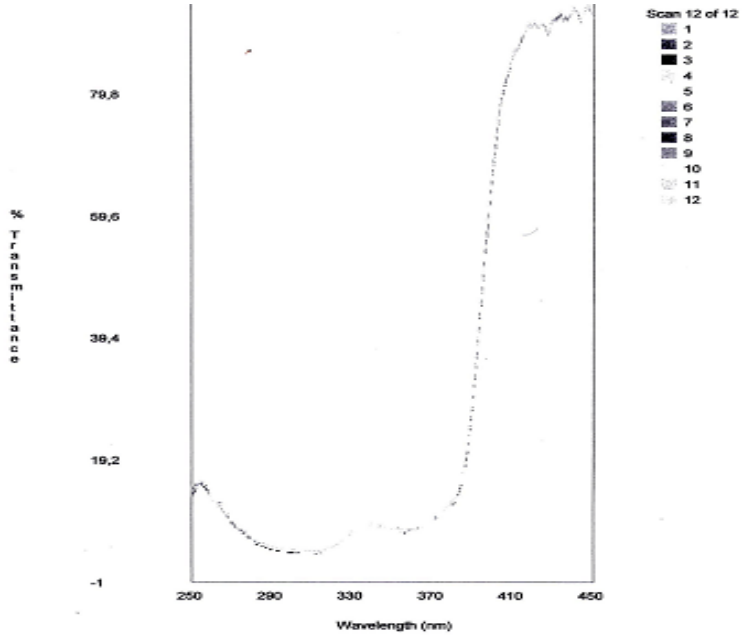

Fig 1. Transmittance specter of emulsion 025 from Labsphere transmittance analyzer

The emulsion 018 has an SPF=2.43±0.17 (SD=0.13); the emulsion 019 has an SPF 12.28±0..42 (SD =0.11); emulsion 024 has SPF 14.12±1.18 (SD= 0.10); emulsion 025 has SPF 8.14 ±0.28 (SD = 0.37); emulsion 026 has an SPF of 10.27±1.18 (SD= 0.37). In table 3 there are included also the result according to star category. It can be noticed that octocrylene itself and combination of octocrylene and PABA with bees wax gives products that can protect the skin from both UVA and UVB radiation.

Table 3. Values extracted from Labsphere Transmittance Analyzer

Emulsions	**B-018**	**B-019**	**B-024**	**B-025**	**B-026**
SPF medium	2,43±0.17	12,28±0.42	14.12±1.18	8.14±0.28	10.27±1.18
SD	0.13	0.11	0.10	0.37	0.37
COV	2.04	1.97	1.27	2.22	2.22
Star category	too low for UVA claim	good	good	too low for UVA claim	good

As far as it concern the bees wax as a cosmetic ingredient it can be said without a doubt that bees wax has good qualities but it should not be used more than 2 % because it compromise the spreading ability of the emulsion on the surface of the skin, makes the product more consistent.

As it can be noticed in table 3 bees wax has an SPF of its own although it is of minimum value, but in the other hand it protects only from UVB radiation whilst for UVA radiation it is too low to claim such protection. Although from table 3 can be seen that it affects the normal SPF of different filters such as octocrylene and p-aminobenzoic acid. If we analyze the

combination of octocrylene with bees wax compared to the formulation with only octocrylene we see that the SPF is increased by 1.734% (figure 2).

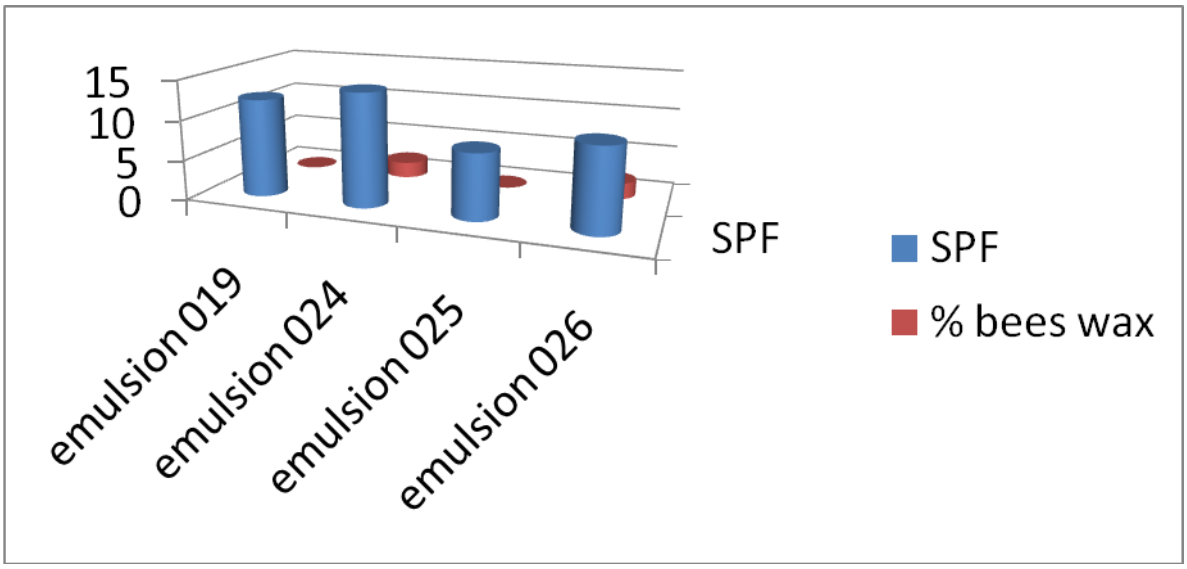

Fig.2 The increase of SPF by the % of bees wax in the formulation

It can be noticed easily in the figure 2 that emulsion 026 with 2% (w/w) bees wax has a higher value of SPF comparing to emulsion 025 that contains only PABA. From table 3 it can be said that this increase goes at 0.836%. Both cases shows an increase of the SPF and the range of that depends on the type of combination.

In table 3 it can be noticed also that bees wax itself or PABA itself cannot protect from UVA radiation.

CONCLUSIONS

Our research shows that beeswax can be used as a primary material in cosmetics for skin products with excellent qualities at 1,5-2% (w/w).

It can be used not only as thickener and emulsifier for Lip balm, cosmetics and medicinal creams, but also as a synergist substance of different filters for sun protection. Bees wax has an SPF of its own at 2,43±0.17 and it affects the SPF of chemical filters. Used at 2% (w/w) it increases the SPF of a sun protection emulsions by 0.84%-1.73%.

This conclusion first makes us think that the research must continue with other formulations, secondly we can decrease the amount of a chemical filter and replace it with bees wax, a natural substance, friendly for the skin.

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Original scientific paper

PHYTOPLASMA DISEASE OF MEDICINAL PLANTS IN SERBIA

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ABSTRACT

Phytoplasmas are important obligate, intracellular, insect – transmitted pathogenic agents, found inside the phloem, in particular in the sieve tube cells of many plant species. In the last decade, phytoplasmas have become a serious problem on medicinal plants in Serbia. Diseased plants exhibit pathological changes which are demonstrated through four typical types of symptoms: phyllody, yellowing and reddening, virescence and proliferation of axillary buds or 'witch's broom'.

Typical phytoplasma symptoms are confirmed on the following species of medicinal plants in Serbia: *Echinacea purpurea, E. angustifolia, Hypericum perforatum, H. barbatum, Plantago major, Saponaria officinalis, Digitalis purpurea, Origanum vulgare, Levisticum officinale, Carum carvi, Trigonella foenum greacum, Melisa officinalis, Petroselinum sativum, Apium graveolens, Valeriana officinalis, Rubus fruticosus, Vaccinium myrtillus, Arnica montana, Calendula officinalis, Cichorium intybus, Salix alba* and *Chamomilla recutita*.

Starting from 2010 to 2014, the presence of phytoplasma was detected in symptomatic medicinal plants in few localities in Serbia using electron microscopy (TEM), and techniques for molecular identification: nested PCR, restriction fragment length polymorphism (RFLP) analyses and sequencing. Sequence homology analysis of 16S rDNAs and *tuf* gene with other phytoplasmas in GenBank database was done using NCBI BLAST.

Clover yellow edge (16SrIII-B) phytoplasma were identified in *Arnica montana*, while Stolbur phytoplasma (16SrXII-A group) were presented in other listed medicinal plants in Serbia (22 species).

***Key words**: medicinal plants, Stolbur, Clover yellow edge phytoplasma*

INTRODUCTION

The trends in herbal cropping, over the last few decades, have led to increased cultivation of medicinal plants, herbs and spices. With these new particular crops, however, unique diseases

and pest problems are emerging. Some of these were previously rare or unknown, like phytoplasma disease.

The first data on the presence of phytoplasma in diseases plants originating from 1967 [1]. Phytoplasmas are today widely spread plant pathogen prokaryotes affecting many plants and crops and responsible of serious diseases on many in several hundred herbaceous and woody plant species and are primary limiting factors for their growing all over the world. On the medicinal plants, they first found in the infected purple coneflower [2] [3].

Phytoplasma symptoms have been observed for the first time on echinacea and St. John's plants in Serbia 2003 in fields located in the Banat regions. Phytoplasmas infected plants exhibit symptoms which suggest a profound disturbance in the normal balance of growth regulators, leading to virescence, yellowing, reddening and proliferation of axillaries buds causing »witches broom« symptoms.

In this paper we present the results of our five-year research on phytoplasma disease on medicinal plants in Serbia.

MATERIAL AND METHODS

The samples of symptomatic and asymptomatic plants were collected on 22 species of medicinal plants in 12 different locality in Serbia from 2010-2014 (Tab.1).

Tab.1 Year of collecting and locality of investigated species

No.	**Species**	Year of collecting	Locality
1.	*Apium graveolens*	2011	Pančevo
2.	*Arnica montana*	2010	Povlen
3.	*Calendula officinalis*	2011	Pančevo
4.	*Carum carvi*	2011	Pančevo
5.	*Chamomilla recutita*	2010	Pančevo
6.	*Cichorium intybus*	2012	Obrenovac
7.	*Digitalis purpurea*	2010	Pančevo
8.	*Echinacea angustifolia*	2010	Pančevo , Indjija, Stara Pazova
9.	*Echinacea purpurea*	2010	Pančevo and Indjija
10.	*Hypericum barbatum*	2010	Pančevo, Indjija, Stara Pazova
11.	*Hypericumperforatum*	2010	Pančevo, Indjija , Stara Pazova

12.	*Levisticum officinale*	2010	Pančevo
13.	*Melisa officinalis*	2011	Pančevo
14.	*Origanum vulgare*	2012	Pančevo
15.	*Petroselinum sativum*	2012	Pančevo
16.	*Plantago major*	2010	Pančevo,Vrdnik, Kovin
17.	*Rubus fruticosus*	2010	Sićevo, Tuleš, Ljubovija
18.	*Salix alba*	2011-2012	Beograd, Obrenovac
19.	*Saponaria officinalis*	2011-2012	Pančevo
20.	*Trigonella foenum greacum*	2012	Inđija
21.	*Vaccinium corymbosum*	2010	Arandjelovac
22.	*Valeriana officinalis*	2010	Pančevo

Sample preparations for electron microscopic investigation were done by the procedure of Hopkins et al. [4]. Tissues from footstalks of leaf-like structures from flowers and stems of symptomatic plants: chamomile, echinacea, St.John's worth, lovage, plantain and rubus, were processed for TEM analysis. After collections, samples of 2 mm in size were fixed with 5.0% glutaraldehyde in 0.1mol/L potassium phosphate buffer (pH 7.2) for at least for 2 days at 4°C and, subsequently, post-fixed in 2.0% osmium tetroxide in the same buffer. The specimens were dehydrated by an ethanol series. Ultra-thin sections were double-stained with uranyl acetate in 70% ethanol.

The total nucleic acids were extracted from the midribs of leaves according Angelini et al,. [5]. A nested PCR using the primer pair P1/16S-Sr followed by R16F2n/R2 was performed to detect phytoplasmas in investigated samples. DNAs extracted from asymptomatic plants were used as a negative control.

Restriction fragment length polymorphism (RFLP) analyses of R16F2n/R2 amplicons were carried out by single digestion with *Alu*I and *Tru*I. The resulting RFLP patterns were compared with patterns of Stol, AY, FD-C and CYE phytoplasmas. *Hpa*II and *Hha*I restriction endonucleases were used for amplicons showing different patterns after digestion with *Alu*I and *Tru*I. Amplification of elongation factor Tu (*tuf* genes) were performed using universal primers fTuf1/rTuf1 for the first PCR and fTufAY/rTufAY for the second amplification in nested PCR [6]. The nested amplification products of the *tuf* gene were digested with *Hpa*II and compared with pattern of Stol phytoplasma. All amplifications were performed using Dream Taq Green PCR Master Mix (Thermo Scientific, Vilinius, Lithuania) and products were separated by electrophoresis in 1.2% agarose gels. RFLP products were separated on 2.3% agarose gels.

Four representative amplicons of 16S rDNA 1,2kb in length, showing different RFLP patterns, were purified and subjected to sequence analysis (IMGGI Sequence Service,

Belgrade, Serbia). Sequence homology analysis of 16S rDNAs with other phytoplasmas in GenBank database was conducted using NCBI BLAST.

RESULTS AND DISCUSSION

Infected plants generally showed phyllody (Fig.1), yellowing and reddening (Fig.2), virescence and proliferation of axillary buds or 'witch's broom' (Fig.3).

Symptomatic plants died within one or two months. The percentage of infected plants is lower in the first year of observation, and growing in the next years. Manifestations of symptoms on the plant host were described on Tab. 2.

Fig. 1. Phyllody on *Echinacea* sp.

Fig. 2. Yellowing and reddening: *L.officinale, H.barbatum, M.officinalis, P.major, S.officinalis, V.officinalis*

Fig. 3. Virescence: *A.montana*, and proliferation of axillary buds or 'witch's broom': *C intibus, S.alba, C.officinalis, C.rectuita*

Phytoplasma like structures were detected in the sieve tube elements of all investigated symptomatic plants. Plant cells were filled with spherical or pleomorphic particles ranging in size from 75 x 500 nm in diameter (Fig.4), which corresponds to the dimensions already cited in literature [7,8,9,10,11,12,13,14].

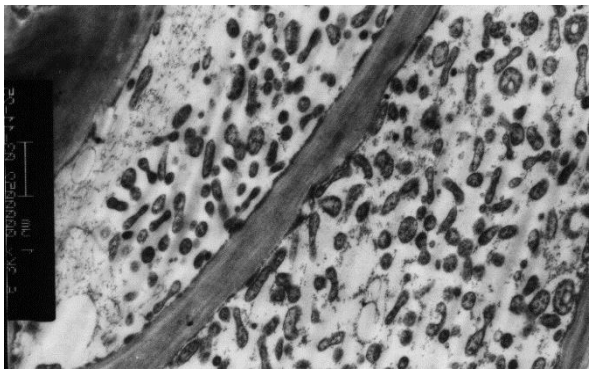

Fig.4 Ultra-thin section of a sieve tube cell *Levisticum officinale*

Tab. 2. Symptoms exhibited in medicinal plants and Phytoplasma detection using 3 methods

Species	Symptoms*	Detection methods		
		Electron microscopy	Sequencing	RFLP
Apium graveolens	Y,R,W	/	/	Stol.
Arnica montana	V, P(WB), SF, R	/	JX297491	CYE
Calendula officinalis	P(WB), SF	/	KJ174507	Stol
Carum carvi	Y,R	/	/	Stol.
Chamomilla recutita	P(WB),W	+	/	Stol
Cichorium intybus	P(WB), SF, R	/	KF661322	Stol.
Digitalis purpurea	R,W	/	/	Stol.
Echinacea angustifolia	V/PH,Y,R	+	/	Stol.
Echinacea purpurea	V/PH,Y,R	+	/	Stol.
Hypericum barbatum	Y,R	+	/	Stol.
Hypericum perforatum	Y,R	+	/	Stol.
Levisticum officinale	Y,R	+	/	Stol.
Melisa officinalis	Y,R	/	/	Stol.
Origanum vulgare	R,W	/	/	Stol.
Petroselinum sativum	Y,RW	/	/	Stol.
Plantago major	R,W	+	/	Stol.
Rubus fruticosus	Y,R	+	/	Stol.
Salix alba	P(WB)	/	/	Stol.
Saponaria officinalis	R,W	/	JX866951	Stol.
Trigonella foenum greacum	Y,R,W	/	/	Stol.
Vaccinium corymbosum	Y,R,	/	KC960486	Stol.
Valeriana officinalis	Y,R	/	/	Stol.

* P (WB) – proliferation “witches broom”, SF – sterility of flowers, Y – yellowing, R – reddening, V/PH - virescence / phylody, W – wilting

The PCR method provided the R16F2n/R2 amplicons from symptomatic plants, whose processing by restriction endonucleases (*Alu*I and *Tru*I) enabled the identification of phytoplasmas, as is shown in Tab.2. RFLP method indicated that most of them belong to the Stolbur phytoplasma (subgroup 16SrXII-A). Some of them have already been published

[12,15, 16,17,18,19]. Only phytoplasma from *Arnica montana* belongs to the 16SrIII-B as already reported [20].

The 16S rDNA sequences of representative samples have been deposited at GenBank (Tab.2) and compared using the BLAST protocol with those of other phytoplasmas in GenBank. The sequence analyses confirmed *Arnica montana* as a host plant for the Clower yellow edge phytoplasma, while all other investigated plants are confirmed as hosts for the Stolbur phytoplasma. These obtained data are correlated with data of the Stolbur phytoplasma presence in other species of cultivated plants. Stolbur is the most common phytoplasma in Serbia, as already detected in corn [21], grapevine [22,23], blackberry [24], alfalfa [17], blubbery [25], potato [26], celery [27].

The presence of phytoplasma pathogens have an important influence on the secondary metabolites in plants, they usually reduced the content of essential oil [28]. This fact is very important, because the damages caused by phytoplasma in medicinal plants are of significant economic importance and we should give them more attention.

CONCLUSION

Presence of phytoplasmas is proved in all location on 22 species of medicinal plants, using three indepenent methods: electron microscopy, sequencing and RFLP of 16S rDNA.

Stolbur is the most common type of phytoplasma on medicinal plants in Serbia.

Damages caused by phytoplasma on medicinal plants are significant, but insufficiently studied.

ACKNOWLEDGEMENTS

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Original scientific paper

FUNGI ASSOCIATED WITH CARAWAY FRUIT IN SERBIA

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ABSTRACT

Caraway (*Carum carvi* L., Apiaceae) is one of the important and popular aromatic and spice crop. It is grown for fruit (*Carvi fructus*), which containes 2-7% of essential oils (*Carvi aetheroleum*). Carawey is used as spice, preservative in the food industry, as wel as for medicinal and cosmetic purposes.

The data on carawey diseases are missing in Serbia. Consequently, the fungi associated with *Carvi fructus* were studied in 2011 and 2012. The *Carvi fructus* on annual and biennial crops were collected from four localities (Ostoićevo, Mošorin, Kulpinand Pančevo) in Vojvodina Province.

Twenty four fungi belonging to 17 genera on caraway fruits were found. The dominat species were *Alternaria alternata* and *A.solani* (up to 75% of contaminated fruits). Fungi from the genus *Fusarium* (*F. oxysporum*, *F. equiseti*, *F. solani*, *F. acutatum* and *F. avenaceum*) were presented in 2-7% on carawey fruits. The presence of other fungi (*Ulocladium* spp., *Aspergillus niger* and *A. flavus*, *Penicillium* spp., *Epicoccum purrpureus*, *Cladospirium cladosporioides*, *Trichotechium roseum, Trichoderma. viride*, *Mucor* spp., *Rhysopus* spp., *Sclerotinia sclerotiorum, Chetomium spp., Botryris cinerea* and *Colletotrichum* sp.) was 1-4%. Besides the above mentioned fungi, *Phomopsis* sp. and *Ascochyta* sp. were found on annual and biennial carawey in two localities in both years.

The presence of *Fusarium* spp., *Alternaria* spp., *Penicillium* spp. and *Aspergillus* spp.is very imortant, because they are known as toxin-forming fungi. Also, *Phomopsis* and *Colletotrichum* species are described as dengerous pathogens on caraway.

***Key words**: caraway, Carum carvi, Carvi fructus, mycopopulation, Serbia*

INTRODUCTION

Caraway is one of the oldest aromatic and medicinal plant. It is grown for fruit (*Carvi fructus*), which contained 2-7% of essential oils (*Carvi aetheroleum*). Caraway is used as spice, preservative in the food industry, as wel as for medicinal and cosmetic purposes.

Many fungal species are known to attack caraway and they could caused a great damage in crop production by decresing the quality and quantity of the herbal material. Seed health is an impo rtant factor for successful cultivation of crops. Seed-borne and seed- transmitted fungi are responsible for the spread of diseases in crops.

The mycobiota of seeds of medicinal plants is well documented [1, 2, 3, 4, 5], but data on fungal species associated with caraway fruits are missing in Serbia. Consequently, the mycobiota of *Carvi fructus* were studied in 2011 and 2012.

MATERIAL AND METHODS

The schizocarps of annual crops were collected in three localities (Ostoićevo, Mošorin, and Kulpin) andbiennial in one locality (Pančevo) all in Vojvodina Province in 2012 and 2013. Samples were analyzed for the presence of fungal flora on and inside the shizocarps using blotter and pure culture (potato dextrose agar /PDA/ and water agar with carnation leaf pieces /CLA/). Four hundred seeds (16 trials, each with 25 schizocarps) from each locality were sterilized with NaOCl for 3 minutes and then rinsed with sterile water and transferred to the filter paper on Petri dishes, 10 cm in diameter. Fifty seeds from each locality were transferred to the PDA medium following the seed surface sterilization. The same amount of schizocarps were not sterilized. After the eight-day incubation at 25°C, parts of the mycelia taken from well-developed colonies was transferred to the PDA in order to be further examined [6].

Fungal development from each schizocarp were estimated and identified based on their morphology and cultural characteristics according to different manuals for fungal identification.

RESULTS AND DISCUSSION

Twenty four fungi belonging to 17 genera on caraway fruits were found (Table 1). *Alternaria alternata, Aspegillus niger, A. flavus, Fusarium oxysporum* and *Sordaria fumicola* were present in all localiteis in both years. The dominat species were *Alternaria alternata* and *A.solani* (up to 75% of contaminated fruits) (Fig. 1a-b). Fungi from the genus *Fusarium* (*F. oxysporum, F. equiseti, F. solani, F. acutatum* and *F. avenaceum*) were present in 2-7% (Fig. 1c-f). The presence of other fungi (*Ulocladium* spp., *Aspergillus niger* and *A. flavus, Penicillium* spp. (Fig. 1c), *Epicoccum purrpureus, Cladospirium cladosporioides, Trichotechium roseum, Trichoderma viride*, *Mucor* spp., *Rhysopus* spp., *Sclerotinia sclerotiorum, Chetomium spp., Botryris cinerea* and *Colletotrichum* sp. /Fig. 2 a-b/) was 1-4%. Besides the above mentioned fungi, *Phomopsis* sp. (Fig. 2c-g) and *Ascochyta* sp. were found on annual and biennial caraway fruits in two localities in both years. Many of the above mentioned fungi have been described on caraway fruits [7, 8, 9, 10].

Table 1. Fungi associated with caraway fruits in 2011 and 2012

Fungal species	Locality							
	Ostojiæevo		Mošorin		Kulpin		Panèevo	
	2011	2012	2011	2012	2011	2012	2011	2012
Alternaria alternata	+	+	+	+	+	+	+	+
Alternaria solani.	-	-	+	+	+	+	-	-
Aspergillus niger	+	+	+	+	+	+	+	+
Aspergillus flavus	+	+	+	+	+	+	+	+
Fusarium oxysporum	+	+	+	+	+	+	+	+
F. equiseti	-	-	+	+	+	+	-	-
F. acuminatum	-	-	-	-	-	-	+	+
F. avenaceum	+	+	-	-	-	+	+	+
F. solani	+	+	+	+	-	-	-	-
Ulocladium sp.	+	+	+	-	+	+	+	-
Penicillium spp.	+	+	-	-	+	+	+	+
Cladosporium cladosporioides	+	+	-	-	+	-	+	+
Trichothecium roseum	+	+	+	+	+	+	+	-
Trichoderma viride	+	+	+	+	-	-	-	-
Mucor sp.	+	+	+	+	+	-	+	+
Rhisopus sp.	-	-	+	+	+	+	+	+
Chetomium sp.	+	+	+	-	+	+	+	+
Botrytiscinerea	-	-	+	+	-	-	+	+
Sclerotinia sclerotiorum	+	+	+	-	+	+	+	+
*Colletotrichum*spp.	-	-	-	-	+	-	+	-
Phomopsis sp.	-	-	-	-	+	+	+	+
Ascochita sp.	+	+	-	-	-	-	+	+
Physarum sp.	+	+	-	-	-	-	+	+
Sordaria fumicola	+	+	+	+	+	+	+	+

The presence of *Fusarium* spp., *Alternaria* spp., *Penicillium* spp. and *Aspergillus* spp.is very imortant, because they are known as toxin-forming fungi. Also, *Phomopsis* and *Colletotrichum* species are described as dengerous pathogens on carawey [11, 12, 13, 14].

CONCLUSION

Twenty four fungi belonging to 17 genera on carawey fruits were found in 2011 and 2012. Alternaria alternate and A. solani were predominant fugnal species on the caraway feuits. From all five identified species from the Fusarium genus, the most widespread species was F. oxysporum.

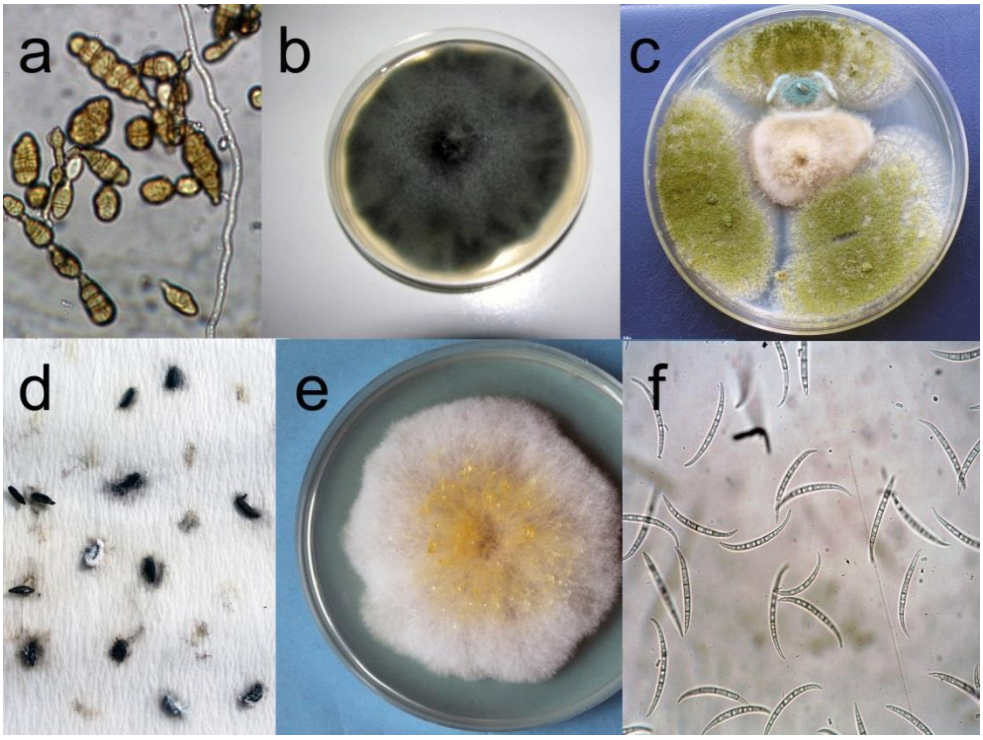

Fig. 1. Conidia (a) and culture (b) of Alternaria alternata on PDA, Penicillium sp., Aspergillus flavus and Fusarium sp. on PDA (c), mycelia on caraway seed (d), colonyon PDA (e) and conidia (f) of Fusarium avenaceuum.

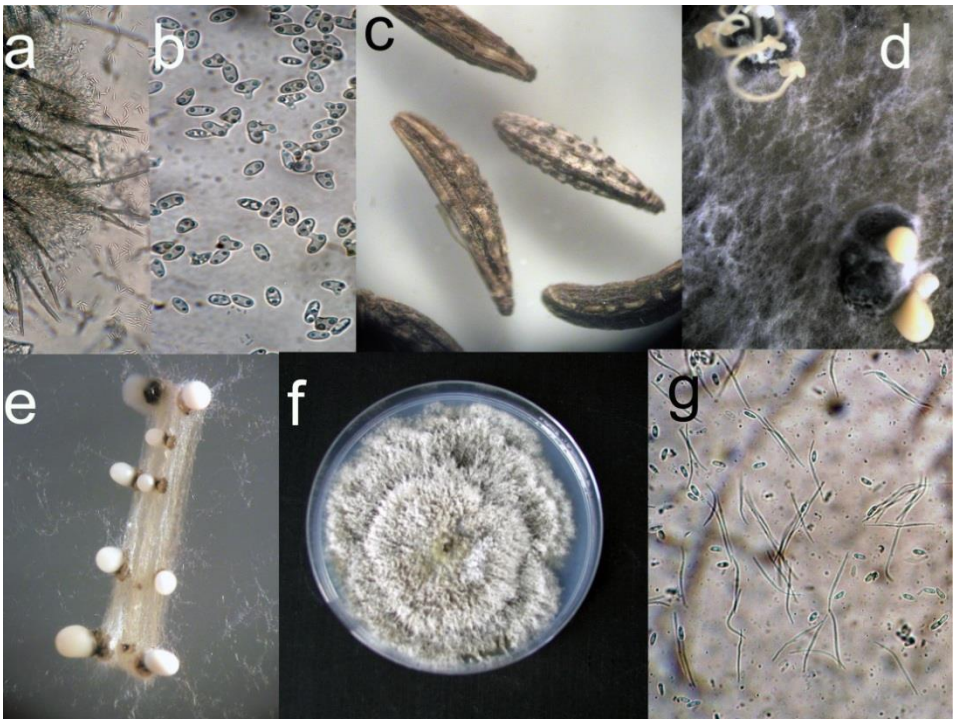

Fig. 2.Colletotrichum sp.: setose acervulum (a) and conidia (b), Phomopsis sp.: pycnidia on schizocarps (c), on PDA (d) and CLA (e), culture on PDA (f), α and β pycnidiospores (g).

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Original scientific paper

NAPHTODIANTHRONE PRODUCTION IN *HYPERICUM PERFORATUM* L. TRANSGENIC SHOOTS

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ABSTRACT

St. John's wort (*Hypericum perforatum* L.) has received considerable interest in recent years due to the increased market demand for *Hyperici herba* crude material as a source of bioactive pharmaceuticals. *Hypericum* extracts contain a complex mixture of bioactive metabolites, mainly naphthodianthrones (hypericin and pseudohypericin), phloroglucinols (hyperforin and adhyperforin) and flavonoids with a broad spectrum of biological effects. As a consequence of the great commercial potential of this species, attempts have been focused on improvement of secondary metabolite production by application of *in vitro* culture methods. Although *H. perforatum in vitro* cultures are known to produce naphtodianthrones, there has been little work to investigate whether these compounds are inducible by genetic transformation. The main objective of this study was to establish an efficient *Agrobacterium rhizogenes* A4-mediated transformation system that would result in the rapid formation of *H. perforatum* transgenic shoots for the purposes of studying the naphtodianthrone production. Naphtodianthrones in control and transformed shoots were analyzed using high-performance liquid chromatography (HPLC) coupled with diode-array detection (DAD) and tandem mass spectrometry (MS^n) with electrospray ionization (ESI). Chromatographic analyses revealed that pseudohypericin, hypericin and protopseudohypericin were the only naphtodianthrones identified in both control and transgenic shoots. The concentration of hypericin and pseudohypericin was about 12-fold higher in transformed shoots compared to control. Regarding the quantitative aspect, pseudohypericin was found as the main naphthodianthrone in transgenic shoots, usually present in 3.5-fold higher amounts than hypericin. In addition, a 3-fold increase of protopseudohypericin was also found in transgenic shoots. Transgenic shoot cultures accumulated significantly higher levels of total naphtodianthrones (113.74±6.13 mg·100g^{-1} DW) compared to control shoots (10.67±0.88 mg·100g^{-1} DW). The direct mode of shoot regeneration, as observed in *H. perforatum*, is a desirable trait when the aim is to obtain genetically stable and viable plants for the purposes of studying the production and accumulation of bioactive compounds. Therefore, *H. perforatum* transgenic shoots could be considered as a source for rapid and increased production of naphtodianthrones.

***Key words**: Hypericum perforatum L., naphtodianthrones, transgenic shoots.*

INTRODUCTION

Over the past decade, medicinal plants have received considerable interest for their phytomedicinal bioactive compounds. Among them, *Hypericum perforatum* L. has been considered according to its biochemical characteristics and secondary metabolite production. *Hypericum* species contain a number of biologically active compounds naphtodianthrones, phloroglucinols, flavonoids, procyanidins, tannins, essential oils, amino acids, phenylpropanoids, xanthones and other water-soluble components [1]. The importance of *H. perforatum*, as a medicinal plant is to the presence of hypericin and pseudohypericin, and their precursors: protohypericin, protopseudohypericin and cyclopseudohypericin [2]. Protohypericin and protopseudohypericin (protopigments) are converted into hypericin and pseudohypericin (pigments) under the action of light. The content of hypericin and pseudohypericin (0.03-0.3 %) varies from species to species and is sometimes also found to vary within the same species, depending on the plant developmental stage, location or harvesting time. It has been reported that production of hypericins by *H. perforatum* plants is strongly dependent on physiological, genetic and environmental factors [3].

Naphtodianthrones such as hypericin and pseudohypericin are localized in the small black glandular structures located on flower petals, leaves and stems [4]. The biosynthesis and accumulation of hypericin is coupled with the morphogenesis and formation of dark red-colored oil glands on the leaves of intact plants [5]. Hypericin and pseudohypericin are photodynamic pigments, produced from dimerized emodin anthrone, presumably via phenol oxidation further oxidized in hypericins [6]. Photodynamic hypericin activities displayed under the influence of light are used for therapy in various diseases. Hypericin has been widely studied in recent years due to its inherent antidepressant, antiviral and antimicrobial properties, as well as its appreciable pro-oxidant activities and potential as an active photosensitizer in photodynamic therapy of cancer [7]. Photoactivate hypericin generates reactive oxygen species (ROS) and its photocytotoxycity properties have been proposed as photochemotherapeuticum [8]. These properties allow hypericin to acts as an antiviral agent. Attention has been focused on its use against human immunodeficiency virus type 1 (HIV-1), [9] and to enhance radiolytic sensitivity of tumor cells [10]. Hypericin has also antidepressant properties and its action alters the monoamine neurotransmission in the central nervous system [11].

Phytopharmaceutical preparations of *Hypericum* are usually produced from field-grown plants. The limited area of occurance of this plant, seasonal harvesting, loss of biodiversity, variability in quality, and contamination issues, trigger to search alternative methods for hypericin production. In phytopharmaceutical industry, one solution could be the production of micropropagated plants, in sterile and standardized conditions. Several studies have been carried out to investigate the effects of different biotic and abiotic factors on the accumulation of secondary metabolites in *Hypericum in vitro* cultures. We have previously studied the overproduction of phenylpropanoids and naphtodianthrones in *H. perforatum in vitro* cultures upon treatment with phytohormones [12] and various elicitors, such as jasmonic acid [13], *Aspergillus flavus* mycelia extract [14] and salicylic acid [15]. In addition, various *in vitro* cultures from *H. perforatum* have been tested for their ability to produce naphtodianthrones and acyl-phloroglucinols upon treatment with mannan, β-1,3-glucan, pectin, jasmonic acid, methyl jasmonate, salicylic acid, fungal elicitor *Phytophtora cinnamoni* and bacterial elicitor *Agrobacterium tumefaciens* [16, 17, 18, 19]. Even if some biotic and abiotic factors can regulate hypericin and pseudohypericin production, the role of genetic transformation need further investigations.

Plant genetic transformation offers opportunity to introduce new qualities into medicinal and aromatic plants. Until now, only *A. rhizogenes*- [20, 21, 22] and biolistic-mediated [23] transformation procedures for few *Hypericum* species have been applied. Wild agropine strain *A. rhizogenes* ATCC 15834 was used in the first successful transformation of *H. perforatum* [20]. Also, an efficient transformation protocol of this species was reported with *A. rhizogenes* A4M70GUS [21]. Two other *Hypericum* species (*H. tomentosum* and *H. tetrapterum*) were successfully transformed with *A. rhizogenes* ATCC 15834 and A4 [22]. Recently, we have developed an efficient *A. rhizogenes* A4-mediated transformation system for *H. perforatum* which lead to the formation of hairy root (HR) cultures [24]. *H. perforatum* HR exhibited high potential for spontaneous regeneration into whole transgenic plants [20, 21]. Studies on successful regeneration of HR have stimulated interest in developing procedures for analyses of phytochemical composition in transgenic plants. However, only few studies have been focused on secondary metabolite production in *H. perforatum* HR [24] and HR-regenerated plants [20, 25, 26]. Even if work had been carried out on these topics, our study provided complementary information on the identification and quantification of naphtodianthrones in *H. perforatum* transgenic shoots.

The main objective of this study was to establish an efficient *Agrobacterium rhizogenes* A4-mediated transformation system that would result in the rapid formation of *H. perforatum* transgenic shoots for the purposes of studying the naphtodianthrone production. Naphtodianthrones in control and transformed shoots were analyzed using high-performance liquid chromatography (HPLC) coupled with diode-array detection (DAD) and tandem mass spectrometry (MS^n) with electrospray ionization (ESI).

MATERIAL AND METHODS

Plant material

Seeds from *H. perforatum* were collected from wild plants growing in a natural population in the National Park Pelister at about 1394 m asl. Voucher specimen (number 060231) of *H. perforatum* is deposited in the Herbarium at the Faculty of Natural Sciences and Mathematics, University "Ss. Cyril and Methodius"-Skopje, Republic of Macedonia (MKNH). As previously reported [12], seeds were surface sterilized and cultured on MS macro and oligoelements [27], B_5 vitamin solution [28], supplemented with 3% sucrose and solidified with 0.7% agar. *In vitro* cultures were maintained in a growth chamber at 25±1°C under a photoperiod of 16 h light, irradiance at 50 $\mu mol \cdot m^2 \cdot s^{-1}$ and 50 to 60% relative humidity.

Establishment of transgenic shoots

Establishment of *H. perforatum* HR cultures was described in our previous study [24]. Briefly, HR cultures were induced from root segments of *in vitro* grown seedlings, after co-cultivation with *Agrobacterium rhizogenes* strain A4. The transgenic nature of HR cultures was confirmed by PCR analysis of the presence of *rol*B sequences from T_L-DNA of *A. rhizogenes* Ri plasmid [24]. Transformed roots were maintained by subculturing at 28-day intervals on MS/B_5 hormone-free medium in the dark. One HR line exhibiting the highest growth potential was previously selected for HPLC/DAD/ESI-MS^n analysis [24]. In this study, we used the same HR line for establishment of transgenic shoot cultures. For this

purpose, HR segments (about 1.5 cm length without root tip) were excised and cultivated on solid MS/B_5 medium without phytohormones. The cultures were maintained in a growth chamber at 25±1°C under a photoperiod of 16 h light, irradiance at 50 μmol·m^2·s^{-1} and 50 to 60 % relative humidity. A control experiment was set up with root segments apart the root tip obtained from *in vitro*-grown seedlings. After 4 weeks, HR and control explants exhibited high potential for spontaneous shoot regeneration. Emergent control and HR-regenerated shoots of size 1.5-2.0 cm were excised from the root tissues and transferred on solid MS/B_5 medium supplemented with 0.2 mg·L^{-1} N^6-benzyladenine (BA) for incessant shoot growth. Shoot cultures were monthly subcultured on the same medium and maintained under a 16/8 h light/dark photoperiod. After the 3rd subculture, control and transgenic shoots were used for evaluation of naphtodianthrone composition. Thereafter, control and transgenic shoots were harvested, frozen in liquid nitrogen and then stored at -80°C, until phytochemical analysis.

HPLC/DAD/ESI-MS^n analysis

Naphtodianthrones were extracted from powdered plant material (0.2 g) with 80% (v/v) methanol in ultrasonic bath for 30 min at 4ºC. Methanolic extracts were centrifuged (15 min at 12000 rpm) and the supernatant was filtered through Sep-pack C_{18} cartridges before HPLC analysis. The HPLC system was equipped with an Agilent 1100 series diode array and mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser and G1315B photo-diode array detector, controlled by ChemStation software (Agilent, v.08.03). Chromatographic separations were carried out on 150x4.6 mm, 5 μm XDB-C18 Eclipse column (Agilent, USA). The mobile phase consisted of two solvents: water-formic acid (A; 99:1, v/v) and methanol (B) in the following gradient program: 90% A and 10% B (0-20 min), 80% A and 20% B (20-30 min), 65% A and 35% B (30-50 min), 50% A and 50% B (50-70 min), 20% A and 80% B (70-80 min) and continued with 100% B for a further 10 min. Each run was followed by an equilibration period of 10 min. The flow rate was 0.4 mL·min^{-1} and the injection volume 10 μL. All separations were performed at 38°C. Formic acid (HCOOH) and methanol (CH_3OH) were HPLC grade solvents (Sigma-Aldrich, Germany). The HPLC-water was purified by a Purelab Option-Q system (Elga LabWater, UK). The commercial standards hypericin and pseudohypericin (Sigma-Aldrich, Germany) were used as reference compounds. Peak areas were used for quantification at 590 nm where naphtodianthrones exhibited an absorption maximum. The HPLC system was connected to the Agilent G2445A ion-trap mass spectrometer equipped with electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v.6.1.). Nitrogen was used as nebulizing gas at a pressure-level of 65 psi and the flow was adjusted to 12 L·min^{-1}. Both the heated capillary and the voltage were maintained at 350°C and 4 kV, respectively. MS data were acquired in the negative ionization mode. The full scan mass covered the mass range from *m/z* 100 to 1200. Collision-induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with voltage ramping cycle from 0.3 up to 2 V. Maximum accumulation time of the ion trap and the number of MS repetitions to obtain the MS average spectra was set at 300 ms and 3, respectively. Identification of the component peaks was performed by the UV/Vis, MS and MS^2 spectra and retention times of the abovementioned available standards.

Statistical analysis

The experiments were independently repeated twice under the same conditions and all analyses were performed in triplicate. The data were expressed as mean values with a standard deviation (±S.D). In statistical evaluation, the Student's *t* test was used for the

comparison between two independent groups using SPSS statistical software program (SPSS version 11.0.1 PC, USA, IL). Differences were considered significant at $p<0.05$.

RESULTS & DISSCUSION

Establishment of transgenic shoots

HR induction and shoot regeneration are important prerequisites for successful production of transgenic plants using *A. rhizogenes.* In our laboratory, *H. perforatum* HR cultures were induced by co-cultivation of root explants with *A. rhizogenes* strain A4 in order to study the production of phenolic compounds [24]. *H. perforatum* transgenic shoots were regenerated from HR explants on MS/B_5 medium without phytohormones under a 16/8 h light/dark photoperiod. Control root explants obtained from *in vitro* grown seedlings were also cultured under the same conditions. HR and control explants did not exhibit a significant difference in the regeneration frequency (about 65%). Transgenic shoots evaluated in our study showed normal size and morphology like those regenerated from control roots. In order to stimulate further growth of transgenic and control shoots, they were cultured on MS/B_5 medium supplemented with 0.2 $mg \cdot L^{-1}$ BA medium. After 20-30 days of culture on this medium, multiple shoots were obtained directly from apical or axillary buds. Various potential for regeneration of transgenic shoots from *H. perforatum* HR cultures was already reported in the literature [20, 21, 25]. In this view, the regenerative potential of *H. perforatum* HR cultures differs with respect to the light requirement; consequently, shoot regeneration can be promoted by light [20] or is light independent [21]. With regard to the shoot morphology, *H. perforatum* HR-regenerated shoots have shown either a normal [21] or altered phenotype [20]. As presently established, transgenic shoots had normal morphology like those regenerated from *H. perforatum* HR induced by infection with *A. rhizogenes* A4M70GUS [21]. On the other side, transgenic shoots regenerated from *H. perforatum* HR transformed with *A. rhizogenes* ATCC 15834 exhibited typical "HR phenotype" including dwarfism, shorter internodes, increased branching, reduced apical dominance, small and wrinkled leaves [20]. The direct mode of shoot regeneration, as observed in *H. perforatum*, is a desirable trait when the aim is to study the production and accumulation of bioactive compounds in transgenic plants.

HPLC/DAD/ESI-MS^n analysis

The HPLC/DAD/ESI-MS^n technique was used to analyse the naphtodianthrone production in *H. perforatum* control and transgenic shoots. Among the class of naphtodianthrones, hypericin (**H**), pseudohypericin (**PH**) and protopseudohypericin (**PPH**) were identified in control and transgenic shoots (Table 1, Fig. 1). The HPLC-MS analysis of naphtodianthrone compounds (**H**, **PH** and **PPH)** have given collision induced fragment ion spectra identical to those reported by Piperopoulos et al. [29] and Piovan et al. [30], allowing a clear identification of these compounds in the samples. The UV/DAD spectra of compounds **PH** and **H** showed identical absorption maxima typical for pseudohypericin and hypericin, respectively [31]. In addition, the identity of these compounds was verified by comparison of their ESI mass spectrum and the HPLC retention time with an authentic standard of hypericin. The UV and mass spectra of compound **PPH** were consistent with those of protopseudohypericin [31]. Regarding the quantitative aspect, the concentration of identified naphtodianthrone pigments was about 12-fold higher in transgenic shoots (Fig. 1b) compared

to control (Fig. 1a). Pseudohypericin was found as the main naphthodianthrone in transgenic shoots, usually present in 3.5-fold higher amounts than hypericin (Fig. 1b). Although, *H. perforatum in vitro* cultures are known to produce naphtodianthrones, there has been little work to investigate whether these compounds are inducible by genetic transformation. In this view, only few studies have been focused on hypericin assay in *H. perforatum* HR-regenerated shoots [20, 25, 26]. It is important to point out that hypericin content in *H. perforatum* transgenic shoots evaluated by Bertoli et al. [25] was similar to that reported here. On the other hand, Koperdáková et al. [26] showed much lower total hypericin content in *ex vitro* cultivated *H. perforatum* transgenic plants due to reduced number and size of dark glands on leaves and petals. Even though *Hypericum* transgenic shoots are suitable for production of hypericin and pseudohypericin, results from our previous study [24] clearly showed the absence of naphtodianthrones in transgenic roots. In the genus *Hypericum*, the roots have never been used as a source of hypericin and naphtodianthrones were mentioned to be present just in traces or not detected at all [32]. However, bioreactor-cultured adventitious roots of *H. perforatum* have shown a capability for hypericin production [33]. Recent studies have been carried out to determine the possible sites of hypericin biosynthesis [34, 35]. These authors analysed the expression level of the *hyp-1* gene encoding for the phenolic coupling protein which is assumed to be involved in conversion of the precursor emodin to hypericin. The gene is expressed in all organs of *in vitro* grown plants with the highest level of *hyp-1* mRNA found in the roots [35]. These authors indicated that the final stages of hypericin biosynthesis take place in different plant parts, mainly in roots, which are not essentially associated with the dark glands and primarily serve for hypericin accumulation. In contrary, recent histochemical analysis showed the presence of dark-red globules rich with hypericin in all *H. perforatum* organs, including leaf, stem and root [36]. These findings suggested that the black glands are not the only site of naphtodianthrone accumulation and hypericin is probably generated in mesophyll cells or in tissue of the root and/or stem and subsequently is transported to the glands. Transgenic shoots evaluated in this study showed apparition of dark glands on the margins of leaves and exhibited high yield of naphtodianthrones during the first month of cultivation. It is worth noting that transgenic shoots produced hypericin and pseudohypericin content up to 5-fold higher than the best reported from our previous studies [12, 15] with *in vitro* multiplied shoots. This is the most important advantage of transgenic shoots as an *in vitro* model for studying the biosynthetic pathways of naphtodianthrones within a short cultivation time. Altogether, these results indicated that *H. perforatum* transgenic shoots represent a promising experimental system for enhanced production of naphtodianthrones.

Figure 1 HPLC-DAD chromatograms of *Hypericum perforatum* control (a) and transgenic shoots (b) monitored at 590 nm for detection of naphtodianthrones. Compound symbols correspond to those indicated in Table 1.

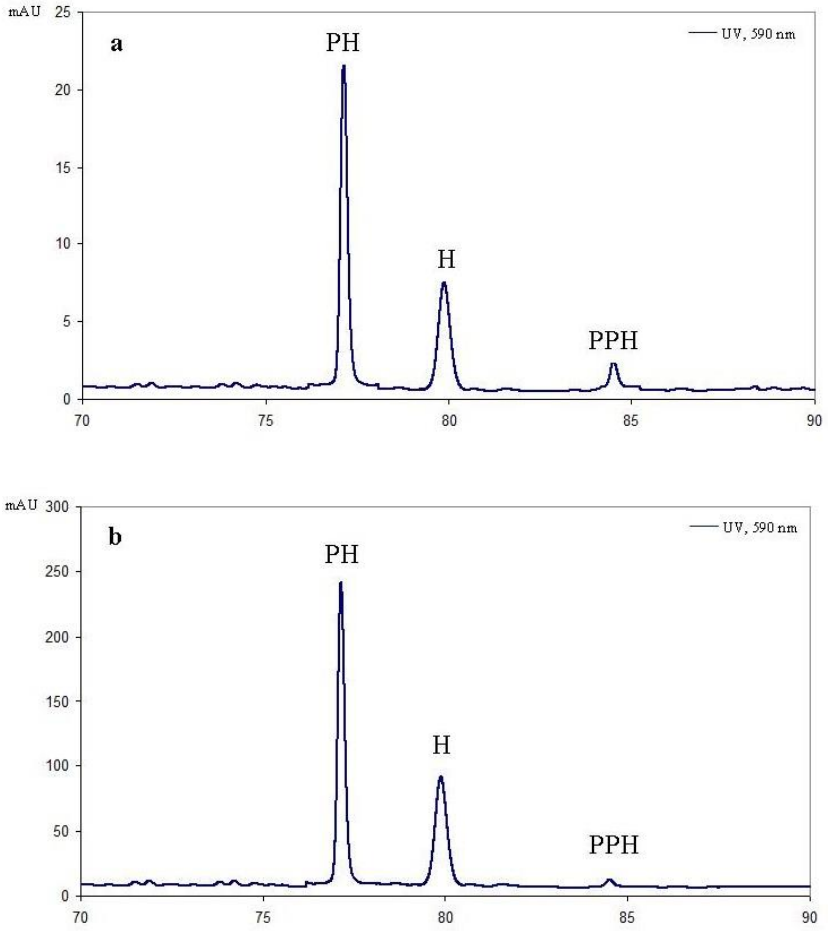

Table 1 HPLC/DAD/ESI-MS^n data of the identified naphtodianthrones in *H. perforatum* control and transgenic shoots.[a]

Peak	Compounds	t_R (min)	UV (nm)	$[M–H]^-$ (*m/z*)	$–MS^2 [M–H]^-$ (*m/z*)	Control shoots mg·100g^{-1} DW±S.D.	Transgenic shoots mg·100g^{-1} DW±S.D.
PH	Pseudohypericin	76.7	288, 325, 465, 580	519	487, 421	7.84±0.82	85.76±4.23*
H	Hypericin	78.2	288, 325, 465, 580	503	**405**	2.14±0.02	25.83±1.89*
PPH	Protopseudohypericin	85.3	285, 375, 550	521	**423**	0.69±0.04	2.15±0,01*
Total						**10.67±0.88**	**113.74±6.13***

[a]DW dry weight, t_r retention time. MS^2 ions in bold indicate the base peak. For information on peak letters see Figure 1. *Denoted values indicating significant differences between data ($p<0.05$).

CONCLUSION

In conclusion, we have developed an efficient system for regeneration of *H. perforatum* HR cultures which leads to the formation of transgenic shoots producing naphtodianthrone compounds. Chromatographic analyses revealed that pseudohypericin, hypericin and protopseudohypericin were the only naphtodianthrones identified in both control and transgenic shoots. The concentration of hypericin and pseudohypericin was about 12-fold higher in transformed shoots compared to control. Pseudohypericin was found as the main naphthodianthrone in transgenic shoots, usually present in 3.5-fold higher amounts than hypericin. In addition, a 3-fold increase of protopseudohypericin was also found in transgenic shoots. The direct mode of shoot regeneration, as observed in *H. perforatum*, is a desirable trait when the aim is to obtain genetically stable and viable plants for the purposes of studying the production and accumulation of naphtodianthrones. Therefore, *H. perforatum* transgenic shoots represent a promising experimental system for obtaining extracts with qualitatively and quantitatively standardized amounts of naphtodianthrones. Further studies are necessary to exploit the biosynthetic potential of transgenic shoots, focusing on the production of other specific bioactive metabolites.

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Original scientific paper

IN VITRO CULTURE OF MATURE ZYGOTIC POMEGRANATE EMBRYOS (*PUNICA GRANATUM* L.)

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ABSTRACT

In Albania there are some spontaneous populations, as well as autochthonous varieties of pomegranate. This important tree, except for the use of tasty fruits, is also evaluated for its pharmacological properties and medicinal bioactivities of compounds. The objective of this study is to establish an *in vitro* germination and cultivation protocol for two pomegranate varieties (spontaneous and autochthonous Devedishe) using zygotic embryos. A high rate of germination was obtained when isolated embryos were cultured *in vitro*, a result alike those reported in the literature with traditional dormancy breaking treatments. First stage of i*n vitro* development of embryos was optimal in a full-strength Murashige and Skoog media without PGRs. The maximal growth percentage of embryos occurred for Devedishe variety (80%) in comparison with spontaneous variety. It is also observed spontaneous rooting in organogenesis induction media. This is due to the ability of mature zygotic embryo to be autonomous, especially in synthesizing phytohormones. During subcultures, in a high micropropagation coefficient resulted the use of MS media supplemented with plant growth regulators (mg l^{-1}) cytokinin BAP 0.5 and auxin NAA 0.1. In this stage, a great number of shoots was produced from the initial seedlings after 14 days of culture. Devedishe variety reacted better in this stage as well. The future of this fruit depends on the selection of high quality varieties and micropropagation of elite pomegranate genotypes seen as effective method for its *in vitro* germplasm conservation.

Keywords: pomegranate, zygotic embryo, micropropagation, phytohormones

INTRODUCTION

Establishment of a tissue culture protocol for plantlets regeneration through micropropagation is an essential prerequisite for the potential applications of clonal propagation, genetic transformation and preservation of plant germplasm.

Pomegranate (*Punica granatum* L.) is generally known as a distinct family (Punicaceae), which comprises only one genus (*Punica*) and two species *P. granatum* and *P.protopunica*. *Punica granatum* was cultivated and naturalized over the whole Mediterranean region since

ancient times. The pomegranate is one of the oldest edible fruits and an excellent tree for growing in arid zones due to its resistance to drought conditions [20].

It is cultivated throughout the tropical and subtropical regions of the world for its delicious and highly nutritive fruits. Apart from fruits, stem bark contains alkaloids useful to control dysentery and diarrhea [6].

This important tree, except for the use of tasty fruits, it is also evaluated for its pharmaceutical properties, medicinal bioactivities of compounds and like ornamental plants. Some parts of the pomegranate tree (leaves, immature fruits, fruit rind, and flower buds) have been used traditionally for their medicinal properties and also for tanning of leather [4].

The seeds along with the fleshy pulp are dried and used as condiment. The fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols, and a fair source of iron. The several uses of the minor fruit plants (alimentary, ornamental, forestry and medicinal), where the pomegranate belong, as well as the socio-economic impact, justifies the need to preserve these traditional genetic resources, that include conservation of agro-biodiversity, production of organic food, conservation of traditional landscapes and the related bio-diversity. In this context, the pomegranate represents a very interesting fruit plant for both economic and scientific reasons.

Several protocols have been developed for regeneration of *Punica granatum* L. plantlets through either indirect organogenesis [12, 16] or through embryogenesis from various seedling explants [3, 5, 8, 15].

The main objective of this study was to develop an efficient plant regeneration system via zygotic embryo *in vitro* culture of two Albanian pomegranate varieties (spontaneous and autochthonous Devedishe).

MATERIAL AND METHODS

Plant material: As primary explants were used zygotic embryos isolated from mature dried seeds of two Albanian pomegranananate varieties (spontaneous and autochthonous Devedishe). Three weeks after embryo culture, from regenerated seedlings were isolated root segments, which were used as explants in order to induce somatic embryogenesis or indirect organogenesis.

Sterilisation and desinfectation procedures: Before sterilization, dried seeds were washed carefully with tap water and then are shaken for 5 min. in Bavistine 0.2%, were rinsed three times with H_2O_2, followed by 2 min. treatment with $HgCl_2$ 0.1%. After that, the explants were rinsed five times with sterilized H_2O_2, followed by the final treatment with ethanol 70% for 30 sec. Then the seeds were left for 24 hours in sterilized H_2O_2 in order to facilitate the isolation of zygotic embryos.

Organogenesis induction: For both varieties of zygotic embryos was used Basal MS media [11] without phytohormones or PGRs, combined with 3% sucrose and 0.7% agar. pH value was established in 5.6.

Subculture stage: For plantlets micropropagation, regenerated from the first stage of culture, was used MS basal media and were compared two variants of PGRs concentrations: MS I

which contained cytokinin BAP (6-benzylaminopurine) 0.5 mg l^{-1} and auxin NAA (1-naphtaleneacetic acid) 0.1 mg l^{-1}, and MS II which contained only BAP 0.5 mg l^{-1}.

***In vitro* chamber conditions:** the cultures were maintained in the growth chamber at temperatures of 25° ± 2°C in a 16 h/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m^{-2} s^{-1}.

Statistical analyses: All experiments are repeated at least three times. Experimental data is elaborated by Tucey-Kramer, Student's methods and the analyse of variance (ANOVA) with JMP 7.0 statistical software.

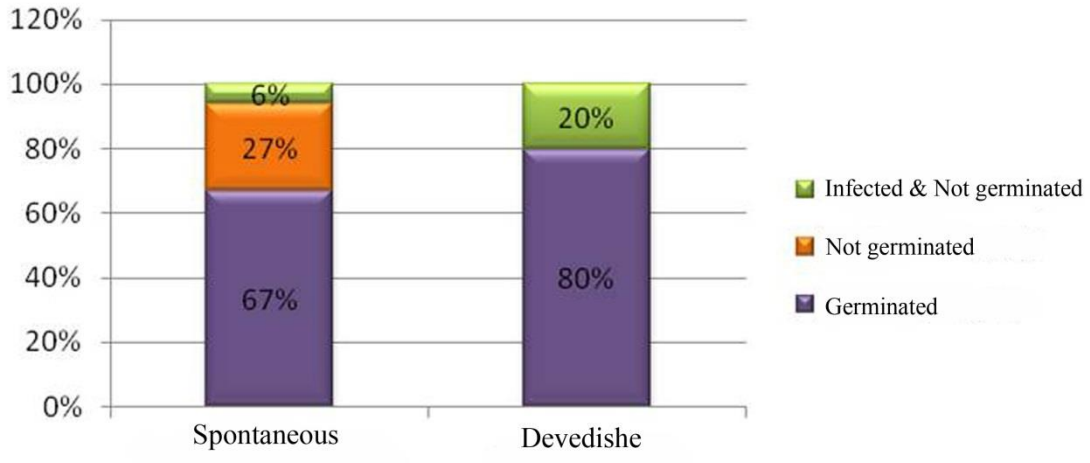
RESULTS AND DISCUSSION

Zygotic embryo culture for organogenesis induction (Stage I)

Zygotic embryos were isolated aseptically and were inoculated in basal MS media in order to induce organogenesis (Fig. 1a). The seeds of spontaneous pomegranate population were smaller and the seed coat was thinner than in seeds of Devedishe population. Therefore the isolation of zygotic embryos from the seeds of spontaneous population of pomegranate was more difficult.

Plantlet regeneration was observed 4–5 days after embryos inoculation (Fig. 1b). Germination and infections percentages for both pomegranates varieties are given in Graphic 1. From the data obtained is observed that maximal growth percentage of embryos occurred for Devedishe variety (80%) in comparison with Spontaneous variety in which is observed a greater percentage of infections during cultivation.

Three weeks after inoculation was observed the development of a vigorously root system (Fig. 1c). This is due to the ability of mature zygotic embryo to be autonomous especially in synthesizing phytohormones. In this case, the basal media only stimulates the growth and regeneration process.



Graphic 1. Germination and infections percentages for both varieties a week after inoculation

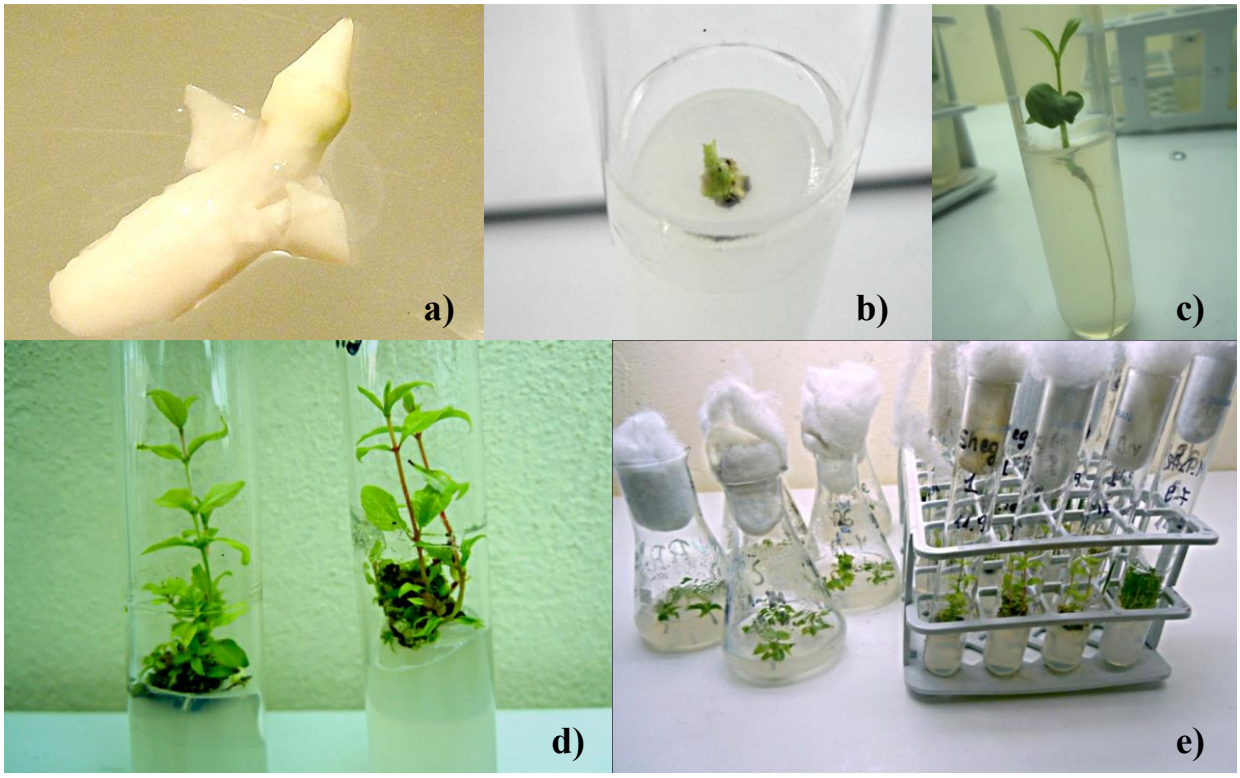
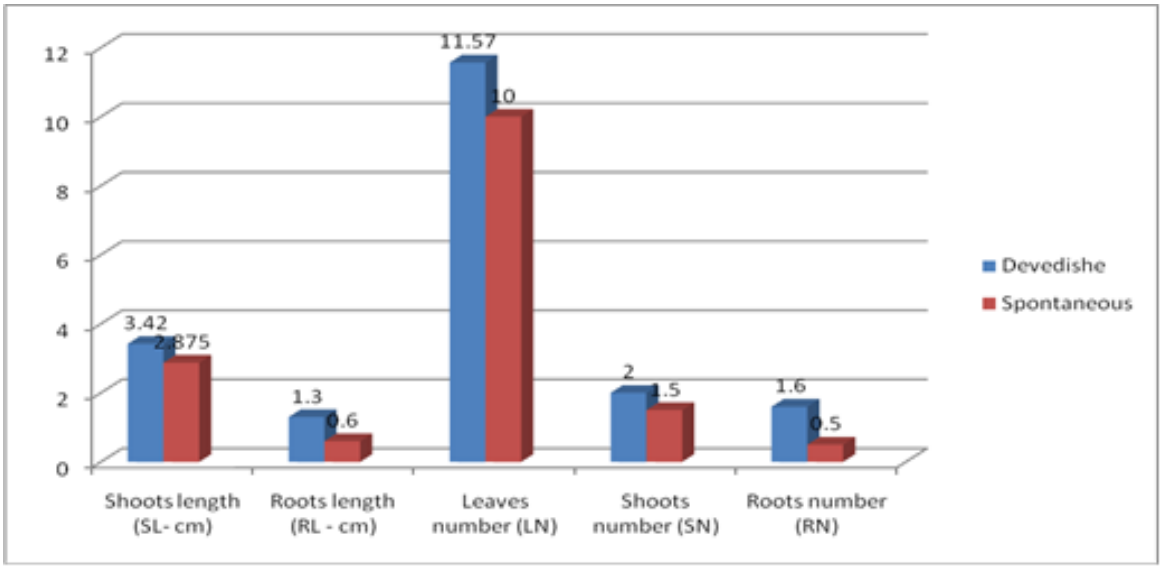


Figure 1. Stages of embryo culture: **a)** Isolated zygotic embryo **b)** Germination of zygotic embryo **c)** Roots formation during embryo culture **d, e)** Micropropagation of plantlets

During the first stage (organogenesis induction) were evaluated some biometric parameters such as shoots length (SL), roots length (RL), leaves number (LN), shoots number (SN) and roots number (RN) for both varieties. All the data are presented in Graphic 2.

Evaluating the biometric parameters, it must be concluded that during the first stage of embryo culture, the plantlets from Devedishe variety react better for all parameters measured in comparison with the plantlets of Spontaneous pomegranate variety.



Graphic 2. Dynamics of biometric parameters during first stage of embryo culture

These differences could be eventually due not only with the specific reaction of different pomegranate varieties during *in vitro* culture, but even with the embryo genetic capacity for each variety. It is well known that, for cultivated pomegranates varieties, different hybridizations and genetic improvements are made not only for the increase of production, but even for improving the fruits quality and, consequently, the quality of embryos. Our data fit well to the assumption that regeneration in pomegranate is genotype specific [10, 21].

Basal MS media without PGR's, resulted effective for pomegranate embryo culture. Oftentimes, when using mature zygotic embryos, PGRs or phytohormones are not necessary because the embryo has a considerable size and is in an autotrophic phase. As reported by other authors [18], there is no specific need for additional amounts of PGRs in the nutrient media for a large broad of wild plants.

In many cases, cultivation of zygotic embryo is used to avoid successfully post zygotic incompatibility in many woody plants [19].

Several other authors report successful micropropagation of pomegranate using nodal explants [17], internodes, hypocotyls and leaf explants [4], cotyledons [22] etc.

Subculture stage (Stage II)

Four weeks after embryo culture, all regenerated plantlets from Stage I were transferred onto fresh media for further micropropagation. For subculture stage were compared two nutrient media (both MS), which differed from PGRs concentration, mentioned below as MS I (BAP 0.5, NAA 0.1) and MS II (BAP 1).

During this stage is evaluated the effect of nutrient media (MS I and MS II) in shoots length (SL), leaves number (LN) and shoots number (SN) dynamics within a variety (Table 1, Graphic 3). Also is evaluated the dynamics of each parameter (SN, SL, LN) between varieties for each media used (Table 1, Graphic 4).

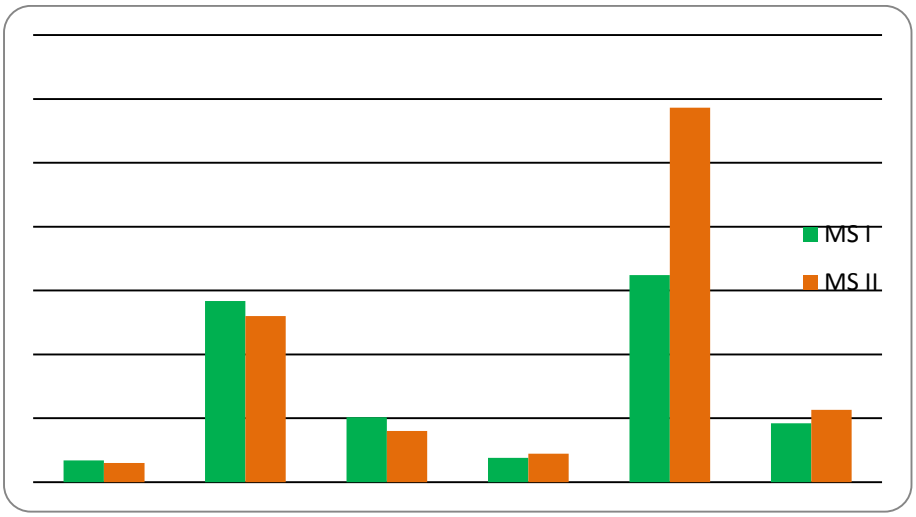
As it could be seen from Table 1, both variety do not differ statistically between them during cultivation on MS I and MS II regarding to shoots length and leaves number parameters. The effect of PGRs concentration for these parameters and for both varieties does not have any significant effect.

From the presented data, only LN (leaves number) parameter shows some significant differences. It is higher for Spontaneous pomegranate variety during cultivation on MS II (29.33) comparing to its cultivation on MS I (16.20) or to Devedishe variety in both media used (14.18 and 13.0, respectively).

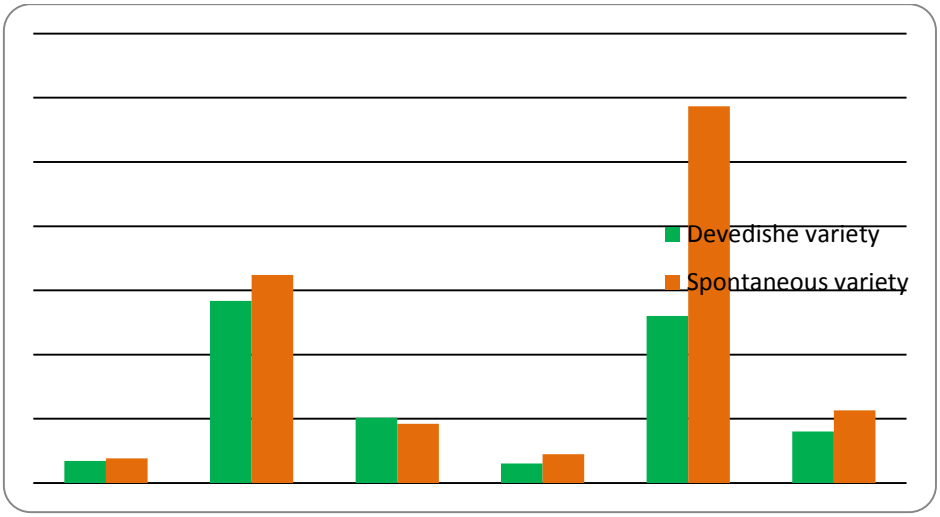
Table 1. Comparison of biometric parameters between Devedishe and Spontaneous varieties of pomegranate during subculture stage for MS I and MS II media

	Devedishe variety		Spontaneous variety	
	MS I (BAP + NAA)	**MS II (BAP)**	**MS I (BAP + NAA)**	**MS II (BAP)**
Shoots length (cm)	1.70 ± 0.25 A	1.51 ± 0.47 A	1.90 ± 0.74 A	2.23 ± 0.75 A
Leaves number	14.18 ± 1.94 B	13.00 ± 2.77 B	16.20 ± 2.35 B	29.33 ± 4.80 A
Shoots number	5.09 ± 0.94 A	4.00 ± 0.77 A	4.60 ± 1.01 A	5.66 ± 1.33 A

Note: The values not connected by the same letter (for each parameter between variety and PGRs concentration) are very different between them



Graphic 3. Comparison of measured parameters within variety during cultivation of explants on MS I and MS II



Graphic 4. Comparison of measured parameters between varieties during cultivation of explants on MS I and MS II

MS I has a 5:1 ratio of cytokinin and auxin, meanwhile MS II has only BAP but in a higher concentration (1 mg l^{-1}) than in MS I (0.5 and 0.1 respectively). From the results of this study, there is no significant differences when is used MS I or MS II, except for LN parameter. It is a well known fact that cytokinins stimulate lateral growth and inhibit apical dominance. From many reports, NAA and BAP combinations are necessary and effective for micropropagation of many fruit tree species [14, 24]. This is due to the fact that synthesis and function of cytokinin, auxin and ethylene are closely related [9]. Other authors suggest that auxins and cytokinins increase cell division and cell enlargement caused by an increase of water uptake as a result in an increase in the osmotic potential of the cell [2]. Cytokinins play an important role in plant growth and development by stimulating cell division and differentiation [7].

The combination of BAP and NAA resulted effective in micropropagation of pomegranate from nodal explants [4, 17]. Meanwhile, the highest percent of shoots regeneration and maximum number of shoots was recorded on a medium containing BA during *in vitro* propagation of pomegranate from cotiledonary explants [13] or shoot tips [10].

Other authors report that for micropropagation of *Vitis* sp. the most important is BAP concentration in the media rather than NAA concentration. They found that the most effective concentration of BAP was 1 mg l^{-1} [1]. These findings must be taken place due to the balance between endogenous and exogenous plant growth regulators.

CONCLUSIONS

- *In vitro* embryo culture results effective for micropropagation of pomegranates varieties. Basal MS media without PGRs resulted effective for these types of explants which developed throughout direct organogenesis.
- During organogenesis induction is observed that the plantlets from Devedishe variety react better for all parameters measured in comparison with the plantlets of Spontaneous pomegranate variety.
- During subcultures was observed not only the production of a considerable number of plantlets, but even increase in length of secondary and tertiary adventitious shoots.
- During subcultures there is no significant differences when is used MS I (containing BAP and NAA), or MS II (containing only BAP), except for LN (leaves number) parameter.

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Original scientific paper

MID-TERM *IN VITRO* CONSERVATION OF MYRTLE (*MYRTUS COMMUNIS* L.) – A VALUABLE MEDICINAL PLANT

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ABSTRACT

The myrtle (*Myrtus communis* L.) is a shrubby species, typical of the Mediterranean area and very common in Albanian Flora. Interest for this species is related to its uses as aromatic medicinal plant in medicine, food, handicraft, cosmetic etc. *In vitro* conservation of plant genetic resources is becoming a complementary approach to the conventional conservation methods. The objectives of the present investigation were to find out a medium-term *in vitro* preservation protocol of myrtle. Effect of reduced sucrose and Murashige and Skoog salts concentrations, elimination of PGRs from nutrient media and combination of low temperature and light regime on a collection of 30 days old of *in vitro* myrtle microcuttings have been examined for different periods. To test the regeneration of the conserved cultures, they were transferred onto fresh culture medium. The highest survival (98.0 %) and regeneration rates (88.6 %) were found in cultures stored at low temperature (4^{o}C) combined with reduced light regime for the period of 3 months. The maximal time of conservation without subculture on 4^{o}C was 10 months, in reduced sucrose and MS salt (1/2MS) concentrations and in nutrient media without PGRs was up to 5 months. Hence the shoot tips of myrtle can be successfully stored *in vitro* for medium terms at reduced incubation temperatures.

Keywords*: myrtle, minimal growth, germplasm, storage*

INTRODUCTION

Albania has a great number of medicinal plants species distributed along its territory. These plants have been used in pharmacology and popular medicine since the early days of civilization. Unfortunately, many of the species are threatened or endangered, mainly due to habitat destruction and predation.

The myrtle (*Myrtus communis* L.) is a typical species of Mediterranean Flora, shrub or small tree, very common in regions with warm climate, especially in the Southern and Central Albania to 500 m above sea level [12]. As a rustic plant, it can grow on poor and dry soils (Qosja *et al.,* 1992).

Interest for this species is related to landscape and ecological value and the uses of myrtle as aromatic plant in medicinal, food, industrial, handicrafts and cosmetic fields. Myrtle occupies a prominent place in the writings of Hippocrates, Pliny, Dioscorides, Galen, and the Arabian writers. The leaves and branches are used to make medicine. People take myrtle for treating lung infections including bronchitis, whooping cough and tuberculosis. They also take it for bladder conditions, diarrhea, and worms [8]. The liqueur produced from the berries shows antioxidant capacity values, comparable to those of red wine [25]. The myrtle is a sacred plant tied the Goddess Aphrodite and in ancient time it symbolized the fertility. The tradition about the use of plants was conserved until the 50's, but many uses were progressively abandoned, because of the change of life style. However, recently, the tradition value has been rediscovered, it represents a fundamental aspect of the Mediterranean area. Recent developments have found success in the field of the decorations. During the last years, the myrtle is used in Albania as ornamental plant for indoor decoration and gardening and as aromatic – medicinal plant.

During the last few years, *in vitro* culture techniques have been developed into a successful and rapid mean of asexually propagating a number of plant species. *In vitro* culture is an effective method for ex situ conservation of plant genetic diversity, allowing rapid multiplication from very little plant material and with little impact on natural populations. For safe preservation, the *in vitro* slow growth storage method was developed and is considered an alternate solution for medium term storage of plant germplasm [16]. The aim of medium term storage is to increase the interval period between subcultures by reducing growth. This might be achieved by the use of modified environmental conditions, modified culture medium, growth retardants, osmotic regulators and/or reduction of oxygen concentration [10]. Clonal propagation of myrtle can be successfully achieved by micropropagation and it would be of interest to make a method available for short-term conservation of *in vitro* cultures [3].

The present study involves investigations of slow growth conditions for the storage myrtle shoots cultured *in vitro*. Reduced concentrations of sucrose in medium containing half-strength [14] nutrient salts, basal MS medium without PGRs and combination of low temperature and light regime have been examined.

MATERIAL AND METHODS

- ***Plant material: collection and disinfection***

Cultures of *Myrtus communis* L. were established from apical and lateral buds removed between January and March from adult field-grown plants from Dajti population. Active shoots were cut in two - or three-node sections. The stem sections were washed carefully with water and then are shaken for 5 min in ethanol 70 % followed by 20 min treatment with $HgCl_2$ 0.01% and two drops of Tween 20. Finally, stem sections were rinsed three times with sterile distilled water.

- ***Media composition for in vitro cultivation***

- *Proliferation and subculture medium:* For this purpose was used basal nutrient medium MS [14] combined with two variants of PGRs:

- MS I combined with the cytokinin BAP (6-benzylaminopurine) 0.65 mg l^{-1}; the auxin NAA (1-naphtaleneacetic acid) 0.01 mg l^{-1}; GA_3 (gibberellic acid) 0.1 mg l^{-1};
- MS II combined with BAP 2 mg l^{-1}; NAA 0.05 mg l^{-1}; GA_3 0.1 mg l^{-1};

The media was enriched with sucrose 3% and agar 0.55%. The pH of the media was adjusted to 5.7. After 4 weeks, the developed buds were transferred to fresh media (subculture stage) in order to elongate the shoots. For this purpose was used MS I medium. Measurements of proliferation (%), length of the shoots and leaves number were taken.

- ***In vitro* chamber conditions:** A part of the myrtle cultures in the proliferation stage was grown in the growth chamber at temperatures of 25°±2° C in a 16 h/8 h light/dark regime with cool, white fluorescent light of intensity 43.4 μmol m^{-2} s^{-1}. The other part of the initial explants was maintained in the darkness during a month in order to overcome the polyphenolic oxidation.
- **For *in vitro* conservation, three different methods of minimal growth were tested:**

- *Effect of reduced sucrose and MS salts concentrations*: The cultures were transferred onto half-strength MS I medium without sucrose and supplemented with the same rate of plant regulators and agar as in the multiplication medium. The incubation conditions were the same as in the multiplication stage (in a 16 h/8 h photoperiod).

- *Combination of low temperature and light regime:* The proliferated shoots were incubated at 4ºC in dark conditions. The media under these conditions was the same with the multiplication medium (MS I medium).

- *Absence of phytohormones or growth regulators in the growth media:* The cultures were transferred onto MS medium without growth regulators or phytohormones and supplemented with the same rate of other components as in the multiplication medium (MS I medium). The incubation conditions were the same as in the multiplication stage (in a 16 h/8 h light/dark regime).

The cultures were stored in these conditions for different periods (3 - 5 months) for each method tested. For conservation in low temperature and light regime method, survival and regeneration (%) was evaluated even for 10 months conservation period, meanwhile for the two other methods this evaluation was made for up to 5 months. For each method, there were at least 15 shoots in each replication. Survival of the cultures was assessed on the basis of criteria as suggested by other authors [19], as dead and brown shoots were considered as not survived, while those with vigorous growth and having healthy leaves were considered survived.

For plant regeneration, the survived shoots from each method tested were transferred onto MS I medium and were incubated in light conditions.

- **Data elaboration:** All experiments were repeated at least three times. Experimental data is elaborated by Tucey-Kramer, Student's methods and the analyse of variance (ANOVA) with JMP 7.0 statistical software.

RESULTS AND DISCUSSION

- ***In vitro* cultivation**

Phenols secreted from the cut ends of explants lead to medium browning the 2 days after the culture establishment. This phenomenon can lead to the other problems related to explants growth and organogenesis. For this reason, a part of test-tubes was kept in a 16 h/8 h light/dark regime of *in vitro* chamber, while the other part was maintained in the darkness during a month from the initial period of *in vitro* culture.

One week after explants inoculation in the two media, the organogenesis under the effect of different PGRs concentrations and light regime (cytokinins, auxins, gibberellins) was observed.

MS I nutrient medium showed greater impact on explants proliferation in comparison with MS II nutrient medium (80 % and 37 % respectively) during incubation in a 16 h/8 h light/dark regime, whereas MS II nutrient media showed better results during incubation in dark conditions (Table 1). The positive effect of MS II could be related with the influence of the darkness in the inhibition of polyphenolic compounds production, which, in its turn, affects buds development in proliferation stage. It is assumed that this effect is related to the increase amount of auxin (5 times higher in MS II than in MS I).

Table 1. Influence of different light conditions during proliferation of myrtle buds in two different nutrient media

	Light	**Dark**
MS medium I	80 %	12.5 %
MS medium II	37 %	53 %

In any case, the best proliferation percentage was obtained during cultivation on MS I medium in a 16 h/8 h light/dark regime, media and growth conditions in which were transferred the survived shoots for the regeneration of new plantlets.

Besides the proliferation percentage, during the dynamics of development in MS I nutrient media, biometric parameters (shoots length and leaves number) were also measured (Table 2).

Table 2. Dynamics of shoots length and shoots number during proliferation stage in MS I nutrient media

	18 days	**25 days**	**31 days**
Shoots length	1.1 $\pm$ 0.06	1.6 $\pm$ 0.05	1.7 $\pm$ 0.03
Leaves number	4.2 $\pm$ 0.32	7.0 $\pm$ 0.49	10 $\pm$ 0.89

The shoot proliferation and elongation require the combination of the cytokinin BAP with an auxin [4, 21, 22]. In this study, the optimal combination was achieved by the use of the auxin NAA. The presence of gibberellin GA_3 in the proliferation medium improved the shoot elongation (Fig. 1).

Although the absolute amounts of cytokinin BAP and auxin NAA are higher in MS II in comparison with that in MS I, in the later one, the ratio of these PGRs is in favor of cytokinin BAP (cytokinin/auxin = 65). This factor influences positively in the first stage of proliferation, when greater amount of cytokinin BAP are required for buds development.

In many cases, the high concentration of cytokinin results in poor development of explants [17]. This may be a reason for the fact that MS II (with a higher concentration of BAP) is less effective in comparison with MS I.

The release of polyphenolic compounds from the explants basis affects organogenesis and further plantlets development during *in vitro* cultivation of fruit trees. According to other authors [2, 24], lights favors the increase of polyphenolic compounds and stimulates a variation in peroxidases activity during the culture cycles. It is supposed that exists an endogenous regulation of the level of endogenous auxins, which controls the alterations in peroxidases, activity related to changes in light conditions.

During subcultures in MS I nutrient media, were observed similar results as in the proliferation stage (Fig. 1).

Figure 1. Myrtle shoots during proliferation and subculture stage

- ***In vitro* conservation**

The response of *in vitro* cultured shoots stored for 3, 4 and 5 months to each method tested is assessed on the basis of survival and regeneration rates. The survival and regeneration rate of shoots for each method tested differed significantly (Table 3).

From the results it is observed that there are significant differences related to the conservation method used and to the storage period. The most effective method is conservation in 4^{o}C in darkness, which gives better results for all storage periods (Table 3, Graphic 1, Graphic 2).

Data presented in Table 3 show that up to 75.6 % of shoot culture remain healthy and green after 5 months storage on 4^{o}C in darkness. The least survival is observed in shoots inoculated in basal MS media without PGRs for all periods tested.

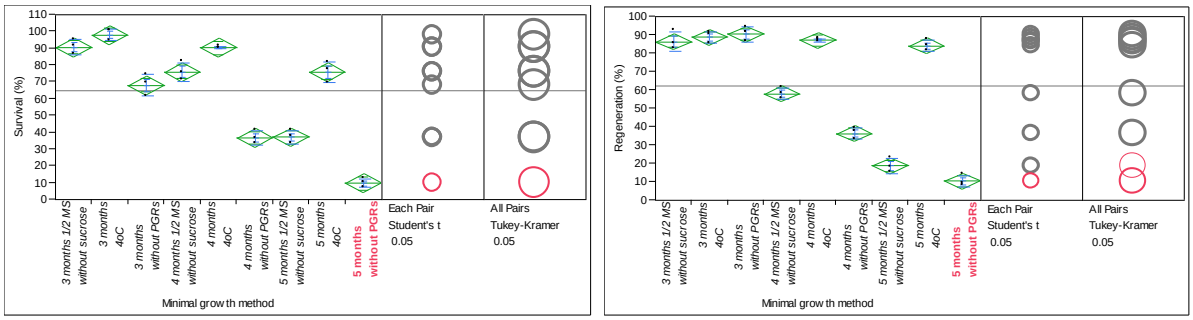
Almost similar pattern is observed for the parameter of regeneration percentage. The maximum regeneration rates were obtained on 4ºC in darkness (84 % in 5 months) while the lowest regeneration rate (9.6 % in 5 months) is observed on basal MS media without PGRs (Table 3, Graphic 1, Graphic 2). It is interesting that for 3 months storage period, conservation on basal MS media without PGRs resulted in the highest regeneration rates in comparison to other methods tested.

The storage period also has a significant effect on survival and regeneration rates. The highest survival and regeneration rates are recorded for 3-months storage, which are significantly different for the other storage periods. The cultured shoots stored for 5 months presents the greatest decrease in both parameters, especially for conservation on basal MS media without PGRs and ½ MS media without sucrose.

Table 3. Survival (%) and regeneration (%) of explants conserved with different methods of minimal growth

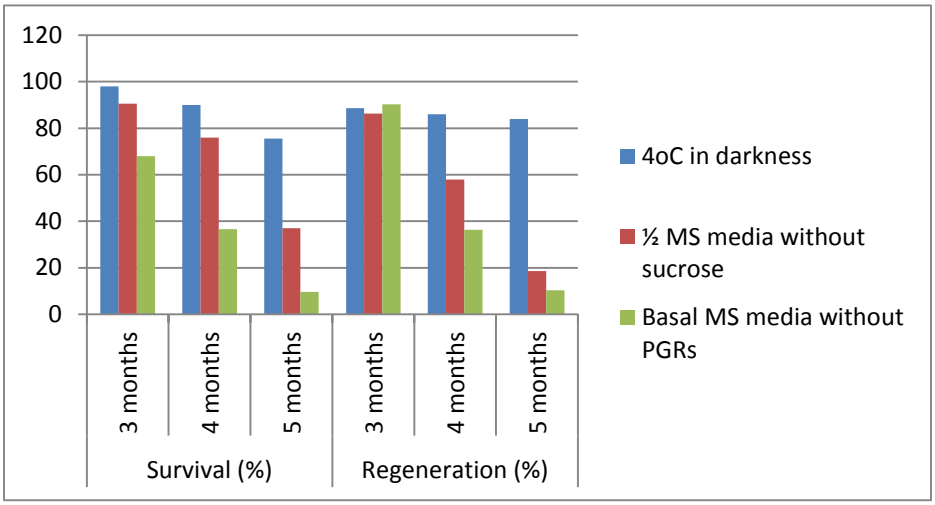
Minimal growth method	***Survival (%)***		
	3 months	**4 months**	**5 months**
4ºC in darkness	98.0 ± 2.00 **A**	90.0 ± 0.33 A	75.6 ± 3.52 **BC**
½ MS media without sucrose	90.6 ± 2.60 **A**	76.0 ± 3.21 **B**	37.0 ± 2.30 **D**
Basal MS media without PGRs	68.0 ± 3.78 **C**	36.6 ± 2.33 **D**	9.6 ± 1.45 **E**
	Regeneration (%)		
	3 months	**4 months**	**5 months**
4ºC in darkness	88.6 ± 1.85 **AB**	86.0 ± 0.57 **AB**	84.0 ± 1.73 **B**
½ MS media without sucrose	86.3 ± 2.96 **AB**	58.0 ± 1.73 **C**	18.6 ± 2.33 **E**
Basal MS media without PGRs	90.3 ± 2.33 **A**	36.3 ± 1.76 **D**	10.3 ± 1.85 **F**

Note: The values not connected by the same letter are very different between them



Graphic 1. One-way analysis of survival and regeneration (%) by storage period of minimal growth

Maintenance on 4°C in darkness is the most effective method because the shoots were stored successfully for longer periods than in the other storage method. Myrtle shoots can be stored under such conditions for up to 10 months without subculture with 31.3 % of survival rate and 36.6% of regeneration rate. Nevertheless, conservation up to 5 months in this conditions results more effective regarding to these parameters. For the two other methods of conservation, the maximum period without subculture is up to 5 months, but the most effective period of conservation might be considered conservation up to 4 months.



Graphic 2. Survival and regeneration (%) of myrtle explants conserved with different methods of minimal growth

Low temperature has been successfully applied to *in vitro* cultures of various plants species for short and medium term storage. From other reports, micro shoots of wild pear (*Pyrus syriaca*) were preserved through slow growth (low temperature) technique [23]. In previous study, temperature in the range of 5 to 10°C has been found suitable for short term *in vitro* storage of meristem cultures of several temperate species.

In the present study, the most effective method of minimal growth results the conservation at low temperature in darkness for different periods (3, 4 and 5 months). These results confirmed the findings of other workers who reported that meristem cultures of pear [26] and apple rootstocks [15, 18] can be stored *in vitro* at low temperatures.

Effectiveness for minimal growth conservation reducing MS salt concentration in basal media without sucrose is also reported from other authors during conservation of *Vitis* sp. [6], *Pyrus* sp. [1, 13], *Coffea* sp. [5] etc.

Plant growth could be reduced even if PGRs concentration in nutrient media is under optimal levels [7]. Reducing PGRs concentration resulted effective in conservation of *Fragaria* sp. [9], meantime eliminating them from nutrient media is reported as an optimal storage method for this specie [20]. This storage method is also reported for conservation of *Ramonda sp.* [11].

CONCLUSIONS

The most optimal nutrient medium is considered MS I medium (cytokinin BAP 0.65 mg l^{-1}, auxin NAA 0.01 mg l^{-1}) with higher ratio BAP/NAA, which favors the buds development in the first stage of *in vitro* culture. Comparing the reaction of cultures to different lighting regime it is concluded that the MS I medium is favorable in light, meanwhile MS II medium is most optimal in darkness.

During subcultures was observed not only the production of a considerable number of plantlets, but even increase in length of secondary and tertiary adventitious shoots in the explants micropropagated on MS I medium.

The shoots of myrtle can be stored successfully for different periods in the tested storage conditions. The most effective storage method resulted the conservation at 4°C in darkness for both survival and regeneration parameters.

With increase in storage period, survival rate as well as regeneration are reduced significantly.

Conservation in 4°C in darkness results effective during *in vitro* short-term storage of *Myrtus communis* L. plantlets up to 5 months (for survival and regeneration parameters).

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Original scientific paper

ESTABLISHMENT OF *GLORIOSA SUPERBA* CELL SUSPENSION CULTURES

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ABSTRACT

Gloriosa superba is well known non-wood forest product that has long been in regular demand amongst practitioners of traditional medicine in tropical African and Asian countries since antiquity. The medicinal importance of the plant is due to the presence of alkaloids (nearly 24 of them) of which colchicine and colchicoside (*O*-glucoside of 3-demethylcolchicine) are the principal ones. Due to action of colchicoside on spindle formation during cell division and less toxicity than colchicine, the plant has been identified as potential source of anticancer agents. Therefore the aim of our study was to increase the amount of colchicoside the expense of colchicine in suspension cultures of *Gloriosa superba*.

The homogeneity of an *in vitro* cell population, the large availability of material, the high rate of cell growth and the good reproducibility of conditions make suspension-cultured cells suitable for the analysis of complex physiological processes at the cellular and molecular levels as well as production and enhancement of secondary metabolites.

In the present investigation, our aim was to establish stable plant cell suspension cultures of *Gloriosa superba*, which provide a valuable platform for the production of high-value secondary metabolites, especially colchicoside. For the first time we have initiated and maintained suspension cultures, starting from sterile explants obtained from *in vitro* germinated seeds and tubers. The effect of some plant growth regulators and their combination on biomass production in suspension cultures was examined. The highest growth rates (54.04%-98.36%) were achieved at liquid Murashige and Skoog (MS) medium with 30 g/l sucrose supplemented with 0.2 mg/l IAA, 0.1 mg/l 2, 4-D, 2 mg/l Kin and 1.0 g/l casein.

We have established optimal conditions of cultivation, which provide the lowest amount even lack of colchicine. High Liquid Pressure Chromatography (HPLC) analysis of 70% EtOH extracts of *in vitro* samples of *Gloriosa superba*, compared to standard prove the lack of non-glycosylated colchicine. A rapidly growing cell line was selected and cultivated in 2-l stirred-tank bioreactor, by batch mode of cultivation for 14 days of culturing.

Key words: *Gloriosa superba, in vitro cell cultures, optimization*

INTRODUCTION

Medicinal plants have been a major source of therapeutic agents since ancient times to cure human diseases. Despite the major advances in the modern medicines, the development of new drugs from natural products is still considered important [1]. In this regard one such plant is *Gloriosa superba*. It is highly valued in both traditional and modern therapies. Alkaloids (colchicine, gloriosine, superbine and salicylic acid) of this plant are used in many rheumatological and immunological diseases, in the therapeutics of modern medicine. It is used in treatment of gout. Nowadays there is a staunch belief that colchicine is used in the treatment of cancer related diseases. It is used to prescribe as regular treatments for familial Mediterranean fever, have low rates of asthma and in treatment of diabetes as well [2]. *In vitro* produced cultures can be used an alternative for meeting out the demand of secondary metabolites within reasonable time and obtain them in large amount. Plant tissue culture refers to growing and multiplying of the cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Tissue culture derived material provides industrial source of different necessary metabolic compounds such as alkaloids, phenols, terpenoids, vitamins and other of compounds which are of medicinal value. Generally used methods of plant tissue cultures for the production or enhancement of the plant products include callus culture and suspension culture mainly [1]. In view of this, present investigation was aimed to establish plant cell suspension cultures of *Gloriosa superba*. Effect of different growth regulators and cultural conditions were evaluated in order to estimate stable and high growing suspension cultures with a focus on higher production of colchicoside at the expense of colchicine.

Accurate and speedy measurement of cell growth and assessment of growth-related bioprocess kinetics are essential to the efficient and rational development of plant cell bioprocess engineering. There are several methods of evaluating growth kinetics of plant cells [3]. In our study to evaluate the plant cell growth kinetics, fresh cell weight as well as fresh weight/dry cell weight ratio and accumulation of polysaccharides in the medium was used. In plant cell suspension cultures, FW/DCW (Fresh Weight/Dry Cell Weight) can be used as an index of cell water content or cell size since the increase of water content in the cell leads to an increased value of fresh weight or cell size. In addition to the growth rate, high concentration of sugar affects the ratio of fresh weight to dry cell weight. The ratio always increases as sucrose concentration drops. Battat et al. (1989) found that it remained fairly constant throughout the exponential growth phase and increased significantly only when the sugar was depleted from the medium [4].

MATERIAL AND METHODS

Plant material and cultivation conditions

In our investigation, cell suspension cultures were initiated from *in vitro* derived explants of tuber like formation from *Gloriosa superba* [6]. These shoots were sub-cultivated from solid to liquid medium. As a suitable liquid medium for inducing suspension cultures appeared modified MS [7] and LS [8] medium supplemented with 30g/l sucrose and different combination of plant hormones (auxins and cytokinins). Cultures were maintained in 100ml Erlenmeyer flasks with 30ml volume of the medium and were incubated under dark conditions or in the light regimen at 25˚C (±1) on a horizontal shaker at 100rmp for 24 days.

Measurement of cell growth

Growth of the cells was determined at 24th day of culturing, repeated twice. In order to estimate growth index (GI) at the end of culturing period, medium was gently decanted and filtered, thus fresh weight of the cells were obtained. Prior to culturing fresh weight of inoculated plant cells was established as initial fresh weight. The results from GI compare the degree of growth affected from different medium ingredients and the effect of light and dark regimen to cell growth. Growth index was presented in percentage according to following formula 1.

Formula 1: Growth index GI performed in percentage (%)

$$GI\% = \frac{FW - IFW}{IFW} x100$$

FW – fresh weight at the end of the process
IFW – initial fresh weight
GI – growth index performed in percentage (%)
DCW – dry cell weight

At the end of the cultivation,, ratio between final fresh weight and final dry weight was also estimated.

Obtaining crude polysaccharides fraction

In the other experiment, accumulation of total polysaccharides in the medium was evaluated. At this second experiment, suspension cultures of *Gloriosa superba* were cultivated at dark regimen on horizontal shaker at 100rmp. Accumulation of polysaccharides was compared for modified MS and LS liquid medium. Suspension cultures incubated on LS medium supplemented with 30g/l sucrose and 2, 4-D (0.2mg/l) were cultured for 19 days and accordingly MS supplemented with 30g/l sucrose and 0.2 mg/l IAA, 0.1 mg/l 2, 4-D, 2 mg/l Kin and 1.0 g/l casein for 22 days. The experiment was performed in three same randomized cohort groups for each of the modified liquid medium. During the experiment were selected five points in which one sample of each of the three same groups was excluded. After filtering, the medium which was carefully collected final fresh cells weight was obtained. Then cells were dried in shade at room temperature for one week and final dry cell weight was obtained. Collected filtered liquid medium was used to obtain crude polysaccharide fraction by precipitation with three times larger amount of cold 95% ethanol at stirring conditions. Precipitate was separated by centrifuge and dry under nitrogen before estimate dry weight of total polysaccharides mixture. For suspensions cultured at modified LS medium, sample were collected at 6th, 10th, 13th, 15th and 19th days of cultivation and at 5th, 12th, 16th, 19th and 22nd days respectively for suspensions cultured on modified MS medium.

HPLC analyses of established suspension cultures of Gloriosa superba

HPLC was performed at Waters 2489 system, which consisted of Waters 1525 binary HPLC pump and detector UV/Vis. The chromatography separations were performed using an analytical Waters XBridge RP-18 (5µm) column (250mm x 4.6mm). Analyses were performed with HPLC grade acetonitrile in water with each containing 0.03% trifluoroacetic acid and flow rate at 1ml/min. HPLC analyses were conducted at UV detection of 254nm.

RESULTS AND DISCUSSION

Plant cell suspension cultures were obtained by transferring *in vitro* derived tuber like formation from *Gloriosa superba* into liquid medium. After several sub-cultivation granular mass was formed onto the edge of *in vitro* explants. Separation of this granular mass free into the liquid medium forms suspension. Modified LS liquid medium supplemented with 30g/l sucrose and 0.2mg/l 2, 4-D and modified MS liquid medium supplemented with 30g/l sucrose and 0.2 mg/l IAA, 0.1 mg/l 2, 4-D, 2 mg/l Kin and 1.0 g/l casein appeared to keep adequate growth value of *in vitro* cultured explants and to provide optimal conditions for establishing suspension cultures. Results for evaluation of GI show that plant cells increase their fresh weight better in the dark conditions. Higher rates of GI were achieved at modified MS medium compare to modified LS medium (diagram 1).

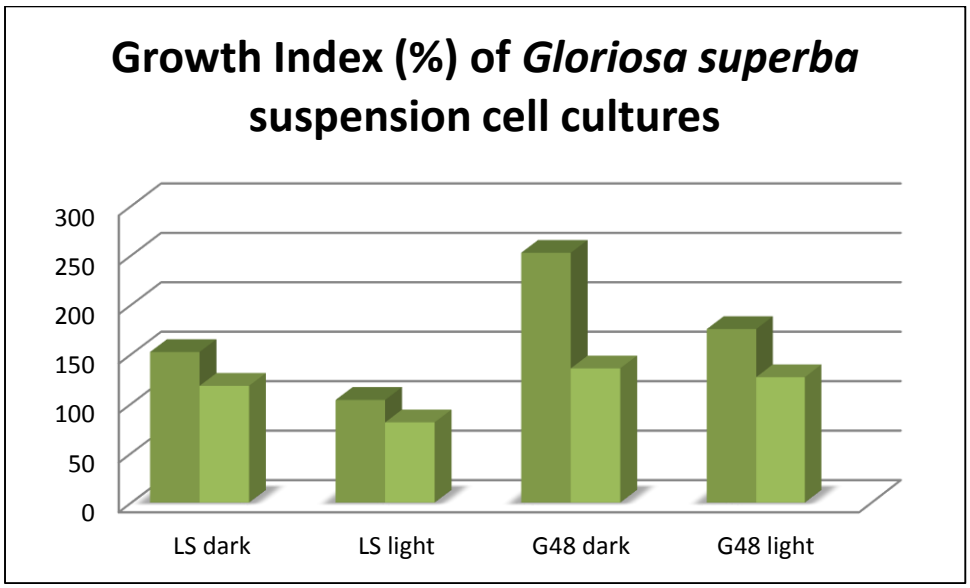

Diagram 1. GI (%) of plant cell suspension cultures of *Gloriosa superba*.

The ratio FW/DCW of suspension cultures at the end of 24th day of inoculation in all three same groups of each modified medium cultured at dark and light regimen appeared to be comparable (Table 1). The FW/DCW can be used as one of the important process variables in the production of useful metabolites by plant cell suspension cultures, since it indicates the status of the cells [5]. Closed values for this variable parameter could be perceived as an indicator for stable established suspensions from *Gloriosa superba*.

Table 1. FW/DCW at 24th day of culturing for both modified medium cultivated at dark and light regimen

FW/DCW at 24th day	**LS dark**	**LS light**	**MS dark**	**MS light**
1st group	10,49	11,7	12,46	10,67
2nd group	10,11	10,72	14	11,76
3th group	9,52	8,75	12,08	12,96

The results from our second experiment concerning the accumulation of polysaccharides in the medium and estimation of ratio FW/DCW from plant cells culturing at modified LS medium is presented in table 2 and figure 1 and from plant cells culturing at modified MS medium accordingly at table 3 and figure 2.

Table 2 Accumulation of polysaccharides from cell suspension cultured at modified LS medium from *Gloriosa superba*

Acculumation of polysacharides from suspension cultures from *Gloriosa superba* - modified LS medium					
	6th day	***10th day***	***13th day***	***15th day***	***19th day***
mean value g/l from the three evaluated groups	0,15	0,17	0,177665	0,227767	0,7478

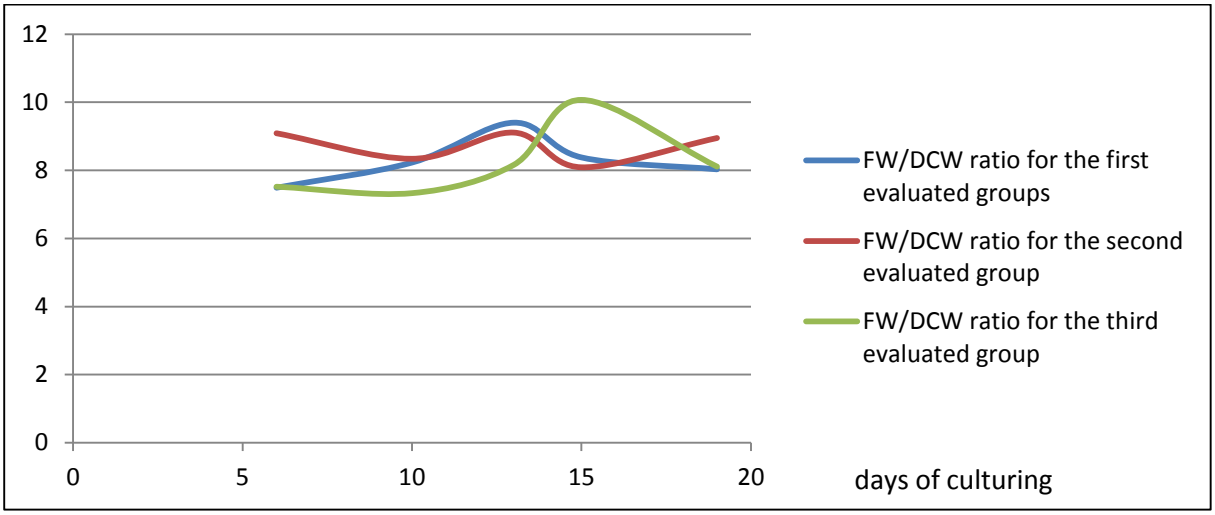

Figure 1. Ratio FW/DCW for cell suspension cultured at modified LS medium from *Gloriosa superba*

Table 3. Acculumation of polysacharides from cell suspension cultured on modified MS medium from *Gloriosa superba*

Acculumation of polysacharides from suspension cultures from *Gloriosa superba* – modified MS medium					
	5th day	***12th day***	***16th day***	***19th day***	***24th day***
mean value g/l from the three evaluated groups	0,32	0,4267	0,506357	0,582233	1,02365

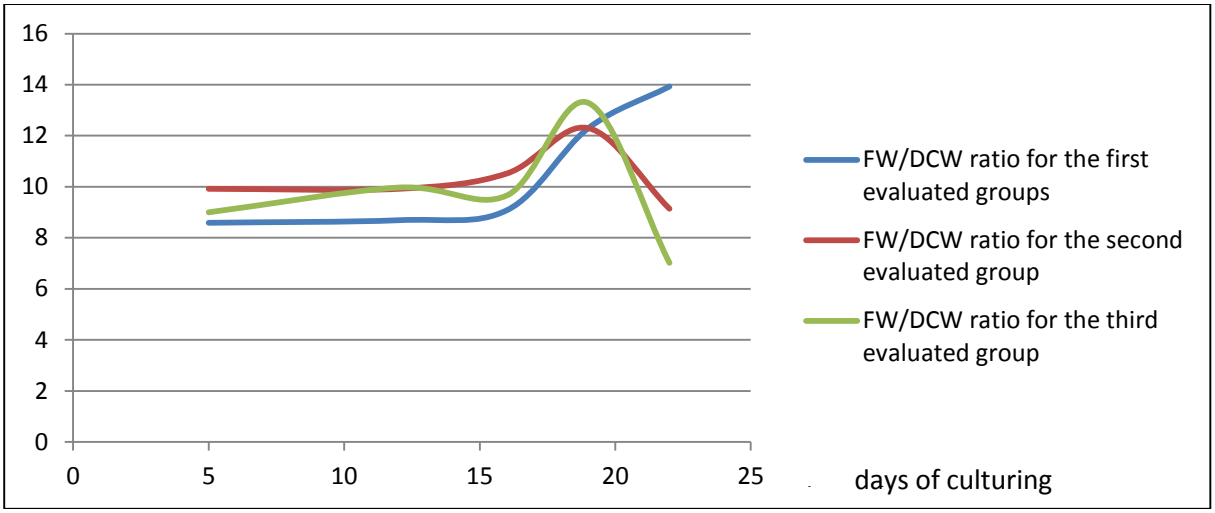

Figure 2. Ratio FFW/FDW for cell suspension cultured on modified MS medium from *Gloriosa superba*

Both types of modified medium, which were used for culturing plant cells during our experiment shown significant increase of the amount of crude polysaccharides released in the medium. For plant cell incubated at modified LS medium, accumulation of polysaccharides happen after 14-15 day and for modified MS medium respectively at 18-20 day of culturing. The rapid increase of polysaccharides could be correlated to changes in the FW/DCW. The FW/DCW maintained a certain value during the exponential growth phase and then increased rapidly in the stationary phase [5]. For established from us suspension FW/DCW ration increases between 13-15 day for plant cell cultured at modified LS medium and at 17-18 day according to modified G48 medium which period responds as stationary phase. But FW/DCW ration decrease significantly as the sucrose concentration increased. This suggests that higher sugar concentration makes the cells more compact [5]. FW/DCW ration depressions at the end of culturing correspond to an increased amount of accumulated polysaccharides.

HPLC analyses for both established types suspension cultures show lack of non-glycosylated colchicine compare to standard. Thus we may conclude that the whole presence of colchicine, main second metabolite to *Gloriosa superba* probably is in its glycosylated form. Further analyses should be focused on estimation of presence of colchicoside.

CONCLUSION

Using the capabilities of modern biotechnology for the first time we have established plant cell suspension cultures from *Gloriosa superba*, important medicinal plant as a potential source of anticancer agents. Optimizing cultural conditions and combinations between different growth regulators we received high growth index to suspensions. Also optimized culturing conditions lead to suspension cultures which have no production of non-glycosylated colchicine. Accumulation of polysaccharides from established suspension cultures of *Gloriosa superba* into the medium was well defined and its correlation between growth index, respectively FW/DCW ratio was revealed. Based on defined kinetic parameters and selection of well growth suspension cultures, our initial investigation to establish plant

bioreactor system of *Gloriosa superba,* producing important secondary metabolites will be extended.

ACKNOWLEDGMENTS

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Additional Papers

Additional Paper

MEDICINAL AND AROMATIC PLANTS FROM THE STUDENICA REGION-REPUBLIC OF KOSOVO

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ABSTRACT

Use of medicinal plants in primary health care and nutrition needs is traditional and imbedded in all cultures. No major problems of acceptability regarding familiarity with the usage of plant products, cultivation methods of many commonly grown plants and technologies required for processing into items of common household uses and value. Medicinal plants have also been used to develop family- based health and livelihood oriented enterprises in rural areas. Medicinal and Aromatic Plants help in: a) preserving the traditional medical knowledge, b) provide easily adaptable enterprising opportunities for unemployed youth and rural poor who can learn the trade from their parents and peers and earn not only for their livelihood but also contribute to the society. Medicinal plants have the potential to fill these needs as they provide green health alternatives and a number of other eco-friendly products of domestic and industrial usage. Found as trees, shrubs, grasses and vines, these plant species abundantly grow in the plains of the eastern region. Its entry into the world food and drug market as the environment friendly botanical products is widely seen as an emerging and new opportunity. The development of medicinal plants-based economic incentives is being increasingly applied to enlist greater participation of people in conservation of forest ecosystems.

Studenica is located in the north-east of Sharri Mountains, and lies at an altitude 600-1723 m asl. In the research conducted during the period 2008-2009 including the updates of 2013 regarding the vascular flora of the Studenica region, there has been found a significant number of medicinal and aromatic plants (MAP's). These plants belong to the following botanical families: *Lamiaceae, Asteraceae, Rosaceae, Fabaceae,* and *Apiaceae*. Some of the medicinal and aromatic plants that grow in this region represent a very important economical asset, including: *Vaccinium myrtilis, Juniperus communis, Primula veris, Sambucus nigra, Malus sylvestris, Crataegus monogyna, Cornus mas, Urtica dioica, Rosa canina* etc. In this paper we present more than 50 medicinal and aromatic species found in this region. The determination of plant species has been performed using standard floristic methods (field research expeditions and determination of species from collected floristic materials).

***Kay words**: Studenica, medical, plants, Sharri Mountains.*

INTRODUCTION

Sharri Mountains are rich with many plant species. Studenica as part of this mountain massif is rich in flora and vegetation too. This diversity was enabled from the historical past, pedological and geological structure, climate and geographical location. Some of indigenous plants of the region are known as endemic, relict and endemorelict which have great scientific importance, Krasniqi, F. (1987), Rexhepi, F. (1974-2003), Millaku, F. (1993), Millaku, F. (ed.) 2013. etc.

Altitude of 600 m to 1723 m has enabled various types of vegetation ranging from the most thermophilous to mesophillous plant communities and up to the subalpine flora and vegetation. Also, the region's geographical position has enabled the research to have an impact on different climates that enable a rich flora and vegetation. Viewing from geological aspect, the main part of the region consists of - Mesozoic and Cenozoic complex cliffs sediments. So, the cliffs of this region consist of: carbonates, some serpentine, silicates and alluviums. The territory explants it by itself that there are spotty climatic conditions, because the region mentioned above lies from 600 m. to 1723 m asl. So the conditions are very changeable. There is a interlacing Mediterranean and continental climate on fluvial valleys and gorges, since the distance from the Adriatic sea is not that big. The winters are characterized by lot of fallings whereas the summers are dry and hot.

The Chinese were the first people to write about plant species that were used in the treatment of various diseases. People of different countries use different types of plants for healing of various diseases. Even Balkan people over the centuries used plants for curing various diseases. In the recent years in southern Europe there are published various works in the field of ethno botany as Pieroni & Giusti (2008), Redzic (2007), Pieroni (2008, 2010), Gentili et al. (2009), Leoniti et al. (2009), Tagarelli et al. (2010). In Kosovo there are to be found about 3,000 plant species, out of which about 400 species are medicinal ones. Suhareka region has a continental climate, and this region is affected by modifiedsubmediteranean climate. In this region dominate oak (++), and beech forests -, and pastures that stretch of 400 meters above sea up to 1750 m (Maja e Studenicës). The average temperature is 13^0C, while the precipitation is 690 mm per year. It should be noted that the preparation of medicines for curing various diseases in this region is made by certain families that have transferred this knowledge from one generation to the next. Even today some of these families keep secret of the preparation of various products. The aims of this study were to document the ethnobotanical knowledge related to the use of plants in local folk medical practices.

MATERIAL AND METHODS

Floristic researches on the Peak of Studenica have been realized during the period 2008-2013. Floristic material is determined at the Department of Biology (Faculty of Mathematics and Natural Sciences - University of Prishtina) in Prishtina during master thesis.

Determination of the species was carried out using adequate botanical literature, as: Demiri, M. (1983), Josifovič, M. (1970-1986), Lakušič, R. (1988), Pajazitaj Q. (2004), Rexhepi F.

(2003), Paparisto, K. et al. (1988, 1992), Qosja, Xh. et al. (1996), Tutin T. G. et al. (1964 – 1976).

Plants were determined to systematic basic unit (the species). Listing of families, genders and species is done in alphabetical order. For each plant species the following information has been provided: species name in Latin, floral element and life form.

RESULTS AND DISCUSSION

Medicinal plants have the potential to fullfill the living needs, by providing health alternatives and a number of eco-friendly products of domestic and industrial usage. Found naturally as trees, shrubs and grasses, these plant species abundantly grow in the plains of this region.

Table 1. Medical and aromatic plants uses recorded on the (Studenica) Suhareka region in the current study

Nr.	Botanical taxon	Used part (s)	Constituents	Administration	Ethno medicine use in different countries of the world	Treated disease (s) or medical uses (s) in Suhareka region
	Achillea millefolium	Herba	Volatile oil, achillein, and achilleic acid, aconitic acid, resin, tannin.	Diaphoretic, astringent, tonic, stimulant and mild aromatic	Diaphoretic, astringent, treat wounds	Anti-haemorrhoidal Stomac disorders Against gases in the digestive organs
	Betula pendula	Leaf and cortex	Flavonoid glycosides, methyl esters, phenylpropanoids, steroidal saponins, methylsalicylate and resin.	Anti-inflammatory, cholagogue, diaphoretic. The bark is diuretic and laxative, and is recommended as a reliable solvent of kidney stones.	Very helpful in treatment of arteriosclerosis, arthritis, cystitis, fevers, gout, rheumatism, and kidney stones.	Antiseptic Anti-rheumatic
	Centaurium erythraea	Herba	Several bitter glycosides, alkaloids, phenolic acids, triterpenes, wax	It acts on the liver and kidneys, purifies the blood, and is an excellent tonic.	traditionally thought to be a “blood purifier” and was used for treatment of indigestion, jaundice, sores, rheumatism and wounds	Fever Digestive diseases blood purifier
	Equisetum arvense	Herba	Silicic acid, calcium, phytosterol, beta-sitosterol, malic acid, vitamin C, volatile oil.	Horsetail has antimicrobial, antiseptic, anti-inflammatory and diuretic properties.	For urinary tract infections, kidney & bladder stones, wounds & burns	Urinary system infections Diuretic
	Galium verum	Herba	Glycosid asperuloside, gallotanic acid, citric acid.	Antispasmodic, astringent, diuretic, foot care, lithontripic and vulnerary.	Abdominal distention, abrasions, arthritis, burns, cancer, cystitis.	Bladder Treatment of burn wounds

	Gentiana asclepiadea	Cortex		Antioxidant, antibacterial, treat digestive problems, fever, hypertension, muscle spasms, parasitic worms, wounds, cancer, sinusitis, and malaria	Influenza, bronchitis, gonorrhea	Remedy for heart strengthening work
	Geranium robertianum	Herba	Flavonoids, tannins, vita. (A, B & C), volatile oil.	Adaptogen, anti-microbial, anti-oxidant, anti-rheumatic, anti-septic, astringent, digestive, diuretic, immune enhancer, sedative, styptic, tonic, and vulnerary.	Bladder conditions, burns, eczema, hemorrhoids, herpes viral infections, inflammations, kidney problems, stomach problems, ulcers.	Urinary system inflammations To becoming pregnant
	Hypericum perforatum	Herba	Flavonoids, Phenolic acids, Tannins, volatile oils, vitamins.	Treatment for depression, alcoholism, gram-pozitiv bacteria.	Remedy for wounds, abrasions, burns, and muscle pain.	To treat infectious diseases of the vagina Cervical cancer Urinary system inflammations Eczemas Skin infections
	Juniperus oxycedrus	Fruit	Tannins, volatile oils, formik acids, glukosid, flavonoids, resin.	Upset stomach,Heartburn bloating, loss of appetite, urinary tract infection, kidney and bladder stones, joint and muscle pain, wounds, other conditions.	Some people apply juniper directly to the skin for wounds and for pain in joints and muscles.	Anti-rheumatic Antiseptic
	Juglans regia	Yang Fruit cortex	Junglandic acid (junglon)	Astringent, antifungicide and antiseptic. help in treatment of various skin conditions, such as acne, eczema, dermatitis, herpes, itching and psoriasis.	Some research suggests that people who eat more walnuts and other nuts might have a lower risk of coronary heart disease and death due to heart problems, high cholesterol.	Young fruits mixed with honey used against harmful bacteria in the stomach and intestine
	Orchis morio	Bulb.	Is mucilage, amounting to 48 %. It also contains sugar (1 %), starch (2.7 %), nitrogenous substance (5 %), and volatile oil.	Orchid has astringent, demulcent, expectorant, and nutritive properties, Used in treatment of gastro-intestinal irritations.	Treatment of irritations of gastro-intestinal canal.	Aphrodisiac

	Origanum vulgare	Herba	Carvacrol, thymol, limonene, pinene, ocimene, and caryophyllene.	Oregano is antioxidant , due to a high content of phenolic acids and flavonoids. In test-tube studies, it also has shown antimicrobial activity against strains of the pathogen Listeria monocytogenes.	In the traditional Austrian medicine *Origanum vulgare* herb has been used internally (as tea) or externall for treatment of disorders of the gastrointestinal.	Bronchitis, Anti-cooling Stomach pain Sedative
	Papaver rhoeas	Seeds	Alkaloids, tannins, painter, mucilage	Corrective, sedative, narcotic, hypnotic, anti-inflammatory	He used to treat a variety of ailments, including eye and lung inflammation.	Sedative Bronchitis
	Pulmonaria officinalis	Leaf	Tannins, flavonoids, saponins, vitamin C, silicic acid.	Used as a remedy against various lung disorders including tuberculosis, asthma and coughs. Lungwort is a, demulcent, diuretic, emollient and expectorant. It can help in treatment of chronic bronchitis, chronic cough, whooping cough and sore throat.	Treatment hemorrhoids, wounds.	Lung disease Bronchitis
	Rubus fruticosus	Fruit	Gallotannins, ellagitannins and flavonoids	Blackberry is astringent, depurative, diuretic, tonic and vulnerary. The plant was usually used to treat dysentery, diarrhea, hemorrhoids and cystitis.	Treatments of wounds, sores, scratches, gum inflammations, ulcers and sore throat.	Anti-inflammatory oral mucosa Astringent
	Teucrium montanum	Herba	Methanol, petroleum ether, chloroform, ethyl acetate, 1-butanol	Used as diuretic, stomachic, analgesic and antispasmodic agent, and also has antibacterial, antifungal, anti-inflammatory and antioxidative activity.	Teucrium montanum help recuperate and regain health and strength back after a longer illness, heavy physical or mental exhaustion	Anti-haemorrhoidal Blood purifier Against cramp in the abdomen Rejuvenating the body
	Urtica dioica	Herba and radix	Chlorophil, salicik acid, vit. (C,E,K_1), tannin, beta-karoten, Fe, Ca, Pand other minerals.	Used for urinary tract inflammation, and kidney stones, for internal bleeding, including uterine bleeding, nose bleeds, and bowel bleeding. diabetes and otherendocrine disorders, stomach acid, diarrhea and dysentery, asthma, lung congestion, rash and eczema, cancer.	In the tradit. Austrian medicine internally (as tea or fresh leaves) for treatment of disorders of the kidneys and urinary tract, gastro intestinal tract, loc. system, skin, cardio-vascular s. hemorrhage, flu, rheumatism.	Anti-rheumatic Anti-anemic Improved appetite Urinary tract and prostate

The table shows that this region is rich in MAP's. MAPs have economic importance and the following are most required at the global market: *Vaccinium myrtillus*, *Juniperus communis*,

Primula veris, Sambucus nigra, Mallus sylvestris, Crataegus monagina, Cornus mas, Hypericum perforatum, Castanea sativa, Achillea millefolium, Urtica dioica, Thymus sp., Rosa canina etc.
There were other authors too, who worked with MAP's in Kosovo, as Rexhepi F. (2003), Millaku F. (2009) and have concluded that Kosovo has been rich in terms of having MAP's. But they were not protected properly so some plants are not to be found anylonger and others are in real danger of extinction.

Plants that are shown in the photo are the ones which are mostly collected. But some of them are endangered due to unfair collection.

CONCLUSIONS

Once we have explored MAP have found that plants were seriously damaged by carelessly during the meeting, as grazing and burning of forests and pastures. Substantial damage to public property has had. Recommend measures to be taken by The Ministry of Environment for the protection of nature and the ministry of agriculture for the protection of MAP. people who do the collecting plants licensed and be notified of MAP collection that day by day their poor fund.

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Additional Paper

TESTING OF SOME ECOTYPES OF SAGE FOR PRODUCTIVITY AND ACTIVE PRINCIPLES QUALITY.

(Sage zonal scheme test 2012-2015, Upper Koplik, Malesi e Madhe).

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ABSTRACT

Through this study, intended to test the three albanian ecotypes of sage such as "Taraboshi","Velipoja" and "Hoti", compared to "Slovenia" variety for productivity and quality of active principles. The differences between albanian ecotypes with the foreign variety,(the variant no.4) in leafy production are more or less at the same level, but in the content of active principles and in total / ha, the differences are significant. Are observed differences in the content of essential oil, for both harvests of the first year altogether up to 41.3-50 l / ha in the local ecotypes and 36.60 l/ha in "Slovenia" variety. Oil yield in Albanian ecotypes is 12-36% higher compared to the"Slovenia" variety.

Key words:*Albanian ecotypes,cultivated sage,aktive principles,essential oil, "Taraboshi" ecotype,"Hoti" ecotype,"Velipoja"ecotype,"Slovenia"variety,oil yeld.*

INTRODUCTION

Undoubtedly that, even though we are a small country, we have a large variety of plant flora, among which, an important part is composed by the medical and aromatic plants with their typical products. Albania is remarkably rich in its biological, ecological and landscape diversity and is home to outstanding natural bio-resources such as a large number of herbs, medicinal, cosmetic and aromatic plants (MAPs).

They are present in the wild in nature and grow throughout the geographical area of the country. Aromatic and medicinal flora of the Albanian regions is noted not only for the large diversity of shapes and types, but also on the high content of aromatic and pharmacological substances. Approximately 30% of the known plant species in Europe, located in Albania, 27 species and 150 subspecies are endemic.

Aromatic and Medicinal Plants(MAPs) is the main non-timber agri-forestry bussines in albania, generating more than 25 mln Euro per year, and as we all know, the chances are that this value can be 10 times higher, involving, mostly as a part time activity, more than 100 000 rural dwellers. In the past, the sector was already an important source of revenue during the planned economy.

MAPs are found all over the country, but collection is more organized in some districts: Malesia e Madhe, Shkoder, Skrapar, Elbasan, Korce, Berat, Permet, and Durres. Sage from Northern districts is generally considered better, while the best oregano and thyme are mainly coming from Central and Southern Albania. However, environmental degradation and loss of biodiversity are considerable. Erosion is a crucial environmental problem in Albania. It results from destruction of the vegetation, inadequate agricultural practices, the country's relief (steep mountain and hillside slopes, high mean altitude above sea level), its geology and vegetation.

The value chain is mainly export-oriented: about 60-65 % of MAPs are shipped to Germany and USA. Exports of MAPs account for more than half of the timber and non-timber forestry products exports and 25% of all agri-food exports. In comparison, the domestic market is much more limited. Albania is a major international player for some products, such as sage, thyme, oregano and winter savoury. In some specific markets and market segment, Albanian products are market leaders, such in the case of sage in USA and wild thyme in Germany.

The efforts of the main operators for expanding their business are now concentrated on widening the range of wild MAPs offered and on investing in MAPs processing for the production of essential oils. The operators have mainly invested in increasing vertically downstream integration, i.e. in better warehouses, sorting/grading equipment and some simple processing facilities, trying to directly access the foreign markets. The main actors did not contribute to develop a system of specialised services or service facilities, so that the core of the chain is made by not sufficiently specialised actors, much bigger than in the past, but still very small for international standards, considering the importance of Albania in MAPs international trade.

Most of the MAPs business is made by wild products. Increasing procurement cost, competition between wholesalers and difficulty to match the market potential only with wild products are increasing the interest of the operators for cultivating some MAPs, such as sage, oregano, thyme,lavender and cornflower.

The increase interest to cultivate medicinal and aromatic plants will bring some positive effects, among which we can mention:

1-More income for growers since it is estimated that the MAPs generate revenue of $ 4000-5000 / ha.

2-Climatic and soil conditions suitable for their cultivation (they can grow in poor and skeletal soils, which, for other arable or vegetable crops is impossible), especially in the mountainous areas.

3-Quality genetic resources, with indications of active principles that best meet the requirements of the standards required by the market of medicinal plants.

4-The increasing demand of the international market for medicinal and aromatic plants.

At present, it is estimated that the total cultivated surface area, should exceed 4500-5000 ha, a cultivated land about 10 times higher than 5-6 years ago.

THE PURPOSE AND OBJECTIVE OF TESTING.

The main purpose of this study is to show the compatibility between collected material plant of sage native ecotypes, the conservation of their natural area and the sustainability of its cultivation in Albania as an alternative crop.

Our main target will be testing some of sage native ecotypes, in view of accessibility and productivity (including qualitative indicators of active principles). This comparison test will be aimed at the recognition of productive characteristics and quality of local ecotypes of sage and foreign variety "Slovenia" under cultivation and adoption of production practices in specific agro-ecological zones of the country.

As a final objective will be the selection of the best ecotype of sage based on the assessment of the suitability and productivity indicators (including qualitative indicators of active principles).

MATERIAL AND METHODS

The materials for testing, will be selected from seedlings prepared from pieces taken from the place of their natural development for the three Albanian native ecotypes, and seedlings from seeds for variety "Slovenia". These selected seedlings will be planted in a regional comparison scheme, with 4 repetitions and 4 variants for each iteration as follows:

1- The "Velipoja" ecotype,
2- The "Taraboshi" ecotype,
3- The "Hoti" ecotype, and

The variety "Slovenia".

The setting of the 4 variants for each repetitive scheme will be randomized. The variety "Slovenia" is selected as the comparative as widely entered in planting schemes in the country these last 2-3 years. Place of implementation: M. Madhe, Center Koplik Municipality, being extended in time from 2012 to 2015. This comparison test is justified by the fact of the expansion of areas planted with sage, since the economic interest of the crop is high, but that importance should also be given to the quality. The place where the testing is done, has been implemented a medium agro-technology in terms of a small farm, will occupy an area of 325 m². Planting date: May 13th 2012 (after planting was conducted an irrigation). Planting distances: 60 x 30 cm, the number of plants per hectare 55,000, the variant surface in harvesting 9 m2. Planting distances: 60 x 30 cm, the number of plants per hectare 55,000, the variant surface in harvesting 9 m2. The autumn harvest: November 2012, (no samples were taken).

a-Evaluation of sage productivity in the second year, first harvest at June 28th , 2013:

Samples were taken in planted surfaces of three native sages and the variety 'Slovenia', set for comparison purpose. Samples were weighed before being dried, measuring at the same time the humidity in harvesting, and after being dried, they were weighed again separately for each ecotypes.

Subsequently has been split leaves from the stalks to draw the ratio of weight leaf / stalk. The examination of essential oil from the leaves of sage was conducted as follows:

1-A quantity of 50 g sage (dried leaves and crushed before) extracted for two hours in a glass Clevenger apparatus.

2-Once is estimated quantity of the essential oil extracted, the calculation is done in ***ml***, versus the material absolutely dry.

3-The extracted essential oil was transferred to an absorption column filled with Al_2O_3 (Aluminium oxide) to remove water and other substances that may have passed during distillation. This clean material will then be used to determine thujone.

4-The amount of thujone can be estimated on the basis of the quantity of hydrochloric acid, HCl, that was released from thujone reaction with hydroxylamine-hydrochloride. Calculations show that 1 ml 0.1 N KOH, is equivalent to 0.01521 gram tujon. The calculation is done in the percentage of essential oil. [Complete Book of Essential Oils Technology (Aromatic Chemicals)].

Nr.	Ecotypes	Essential oil ml/100 gr (dried leaves)	Thujone in % of essential oil	Average yield q / ha (natural dried leaves)	Average yield in %	Essential oil yield in l / ha	Essential oil yield in %
1-	"Velipoja"	3.11	44.1	12.7	107	37	137
2-	"Taraboshi"	3.05	43.1	13.0	112	38	140
3-	"Hoti"	2.75	42.8	12.4	108	33	122
4-	"Slovenia"	2.41	39.2	11.5	100	27	100

Table no. 1: The indicators of oil content and thujone (expressed in % of absolutely dry material) also the indicators of Average yield /ha, the essential oil in l / ha and %.

The differences between Albanian ecotypes, with variety "Slovenia", (Variant No. 4) in leaf production are more or less the same level, but when we evaluate the content of active principles in ml / 100 grams of dried leaves and l / ha, the differences then become significant.

As we see this in the table above, the percentage of essential oil from the autochthonous ecotypes is 22-40% higher compared to the variety "Slovenia".

I did not intend to dwell on this argument because the results are absolutely clear.

b-Evaluation of sage productivity in the second year, second harvest(II), October 12th 2013.

For both harvest (first and second) we have full details regarding the harvested plants for each variant, the newly harvested production in kg, moisture in the harvested plants, dried leaf production in kg, as and yields obtained, but here we will present the most important indicators. In any case, these results will be part of the final doctoral thesis, presenting them on line.

The ecotypes	Essential oil in ml/100 gr (dry leaves)	% of thujone in the essential oil	Average yield q/ha (natural dried leaves)	In % compare to "Sllovenia" variety.	Yield of essential oil l / ha	Yield of esssential oil in %
1."Velipoja"	2.30	41.0	5.70	116	12.88	131
2."Taraboshi"	2.16	39.8	5.10	104	11.00	114
3. "Hoti"	2.02	39.5	4.20	86	8.28	86
4. "Sllovenia"	2.00	37.0	4.90	100	9.60	100

Table no. 2. Indicators of essential oil and thujone, second harvesting (expressed as % of absolutely dry material). Essential oil yield expressed in l/ha and in %, for II harvest.

What catches the eye in second harvest, is the fact that the essential oil yield obtained, is clearly higher (14 and 31 %) on both "Taraboshi" and "Velipoja" ecotypes, while the "Hoti" ecotype is quite below the "Slovenia" variety (86 %).

c-The evaluation of sage productivity in the second year, on both harvests (I+II).

The differences of native ecotypes are also evident even when we analyze the results for both harvests. Thus, the essential oil yield expressed in l / ha for Albanian ecotypes ranges from 41.3 to 50 liters / ha, while for variety "Slovenia" does not exceed the 36.6 liters / ha.

Ecotypes	Average yield q/ha (natral dried leaves, standard 12 %)	In % compare to "Sllovenia" variety	Yield of essential oil l / ha	Yield of essential oil in %
1. "Velipoja"	18.40	112	50	136
2. "Taraboshi"	18.10	110	49	134
3. "Hoti"	16.60	101	41.3	112
4. "Slovenia"	16.40	100	36.6	100

Table no. 3. Essential oil yield expressed in l/ha and in %, on both harvests.

Expressed in%, the essential oil yield for both harvests appears significantly higher than the variety "Slovenia". So "Hoti",Taraboshi and "Velipoja" ecotypes have respectively 12, 14 and 36% more essential oil that "Slovenia" variety.

RESULTS AND DISCUSSION

1- The amount of the essential oil extracted, expressed in ml/100 gram dried leaves, are impressive compared with variety "Slovenia". This ranges from 2.75 ml essential oil/100 gr dried leaves for ecotype "Hoti", at 3.05 for "Taraboshi" and up to 3.11 for "Velipoja", meanwhile "Slovenia" reaches values less than 2.5, exactly 2.41. While the values of thujone (the main component of oil and the component that interests us) at the local ecotypes goes from 42.8-44.1 % when the "Slovenia" is 39.2 %.

2-If these amounts are expressed in liters/ha then looks like there are interestingly values. The content of essential oil goes from 33-38 on the Albanian ecotypes, meanwhile "Slovenia" variety is 27 liters/ha. In percentage the result is much more interesting as it goes 122 % for "Hoti" ecotype , 137 % for "Velipoja" and at 140 % for "Taraboshi", when "Slovenia" as comparator is taken 100 %.

3-The same picture also appears in the case of the second harvest also. Distinguishes here makes "Hoti" ecotype by taking values below the "Slovenia" variety, in the meantime "Taraboshi" and "Velipoja" respectively 14 % and 31 % higher than "Slovenia" variety.

4-To extract synthetic data for both harvests, taking in consideration the given yield expressed as content of naturally dried leaves, always comparing with 'Slovenia" we have:.

expressed in l/ha, in this case we have 41.3 l/ha for "Velipoja" ecotypes, much higher values on "Taraboshi" ecotype about 49 l/ha, and even 50 l/ha for "Velipoja" ecotype, while "Slovenia" gets values 36.6 l/ha.

Expressed in %, and this is the most significant data, we have 112 % for "Hoti" ecotype, 134 % for "Taraboshi" ecotype and even 136 % for "Velipoja" ecotype.

CONCLUSIONS

1. It is important the priority preservation and quality of indigenous ecotypes that represent full advantage in the quality of active principles as we show above.
2. We see as a necessity the seed production and multiplication of native sages, providing not only the continuation of the planted area expansion but also guaranteeing this product in the export market.
3. We recommend the establishment of a chain for seed multiplication of native sage, which should have the starting point at Shkodra ATTC. This center can provide initial production of seeds and seedlings, having as basic principle the preservation of genetic purity for our ecotypes that will subsequently be the product of genetic improvement programs.

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Additional Paper

SAFFRON (*CROCUS SATIVUS* L.) - A NEW AROMATIC AND MEDICINAL PLANT AND ITS CULTIVATION

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ABSTRACT

Saffron (*Crocus sativus* L.) is a plant almost unknown in Albania, and which is not cultivated before. It is a perennial herbaceous plant, with blooming in autumn, which is included in the gender *Crocus*, Iris family (Iridaceae). In the Flora of our country there are described nine species of the genus Crocus, but it is not mentioned C. Sativus L. [2].

Saffron is a sterile Triploid derived from the wild *Crocus*, there it is not able to form seeds [3]. The possible ancestors of *C. Sativus* L, are considered some species of the genus *Crocus*, and closer relationship is thought with *C. cartwrightianus cv. Albus* [5, 6, 7].

At this plant are the dried stigmas, dark-red colour, used as products with aromatic and medicinal value. Saffron is the most expensive herb in the world. Due to its content, it is referred to as a curative plants, and even in the ancient practices it was as medical preparation with effects to the human health [8]. In nowadays saffron is mainly used as a spice in cooking. Its stigmas give a special colour and flavour in foods and beverages. Its use on olive oil, honey, brandy, etc., gives to these products a unique taste.

The value of saffron stigmas is determined by the content of the three key secondary metabolites: crocin, picrocrocin and safranal [7, 8], which are esters responsible for taste values, colour and unique aroma [12, 13].

These special components of saffron, makes it to be one of the most valuable and most expensive spice on the market.

Saffron is one of the newest plants, which through the assistance of a project, has just begun to be cultivated in some areas in Kosovo [10]. The first cultivations are done for testing and promotion purposes, on the summer of 2010. This valuable plant is used in many other sectors, such as pharmaceuticals, food industry (in drinks, sweets, etc), in cosmetics and perfumery, in the industry of colouring (as painting clothes, carpets, etc.).

In the five areas tested, plants are grown and developed normally, and continue to be in the four year of being cultivated. Annual biological cycle passes in the *active* phase that begins in the fall with flowering and blooming of vegetation, which terminates at the end of spring; and *dormant* stage respectively in the summer season, where the vegetation is dried and the corms lies under the ground.

The quality of the harvested product, sensor evaluation (colour and smell) and laboratory tests on chemical composition, resulted as quite good. Stigmas produced contain 25% more crocin and 38.5% more picrocrocin than the standard maximum values. While safranali

stands in average values (31 to allowed limits 20-50). The organic production has made it a more demanded product in the market. The results of the study and the experience of four years, on cultivation in five different areas in Kosovo, show that saffron can be cultivated successfully in the fresh climacteric conditions of our country, and also be produced under the organic production method.

Key words: *Saffron, aromatic plant, medicinal plant, cultivation, organic production*

INTRODUCTION

In recent years there is a tendency to use herbal products and processing industries demands have increased. Our country has favourable climatic-soil potentials for the cultivation of many plant species. Promotion of new species in cultivation gives new opportunities for export products.

One of medicinal and aromatic plants almost unknown, which began to be planted for the first time in the summer of 2010 in Kosovo mainly for testing purposes, is Saffron. It has some special and different characteristics from other plants grown in Albania. From this plant there are used the dried stigmas, which is highly demanded in export markets, and which has the highest price among the herbs. As such it provides good revenue for its producers.

Therefore, with the aim of evaluation of the biological performance of the plant and its product quality, it is developed a study on organic cultivation of saffron in five different ecological areas. Presentation of the results of the four-year study, it is considered as a contribution to the promotion of this new plant, as an opportunity to influence the increase of farmers' revenue and impact on rural economic development.

MATERIAL AND METHODS

Cultivation of Saffron is carried out in five plots, representing different geographical areas in Kosovo. For the first time the cultivation is done in two farms, in the summer of 2010, and then cultivation continued in three other areas, in 2012. Sowing is done in late summer, in the quiet phase of tubers, in agricultural and fallow plots, located in flat, hilly and mountainously areas.

Description of geo-climatic cultivated areas

The plots, where planting of bulbs is done, are situated on five different geographical areas of Kosovo, with continental climate conditions.

The first plantings of corms were made in the summer of 2010 in two villages:

- Dubravë, in central Kosovo, 1.5 ha cultivated, in flat agricultural land.
- Kashanjak (Cërmjan, Gjakova Commune), South-West Kosovo, planted on 15.08.2010, 1.25 ha cultivated with 120000 corms, in hilly fallow plots.

The second plantings were done on 2012, in three villages:

- Krushë e Madhe (Rahovec Commune), South Kosovo, planted on 15.09.2012, 0.55 Ha planted with 126000 corms, in hilly agricultural plots.
- Keqekollë (Prishtina Commune), North-East Kosovo, planted on 12.09.2012, 0.84 ha, hilly fallow and agricultural plots, on an altitude of 895 m above the sea level.
- Bunjak (Novobërda Commune), East Kosovo, planted on 12.09.2012, 1.2 ha, in mountainous agricultural plots, altitude 1000 m above the sea level.

All plots are situated on areas with fresh climacteric conditions, in altitude of about 800-1000 m above the sea level, on a hilly-mountainous position. Plots where plantings are loamy, contain low level of clay, rich in organic matter, with a neutral reaction.

Before being cultivated, the plots were fallow in two areas (Kashanjak and Keqekollë) and agricultural land on three other areas (Dubravë, Bunjak and Krushë e madhe).

Propagating material, corms, originated from the Netherlands, in standard dimensions in diameter 20 - 22 mm and weighing about 5 g. In plots where it is cultivated, are implemented cultivation techniques as recommended by experts [11], where are used low inputs and services are provided in accordance with the requirements of the organic production method.

During the years of cultivation, it is monitored the performance of growth of the plant and followed all stages of plant development as well as production indicators. The product harvested and dried is evaluated through sensor evaluation, for its aroma and colour, and it is analysed in laboratories, for its conformity with the ISO 3632 standards (for the content of three main essences: crocin, picrocrocin and safranal). The product is tested in export and domestic market.

RESULTS AND DISCUSSION

Vegetation phase and biological stages of the plant life cycle

From corms planted in summer the flowering stalks have begun to emerge in the autumn of the same. So plants have begun to bloom since in the first year of planting.

The first flowers have bloomed around October 5th and continued the flowering for about 3 weeks. The blooming and growing of leafs, or vegetative period, begins in October (with blooming) and continues, depending on environmental conditions, until late April or early May of next year.

Thin leaves are quite numerous, appearing in October after the blooming of the flowers, but continue also during or after flowering. They stand collected in batches (approximately 6 to 12 leaves for a bulb), green until spring - a period in which they complete development and growth. In May vegetation dries and then the plant enters into a phase of "sleep", where during the summer months only remain underground bulbs.

So Saffron's life cycle yearly, passes in two phases: the active phase and the second phase, of dormancy. Active phase begins in fall, respectively by the end of August or in September, and ends in May. At this stage the plant has its metabolic activity blooms leaves and floral

axis; at the end of this period occurs the disappearance of "parent" bulb; starts the formation of "daughter" bulbs. As such, Saffron has a vegetative propagation method. During the second phase, which is the dormant phase (that starts by the end of April or beginning of May), the bulbs remain unchanged after reaching full ripeness. Each corm carries one or two corms from which new leaves and floral axis emerges.

Photo 1: *C. sativus* L. plants in the plot on January

Flowering, harvesting and drying of the product

The flowering of plants starts on the first week of October. This varies and depends on the climatic conditions of the area during September, particularly temperature and precipitations (or irrigations done), from where starts the metabolism after the dormant period. The bloomed flowers are delicate; they stay bloomed only 2 or 3 days. Therefore their harvest is done every day in the morning, by harvesting the newly opened flowers. There are harvested all flowers that have bloomed on the plot, because they are not needed for plant reproduction (their inability to form seeds), or other reasons.

After harvest of flowers, they are immediately transported in a room where there are taken off the stigmas, which are dried within the same day. The drying process is carried out immediately in household appliances in baking ovens, at 60 °C temperature.

Photo 2: *C. sativus* L. in plot during blooming (October)

Photo 3: *C. sativus* L. plant in blooming

Photo 4: Flowering stalks

Photo 5: Flower in full blooming

Photo 6: During flower harvest (October)

Photo 7: Harvested flowers

Production indicators, quality and economical evaluation

During the first year, the year of planting, the plant blooms small quantities of flower where there is produced about 0.1-0.2 kg/ha dried stigmas. In the following year, there are more flowers for plant, therefore it is harvested about 1 kg/ha product; while in the third year the production is about 1.5-2 kg/ha dried stigmas.

The content of the product, through the sensorial evaluation (eye witnessed – colour and aroma), resulted to be of high quality. Chemical content analyzed in laboratories shows that stigmas produced contain 25% more crocin and 38.5% more picrocrocin than the maximum limits set by ISO 3632 standard.

While safranali results within the average values (31 to allowed limits of 20-50). Product's qualitative indicators are a guarantee for assuring the market. Producers have traded the product in the market by being identified with the logo of their farm and certification as organic products.

Dried stigmas are introduced in hermetically sealed glass container and kept in indoor conditions, in the dark, for over a year. The product is sold in foreign markets priced 1-2 thousand EUR / kg.

While in the domestic market (in fairs) it is tested and sold in small quantities 1-5 gr, with a very high price, from 5-8 Euros/gr dried stigma. Based on current sales prices from producers to exporters, the annual incomes after the first year of planting reaches about 5 000 EUR/ha and in other years over 10 thousand EUR/ha. The bulbs used for the first planting were purchased at 1 Euro for 8 pcs. Multiplication within the farm in the coming years will be done with the corms, that will be taken out of the now days cultivated plots.

Photo 8: Separation of stigma-s from flowers **Photo 9**: Dried stigma-s on glass jars

Biological evaluation

Plants are normally grown in all areas where are planted. They are in good conditions; the vegetative mass of the plant is already quite developed, compared with previous years. There has been no damage to plants from different climatic factors (lower or higher temperature), etc.

In these years the temperatures and raining were within normal limits. Low temperatures in winter have reached -10 °C, while in summer up to +33 °C. Plants continue to vegetate, although in the fourth of cultivation.

The service done to the plant has mainly been the cleaning from weeds, which is done manually. In fall, during September, before blooming of the stalks, when the rains were lacked, plants are irrigated in order to encourage the awakening of bulbs, to start sooner the period of vegetation and plant growth. Disease problems were not considered as an issue. For preventing the damages caused by animals the plots are fenced. In the flat plots, during winter, there was need for draining.

Organic production of cultivated saffron

Saffron is a very special product, thus buyers have been only interested for its organic production. Therefore producers have implemented organic farming method. This is made possible also because this plant does not have specific demand for agricultural inputs

(pesticides and fertilizers). The product is certified according to international standards of organic production, equivalent to EEC regulation 834/2007. Inspection and certification activities are carried out by Albinspekt, Albanian certification body. The products marketed as organic products are traded (exported) normally.

Biological phases and cycle

Through the data collected, it is drafted this scheme of the biological phases and cycle of cultivated Saffron in Kosovo (Graphic nr 1).

Graph. Nr 1: Biological cycle of saffron

Plant phases	MONTH											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Planting of the bulb												
Awakening of the bulb												
Blooming and harvest												
Vegetation period												
"Dormant" phase												

CONCLUSION

Results of cultivation of Saffron (*Crocus sativus* L.) in five geographical areas in Kosovo, in the altitude of 800 to 1,000 meters above the sea level, on continental climate conditions, in the period 2010-2013, show that this plant is grown and developed normally.

Its content and quality of the product harvested, from plants grown organically, has resulted in very high values and highly appreciated in the foreign market (export).

Although it is presented with a biological cycle in the opposite season with other plants, it is possible to grow it in cool climate areas. With demands and needs for input that it is needed for the cultivation of saffron, it is possible to apply the organic production method. It is a possibility that saffron has to be included in the list of medicinal plants cultivated in similar climatic zones. Cultivation of this new plant provides good income and high efficiency to its producers, compared to other agricultural crops.

ACKNOWLEDGEMENTS

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Additional Paper

INCREASED EXPORT POSSIBILITIES, A NEW TREND FOR ORGANIC CULTIVATION OF MEDICINAL AND AROMATIC PLANTS

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ABSTRACT

The quantity of medicinal and aromatic plants (MAP) in nature is limited, and to respond to increasing demand for these plants, the only option is to cultivate them. The practice followed in the previous years, from the ex-forestry enterprises, has been the cultivation of 3000 ha with 40 different species of MAP [1, 2]. After the 1990, the cultivation of MAP was interrupted.

While in the last years, it is noticed that the surfaces cultivated with MAP-s are increased considerably, by establishing an important branch of the domestic agricultural production.

A new trend of the today's cultivation of MAP-s, is application of organic production method. The demand from the international market is essentially increased, for the certified products. Actually are cultivated 21 MAP, from about 70 MAP-s certified in total.

The application of the organic production method has made possible the increase of the quantities exported. Cultivation of the MAP-s offers the possibilities to fulfil market demand for the quantity, and results with lower costs [2].

Cultivation makes also possible to preserve the MAP-s growed spontaneously in the forests, pastures, etc, as there is no over collection. As such, cultivation of MAP-s contributes to the sustainability of the species.

The climatic and soil conditions, the non-used agricultural areas, the know-how and many other factors, favor the enlargement of the cultivated surfaces and plants [1, 2].

However, there seems to be some limiting factors. One of the factors is that in Albania, the harvest and drying of the flower is handmade, while in Kosovo harvest is mechanically done in artificial way. The handmade work consists on higher costs, delays the service offering on optimal deadlines, while the natural drying does not assures the high quality of the product. The increase in the cultivated surfaces can be reached through improvement of the services

mechanization level and increase on the processing capacities (as artificial drying or distillation machineries) for MAP-s.

Cultivation of MAP-s requires support from the research sector, especially on the selection of ecotypes, improvement of the technologies of cultivation and drying, production of vegetation propagation material, the specialition based on the areas, application of the subvention schemes, training and including in the teaching curricula's .

Key words: *aromatic plants, medicinal plants, cultivation, organic production*

INTRODUCTION

In recent years there is a trend to the use of herbal products, thus requests for medical and aromatic plants from industries.

As for the very good climatic and soil conditions, in Albania there is growing a wide range of species [4].

Cultivation offers good opportunities to grow the quantities of MAP-s for export and decrease the quantities of the spontaneous MAP-s.

Cultivation of MAP-s is a known practice, which after the interruption on 1990, has restarted.

For a greater encouragement of the cultivation of MAP-s, we are here presenting the results of the comparative study.

The certified products according to international standards favorize exports. The increase on the cultivated surfaces, aims to resolve several issues. The present study analyses the limiting factors and gives recomandations for the enlargement of the cultivated surfaces.

The presented publication contributes to the enlargement of the MAP-s and the economical development of the rural areas.

MATERIAL AND METHODS

The study is based on the analyses of the data collected from the actors, involved in the cultivation, processing and exporting chain of the MAP-s. These actors represent producers, pickers and trading companies. The data were obtained through:

- Meetings and discussions as well as questionnaires with the producers and trading companies;
- Documentation and records kept from the producers and trading companies;
- Observations and monitoring during the field visits;
- Photos taken during field and facilities visits, as a unique documentation for the identification of the facts;

- The documented data and scientific and technical publications, archived.

There are analysed the data related to the cultivation conditions and facilities, plants and products produced, technical applications and inputs used during cultivation, marketing issues and economical indicators.

RESULTS AND DISCUSSION

Overview of the inherited cultivations practices

MAP-s cultivation for export has started in Albania since the period of the ex-forestry enterprises. The data of collected during '80s shows that the surface cultivated reached about 3000 ha, where were successfully cultivated about 45 species [1, 2]. Main cultivated plants were Savory (*Satureja Montana* L.), Oregano (*Origanum vulgare*, var. *viridis*), Sage (*Salvia officinalis* L.), Mentha (*Mentha piperita* L.), Rosemary (*Rosmarinus officinalis L.*), Lavender (*Lavandula officinalis Mill.*), White mustard (*Sinapis alba* L.), Basil (*Ocimum basilicum* L.), Coriander (*Coriandrum sativum* L.), Fennel (*Foeniculum vulgare* Mill.), Clarea (*Salvia sclarea L.*), Thyme (*Thymus vulgaris L.*), Oregano (*Origanum vulgare* L.), etc. All these plants were exported as herb, flowers and leaves, or where distillated to produce essential oils. From the distillation of the leaves of Laurel, Sage, Myrtel, needles from Pine and Fir, it is produced about 40 tons essential oils per year [2].

These tradicional plants are presented in smaller cultivated surfaces, on our days. The mainly remaining surfaces and plants, are cultivated with Rosemary (*Rosmarinus officinalis* L.); in Peqin and Ishëm; Lavander (*Lavandula angustifolia* Mill.) in Gramsh, Koplik and some Eastern areas; Laurel (*Laurus nobilis* L.) in Mallakastra and Saranda.

The structure of the organic cultivated plants

The number of MAP-s organically produced is only 21 species, from a total of 70 species that are exported as organically certified (see table nr 1). Successful plants, which surfaces are considerably increased, are: Sage (*Salvia officinalis* L.), Oregano (*Origanum vulgare* L.), Lavander (*Lavandula angustifolia Mill*.), etc. Besides the traditional plants, there are new trends on the cultivation of plants such as Corn flower (*C. cyanus* L.), Gentian (*G. lutea* L.), Common Mallow (*Malva sylvestris* L.), Marigold (*Calendula officinalis* L.*)*, Monarda (*Monarda didyma* L.), etc. [2]. Approximately 300 tons from a total of 2000 tons are certified as organic.

Table 1: Medicinal plants certified organic from cultivation

Nr	Crop (latin name)	Part of plant					
		Herb	**Flower**	**Leaves**	**Root**	**Petals**	**Seeds**
1	*Althaea officinalis* L.		X	X	X		
2	*Calendula officinalis L.*		X				
3	*Centaurea cyanus L.*		X			X	
4	*Foeniculum vulgare Mill.*						X
5	*Gentiana lutea* L.				X		
6	*Helianthus annus L.*					X	

7	*Laurus nobilis L.*			X			
8	*Lavandula angustifolia Mill.*	X	X				
9	*Malva sylvestris L.*	X	X	X			
10	*Matricaria chamomilla L.*	X	X				
11	*Melissa officinalis L.*	X		X			
12	*Mentha piperita L.*	X					
13	*Monarda didyma L.*		X				
14	*Olea europea L.*			X			
15	*Origanum vulgare L.*	X		X			
16	*Primula veris* L		X				
17	*Rosmarinus officinalis* L.	X					
18	*Salvia officinalis* L.			X			
19	*Sideritis syriaca* L.	X					
20	*Taraxacum officinale* F.H. Wigg		X	X	X		
21	*Thymus vulgaris* L.			X			

The first place for the cultivated surfaces is taken by Sage (*Salvia officinalis* L.), followed by Corn flower (*Centaurea cyanus* L.). Sage is cultivated in different areas, but the most intensive area is situated in Malësi e Madhe, with approximately 1000 ha. While Corn flower (*Centaurea cyanus* L.) is cultivated on about 30 ha, mainly in the areas of Korçë, Pogradec and Elbasan. In the last two years, there are produced 100 tons from this plant. Enlargement of the surfaces are a tendency in our days, for the Common Mallow (*Malva sylvestris* L.) in the region of Korça.

The geographical location of new areas where LAB is cultivated

New plantations of MAPs are carried out almost in every district, from Saranda in the South, to Malësi e Madhe, in the North; from the seaside to the altitudes (Korça). These cultivations are carried out on abandoned or fallowed surfaces (Ishëm, Koplik, Elbasan, etc), and on previously cultivated surfaces (Korçë, Elbasan, Lushnjë, Pogradec, etc). As such, the cultivated MAP-s are exploiting the fallow or abandoned land and are substituting the traditional crops. In Korçë, Pogradec, etc the arable crops are substituted by Corn flower, Common Mallow or Sage. Thus, MAP-s are establishing a tendency and are positioned as new cultivated crop.

In some regions within Albania, it is observed a massive cultivation of MAP-s. The most typical region is Malësia e Madhe, which is located in the North-west Albania, on a surface of about 555 km^2 (55 500 ha) [6]. The south part of this region, with a surface of about 7000 ha, located from the mountains until the Shkodra's lake and represented from fields, has not been cultivated for years. In these fields, which are composed from skeletal and stony soils, crops like cereals, vegetables, etc, are no possible to cultivate, as the fields are barren. . This might be one of the reasons, why this region is called "*Pustopojë*", which according to the Slavic toponim means "*desert field*". Duntil '90-s, these surfaces has been cultivated with MAP-s, such as Thyme (*Thymus vulgaris* L.), Lavender (*Lavandula angustifolia Mill.*), Winter savory (*Satureja montana* L.), etc. On nowadays this history is being repeated, as the cultivation of MAP-s is re-started in the region.

The factors of increase of the new plantings

The increase of the surface cultivated with MAP-s has several reasons.

The internal factors, such as climatic and soil conditions, favourite the increase of cultivated surfaces. External factors, such as the demand from the export market, has been also considered as a motivating factor for the increase on surfaces cultivated. Support from projects, donations and public subsidies in the past two years, have been also an important stimulant factor.

The biggest trading companies of the spontaneous MAP-s, are evaluating and implementing the cultivation of new areas. One reason for it is the increased demand from the market for the spontaneous MAP-s and another reason is the low costs of the cultivation of MAP-s compared to collection in the wild.

There are other specific factors, such as the very high price in export markets for plants cultivated, like in the case of Cowslip (*Primula officinalis* L.), which is sold approximately 46 % more than the normal usual price.

The new trends in cultivation of MAPs The biggest companies, the ones mainly trading on the export market, have the know how on the export markets and receive the demand from importing companies. These companies, through the evaluation of the demand, define the species to be planted and evaluate the quantities to be produced. They provide and distribute the seeds to small producers. Another tendency for these companies is the certification of cultivated products. There are about 20 companies that are already certifying their products, through subcontracting small producers for the cultivation activities.

The advantages of the MAP-s cultivation
In this section will be compared, Sage cultivation with their collection from the wild.

In the ecosystem of the soils in the region of Puka, the needed quantity has to be collected on an area of about 10 ha, which is situated in a difficult and remote part of the district.

The same quantity can be produced by cultivation of an agricultural field of about 3.8 ha under irrigation or about 5.5 ha without irrigation.

As such, the cultivation of MAP-s has the following advantages:

- Activates the the agricultural areas left fallow and non used for a years.
- Assists in the enrichment of the biodiversity, in the establishment of green spaces and landscapes, as well as in the preservation of the ecosystem.
- As being plants, which do not need heavy use of agricultural inputs (fertilizers and pesticides), the environment is not polluted and biodiversity is preserved.
- Assures the sustainability of the spontaneous species, through non over collection. The economical interest as such, is concentrated to cultivated plants, and the easiness of production.
- Increase the surfaces of plants, used by bees for collection of pollen, such as Lemon balm (*Melissa officinalis* L.), Basil (*Ocimum basilicum* L.), Coriander (*Coriandrum sativum* L.), Thyme (*Thymus vulgaris* L.), Lavander (*Lavandula angustifolia* Mill.), Rosemary (*Rosmarinus officinalis* L.), etc. [2, 7].

The advantages of cultivated MAP-s compared to the wild collected ones are the following:

- Increased yields for the surface unit and ensures production in the quantities required by the market.
- In cultivation sector, the labour productivity is higher than in collecting plants in natural conditions.
- Improves the quality and hygienic safety of plant products: the labour is focused on a specific geographical area and uses less human labour, contributing to control the process and protect the products.
- Facilitates the workload of personnel working with MAP-s, such as harvesting, cleaning, handling, transport, drying, cutting and distilling; as the work is focused on a lesser areas and in very small territories. This reduces importantly the cost of processed products.
- Facilitates specific processes such as harvesting, transport and processing, through their mechanization.
- Ensures stability in production (no major fluctuations during the years), as the agro-technical factors are more under the control of man.
- Gives the opportunity to select the varieties, populations, ecotypes or clones containing the maximum concentration of essential oils and other important elements, eliminating undesirable ones, which can not be accomplished when plants are collected in nature.
- Contributes to the sustainability of the species grown in the nature. And gives to nature the opportunity to recover from overcollection because it is used vegetative propagation material for cultivation and no plants are collected in the nature. However, this is an issue which has to be controlled further, on the quality of this propagation material

Cultivation of MAP-s as agricultural crops, increases the quantity to be offered in accordance with the market demand.

Advantages of the organic production method

In addition to providing qualitative and safer products [8, 9], organic production method as an added value, increases the possibility of exporting capacities. This, because nowdays, the international market demands mostly certified organic products, regardless of their origin: from spontaneous or cultivated plants.

The tendency of the markets has stimulated the enlargement of the organic cultivated MAP-s. The demand of the market indicates that organic products are a good product to be traded. Actually there are no restrictions by other countries for the quantities of organic products imported, therefore the cultivated field surfaces are freely oriented by market demand.

The analyses of the propagating material

The multiplication of the new plants is done in two ways. In “gamic way” (using seeds) and in “no-gamic way” (with vegetative propagation material: seedlings, cuttings, bulbs, etc). Using seeds are planted: sage, corn flower, common mallow, sunflower, etc. The origin of these seeds is from import, and in the case of sage are also used the domestic ecotypes, which seed can be reproduced. In this sector, selection of the domestic ecotypes, it is not done the appropriate research, and there is space for improvements. For sage, there is done some research to select the best varieties [6]. However, the seed market for MAP-s is not structured

and the biggest companies order the seeds at the foreign companies, in accordance with their personal needs.

While, the organic production method requires an additional element concerning the propagating material: certified propagating material, especially concerning seedlings [8, 9]. This requirement makes the organic producers in a non-safe situation, as within Albania, there are no organic seeds and seedlings, and the imported ones are not organic too. As such, the availability of the organic propagating material might be a restricted factor for the enlargement of the organic certified surfaces.

Comparison with cultivated plants in Kosovo

In Kosovo, there is a significant increase on the surfaces cultivated with Chamomile, Sage, Basil, Oregano, Gentian, Common Mallow, Marshmallow, Saffron, etc. Plantations of MAPs are in blocks, like for example fields with chamomile in blocks up to 10 ha.

While in Albania, the surfaces cultivated are approximately 0.1-0.5 ha.

The structure of the species cultivated, is strongly linked with the market demand, climacteric conditions, experience, etc. For example Saffron (*C. sativus* L.), in Kosovo is planted in several areas and the export has already started [3]. While in Albania, there have just started to be cultivated, and is more on the testing period.

It is obvious that within the two countries, there are differences in the species planted and on the surfaces.

However this analysis will be more complete, if we could also investigate further on the analysis for specific plants. For some species, which are considered as 'easely cultivated', in Albania have not been successful. Like for example Chamomile (*M. chamomilla* L.), in Albania is cultivated only on 0.2 ha in Durrës, while in Kosovo only one producer has cultivated approximately 100 ha [5].

Analysis of factors limiting area expansion

The climate and soil conditions are considered as favorable for growing MAPs As we analysed also above, the expansion of cultivated surfaces in Albania, faces some limiting factors.

In Kosovo are cultivated plants, whose main processes, such as harvest, collection, drying and processing are done using mechanization equipment. While in Albania these main processes are done manually (hand work). If we evaluate the hand work, we calculate that for the harvest of the chamomile flowers, on a surface of one ha, there are needed 50 working days, for one harvest. Also drying is a limiting factor, especially for plants such as lemon balm, oregano, chamomile, etc, that have a big green mass or need fast drying. In Albania, drying is done mainly on natural conditions, with exception of a few greenhouses or storage facilities, which still get the heat source from the sunlight. Some big companies, has purchased drying machinery, by investing on their own, and also this investment is done only on the main storage facility, not on site (collection or cultivation site). In Kosovo, there are introduced drying and harvest machineries, through donations. These machineries make possible faster drying that preserves the qualities of the product, especially the colour; more

homogenous drying and the process is better managed. As such, the limiting factor for the increase on cultivation surfaces in Kosovo, is not the service provision, but the demand from the market. Many programs and projects have assisted the Kosovo producers on training and technical publications, for a better know-how of the plants and also on good agricultural and processing practices, for MAP-s.

Organic production standards and certification

Today, Albanian Medicinal and Aromatic Plants have been certified according to international standards such as equivalent standards to EEC regulation 834/2007, NOP – US National Organic Program, Bio Suisse (Swiss organic standard), etc; from local and international certification bodies. The local certification body, Albinspekt, has established its own standard equivalent to EEC regulation: AOS - Albinspekt Organic Standard, through which are certified about 70% of the Albanian exporters of the MAP-s and main exporters of MAP-s in Kosovo. Albinspekt is known from EU Commission and accredited, therefore the MAP-s certified from it are freely exported in all EU countries.

Certification has given free movement permission to these plants, in all EU countries, US and other part of the world. Besides the free movement obtained from certification, organic production method makes possible to minimize the risks of contamination from inputs or other elements, a very important requirement, especially for the MAP-s used for herbal teas.

Research on cultivated MAP-s

Cultivation, although a reality in Albania and Kosovo, is considered a new branch of agriculture. To achieve success in cultivating the MAP-s we think there shall be worked in these directions:

- To study and improve ecotypes for having a qualitative production.
- To make the regionalization of crops, therefore to define the best areas and specialize employees for improving the quality.
- Develop and improve cultivation and processing techniques.
- Increase the capacity for processing the products, such as the machineries used for distillation and artificial drying.
- Improve the marketing of these products and promote certification as an added value.
- Promote research to improve cultivation of MAP-s and also their quality, in order to precede the problems that may arise.
- The policies and subvention schemes has to be re-evaluated.
- To draft curricula for the schools and introduce as a specific subject in the Faculties of Agriculture (Tirana, Korca), focused on MAP-s.

CONCLUSION

Production of medicinal and aromatic plants in Albania is re-establishing and an important new branch of agricultural production is emerging. Their organic production is already a reality, and has made these products required in the international market.

Certification as an added value has increased the possibility of exporting without any obstacle (limitation). The number of plants in organic farming is currently 21 species from 70 plant species that are exported as certified organic.

Organic production has increased the possibilities of exporting capacities. Cultivation of MAP has contributed to the sustainability of spontaneous plants, because are not harmed from over collection.

The climate and soil conditions are considered as favourable potentials for the increase on the surfaces and species cultivated.

However, increasing cultivated surfaces, is facing some limiting factors, such as using the manual (hand) work for most processes and drying of products in natural conditions. These increase costs, hinder optimal performance of processes and do not provide high qualitative products.

Enlargement of surfaces can be made possible through mechanization of services and introduction of machineries from processing and drying of products. This expansion needs support from research sector, on the improvement of ecotypes, improvement of technologies of cultivation and drying, production of appropriate propagating material, regionalization and instertion of the subject on school curricula's, etc.

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Additional paper

CHEMICAL ANALYSIS OF HYDROLATES OF *SATUREJA MONTANA* PRODUCED FROM STEAM DISTILLATION INDUSTRY IN ALBANIA

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ABSTRACT

Environment pollution from local industries is an issue that we need to consider seriously. Steam distillation industries of medicinal and aromatic plants (MAP) discharge thousands of liters of contaminated waters in the nature. Their chemical composition and their content in organics are not known. In this work these waters have been analyzed for their organic content, which has been later compared to the chemical composition of total essential oils. For this study waters produced during the distillation of *Saturea montana* were used. Two types of waters were analyzed: hydrolates and the waters coming underneath the distillation vessel, waters that do not co-distill with the essential oils. In both cases an amount of waters has been extracted with organic solvent and after solvent evaporation they are analyzed by GC-MS. The identification of volatile compounds has been done with Mass Spectrometer. From the study resulted that these waters contain up to 0.1% organics. Because of their nice smell and beneficial effect in the skin, it has been suggested to the industry their use in shampoos and other detergents. Trials on formulation of such products are undergoing in our laboratory.

***Key words**: medicinal plants, hydrolate, essential oils.*

INTRODUCTION

Aromatic and medicinal plants have been used for centuries for different purposes. They have been the major source of inspiration for the discovery of lots of new medicaments[1]. In the last decades a majority of plants have been studied for their chemical composition. In Albania not so much has been done in this direction. There are a considerable number of endemic or sub-endemic plants in Albania that their chemical composition is unknown. On the other hand an extensive work has been done on the chemical composition of their essential oils[2]. This interest is because of the great importance that has the production and the export of these essential oils in Albania. Thousand of tones of aromatic plants have been steam distilled each year from the industry. During this distillation thousands of hectoliters of contaminated waters are produced and discharged in the nature.

The chemical composition of these waters has never been studied in our country. In the literature there are examples of study of chemical compositions of some hydrolates. From

these studies has resulted that these hydrolates contain a series of polar compounds like Malcohols and phenols[3]. Because of their chemical composition, these waters have been studied for their antimicrobial and anti-oxidative effects in food industry. Trials have been made with positive effects in delaying lipid oxidation in marinades[4].

Because of contents of organics coming from aromatic plants, these waters have a nice aroma. Also, the hydrolates contain distilled waters and for these reasons they have been suggested to be used in the aromatherapy and shampoo industry.

MATERIAL AND METHODS

This work has been done in collaboration with Filipi Company which allowed us to follow the industrial process and collect the samples. In this study we were interested in the waters produced during the distillation of *Satureja montana*. The origin of these plants is not specifically known but they are all local Albanian plants. The steam distillation has been done in industrial vessels of a capacity of 1000 liters. For each batch, samples of 25 liters of hydrolates and waters coming under the vessel, were collected and analyzed in the lab.

For each hydrolate a sample of 2L was taken and extracted with 3x200ml of petrol ether. The remaining water phase was later saturated with salt and then extracted again with petrol ether in order to see if there were still organics on it. After separation and evaporation of organic solvent, the sample was analyzed with TLC and GS-MS. The TLC's were done in silica gel plates with a mixture of hexane/ethyl acetate 5/1 as eluent. GC's were done in a Varian instrument equipped with a ZB-5 column, 30m long. The oven conditions were: the temperature was kept at 80^0C for 1 min, then a gradient of 5^0C/min up to 280^0C.

Waters coming under the vessel were treated in two different ways:

1. A 5L samples was extracted with 3x300ml of DCM, solvent evaporated and analyzed with TLC and GC-MS
2. A sample of 2L of waters was distilled till 1000ml remained. The distillate was then extracted with 3x200ml of DCM and then analyzed with TLC and GC-MS

RESULTS AND DISCUSSION

As described above, 2L of hydrolates taken from the industrial plant were extracted with 3x200ml of petrol ether. The petrol ether was dried over Na_2SO_4, filtered and evaporated to dryness. In the case of hydrolates taken from *Satureja montana* we found after extraction 1.184g of organics with a yield of 0.091%. As we doubted that because of their high polarity some of the organics might still be left in the waters, the remaining waters were saturated with salt and extracted again to give an extra yield of 0.0141%. The combined organics, after being diluted at the right volume, were analyzed with TLC and GC-MS.

The extraction of 5L of under vessel waters gave 4.35g of organics corresponding to a yield of 0.087%. But this direct extraction with solvent was very difficult because of an emulsion formation from the presence of macromolecules like saponins, sugars and chlorophyll. In

order to eliminate the formation of the emulsion we did distill 2L if water up to a volume of 1L. Then, the distilled waters were extracted with DCM. Both these fraction were then analyzed with TLC and GC-MS

For TLC, glass silica gel plates were used and as detector was used vanillin solution. The development was done in a cell filled with a mixture of hexane/ethyl acetate in a ratio 5/1. The Fig. 1 shows the TLC of total essential oil compared with the TLC of hydrolates and waters ender the vessel.

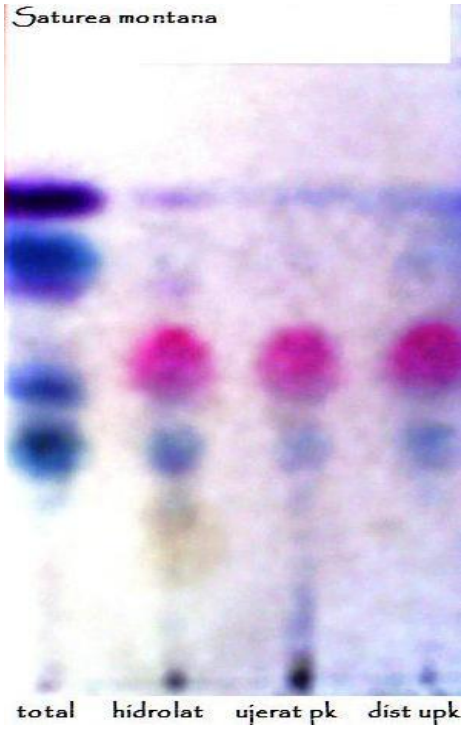

Fig. 1 – TLC of hydrolates extraction of *Satureja montana* – from the left to the right: essential oil, hydrolate, waters under the vessel, waters under the vessel after distillation.

From the TLC we can see that the hydrolates and the waters under the vessel contain components like the total essential oil but in different ratios. There are almost no non-polar organics which comes to the top of the TLC plate. We see that the major spot is a products with a RF=0.6 which have a red coloration. Also from the TLC we could see that the distilled under vessel waters are cleaner on polar products than those from the direct extraction. In order to better determine the chemical composition of this extracts we did a GC-MS of this fraction. The GC is shown below in Fig. 2.

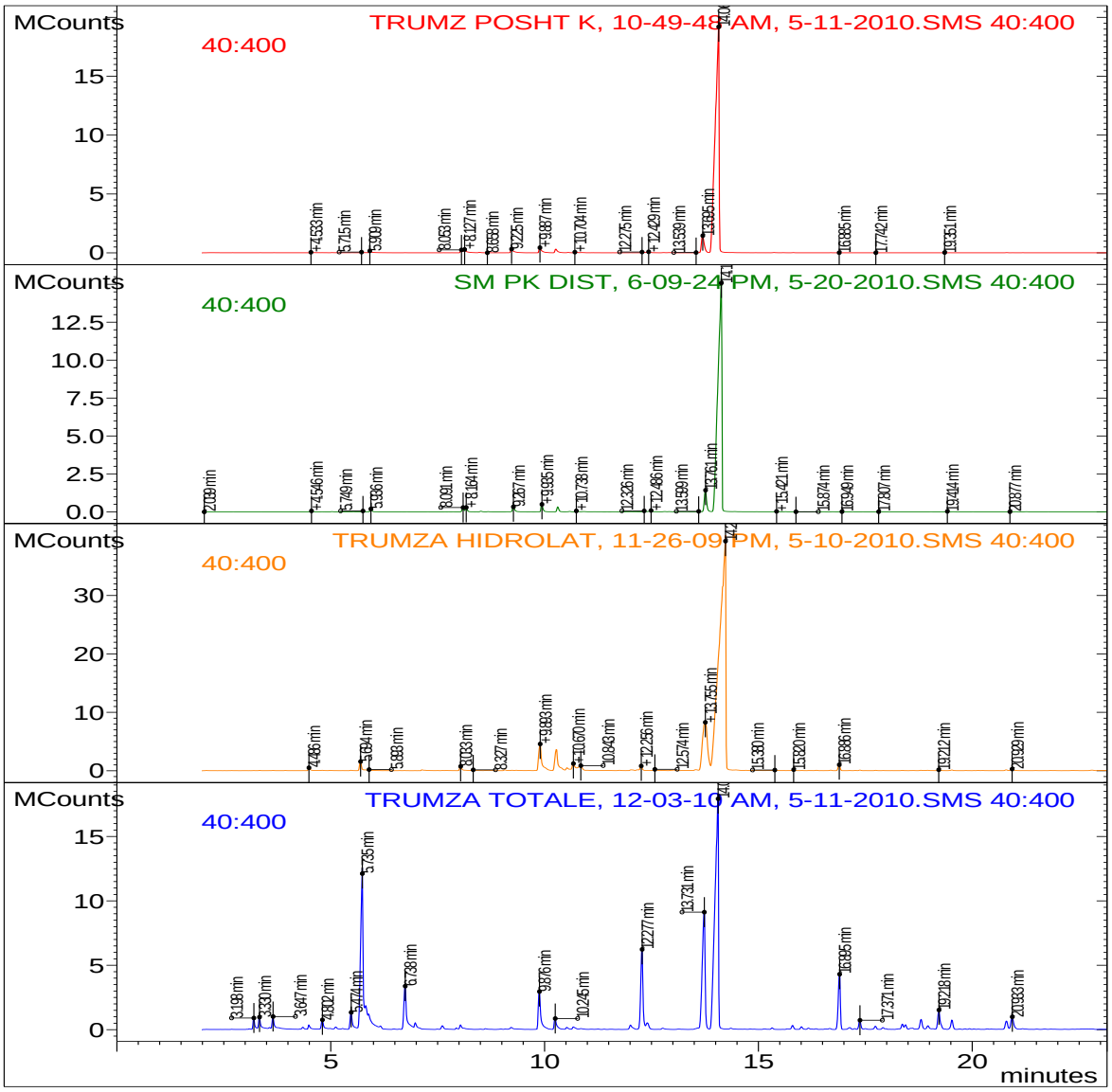

Fig. 2 – GC-MS of extracts taken from hydrolates and total essential oil – from the top down: waters under the vessel after distillation, waters under the vessel, hydrolate, essential oil

From the GC we can see that the substances of these waters are mainly those of higher retention time, which means that they are compounds with higher polarity and therefore higher boiling point. In chromatograms we see that there is a major peak with an RF=14.1. This peak corresponds to the major peak on the TLC, too. The mass-spectrometry of this peak showed that this is 5-Isopropyl-2-methyl-phenol, also known as carvacrol. The distilled and direct extracted under vessel waters, show the same chemical composition as seen on TLC, too. The hydrolate shows almost all the peaks of the total essential oils but in different proportions.

CONCLUSION

As a conclusion we could say that hydrolates are very reach in organics. This organics could be easily isolated via a simple extraction with an organic solvent, and then the waters could be discharged in the nature. The use of salt prior to extraction is not necessary. On the other

hand these hydrolates can be used in shampoos and other detergents. These waters are distilled waters and contain products with nice aroma originated from aromatic plants and they have very beneficial skin effects. Work on formulation of these products is under way in our laboratory.

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Additional paper

ANTIOXIDANT ACTIVITY OF THE METHANOL AND ETHANOL EXTRACTS OF ENDEMIC *THYMUS MALYI* RONNINGER

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ABSTRACT

The genus *Thymus* L. from the family Lamiaceae consists of about 215 species of herbaceous perennials and sub-shrubs widespread in the arid parts of the Mediterranean region.

Thymus malyi Ronninger is an endemic species from the central Balkan which grows on serpentine hills, spreaded on dry, rocky, from the lowland to the mountains.

Essential oils of many *Thymus* species are well known for their antioxidant activity. Since there are no data about antioxidant activitity of extracts of *T. malyi*, in the present study we have investigated and compared possible antioxidant activity of the methanol and ehtanol extracts from *T.malyi* among each other and with artificial food additive BHT using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. Free radical scavenging activity was IC_{50}=0,0370 mg/ml for the methanol extract, and IC_{50}=0,0336 mg/ml for the ethanolextract. The results showed that the methanol and ethanol extracts of *Thymus malyi* Ronninger have significant antioxidant activity, almost the same among each other, and stronger than artificial antioxidant BHT (IC_{50}=0.310 mg/ml), and suggest the use of alcoholic extracts, not only essential oils, for food preserving purpose.

***Key words**: Thymus malyi, antioxidan activity, methanol extract, ethanol extract, BHT*

INTRODUCTION

Thymus L. species are important due to their wide use in traditional medicine, as an antiseptic , antioxidant, anti-inflammatory agents, for anticarcinogenic activities and as a flavouring agents for many kinds of food products [1].The genus *Thymus* L. (Lamiaceae) consists of about 215 species of herbaceous perennials and sub-shrubs, and are widespread in the arid parts of the Mediterranean region [2]. In Serbia, genus *Thymus* is represented by 30 species [3].

Thymus malyi Ronninger is an endemic species from the central Balkan peninsula which grows on serpentine hills, spreaded on dry, rocky, sunny hillsides, from the lowland to the

mountains. *Thymus malyi* is low creeping shrub, with ovate leaves and capitate inflorescence with purple corolla [3].

In contrast to the numerous studies of the esssential oils of *Thymus* species [4-5], there are no data about activities of extracts, including extracts of *T. malyi*, therefore in the present study we have investigated and compared possible antioxidant activity of the methanol and 96% ehtanol extracts from *T.malyi* among each other and with artificial food additive BHT by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. Since artificial additives, like BHT for example, exhibited harmful effects there is an increasing need for natural antioxidants as food preservation agents.

MATERIAL AND METHODS

The plant material (aerial parts) of wild *T. malyi* was collected at the flowering stage in June 2010 in Studenica, Serbia. A voucher specimen (BEOU 16623) of *T. malyi* has been deposited at the herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

The leaves of *T. malyi* were cut up and milled by blender and then extracted with methanol and ethanol (3 x 50 ml), with stirring for 15 min. The extracts were filtered through a filter paper and the filtrates were evaporated to dryness under vacuum.

The antioxidant activity of the *T. malyi* extracts was measured in terms of radical scavenging (hydrogen donating) ability, using the stable radical, 2,2-diphenyl-1-picryl hydrazil - DPPH. The method used was modification of the original method of Blois [6-7]. The decrease in absorbance at 517 nm after 30 min reaction in the dark was determined by UV-VIS spectrophotometer. The percentage of inhibition of the DPPH radical by the samples was calculated according to the equation:

$$I(\%) = A_c - A_u / A_c \times 100$$

where Ac is the absorbance without sample (the same volume of methanol) and Au is the absorbance of the remaining DPPH radical after reaction with antioxidant for 30 min. Five concentrations were used. All samples (concentrations) were done in triplicate. The results were presented as mean ± standard deviation. Comparison of means was analyzed by Student's t test and differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Natural antioxidants have been attracted a big attention as food additives in order to provide protection against oxidative degradation of foods by free radicals and replace harmful artificial antioxidants. In this work the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical-scavenging method was used to determine and compare possible antioxidant activity of the methanol and ehtanol extracts among each other and with artificial food additive BHT. Knowing that different investigated essential oils of *Thymus* species showed antioxidant activity, it was supposed that, perhaps, extracts of endemic *T. malyi* would exhibit signifant

potential for scavenging DPPH radical. Free radical scavenging activity expressed as IC_{50} (concentration which decrease absorption of DPPH for 50%) was 0,0370 mg/ml for methanol extract, and 0,0336 mg/ml for ethanol extract. Artificial antioxidant BHT (3,5-di-*tert*-butyl-4-hydroxytoluene), showed IC_{50} value of 0.310 mg/ml. The results showed that the methanol and ethanol extracts of *Thymus malyi* Ronninger have significant antioxidant activity, stronger than artificial antioxidant, and suggest the use of alcoholic extracts, not only essential oils, for food preserving purpose.

CONCLUSION

This investigation suggested that the plant extracts of *Thymus malyi* could be considered as a new potential source of natural antioxidant and food preserving agents for food industries.

Acknowledgments: We are grateful to prof. Petar Marin for providing a plant material.

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Additional paper

HALACSYA SENDTNERI (BOISS.) DÖRFL. - ANTIOXIDANT ACTIVITY OF THE METHANOL EXTRACT

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ABSTRACT

Halacsya sendtneri is a only species currently described of the genus *Halacsya* of the family Boraginaceae.This perennial tufted plant with a stout and long rootstock is an obligatorz serpentinophzte and grows on open serpentine rocks, along stone blocks at the altitude between 190/1500m. Characteristic species of the endemic communitz in West Serbia *Halacsya sendtneri-Potentilla mollis.*

H. sendtneri is considered a Tertiary relict and endemic species in the central Balkan, particularly in Serbia, Bosnia-Herzegovina, and Albania.

There are no data of the possible antioxidant activity of the methanol extract of *Halacsya sendtneri* by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. Free radical scavenging activity (expressed as IC_{50} - concentration which decrease absorption of DPPH for 50%) of methanol extract was IC_{50} - 0,0480 mg/ml.

The results showed that the methanol extract of *Halacsya sendtneri* have antioxidant effect, and may provide protection on free radicals, so it could be considered as a new source of natural antioxidant.

***Key words**: Halacsya sendtneri, antioxidan activity, methanol extract.*

INTRODUCTION

There is an increasing interest in antioxidants, particulary in those intended to prevent the presumed deleterious effects of free radicals in the human body, and to prevent the deterioration of fats and other constituents of food-stuffs. There is a preference for antioxidants from natural rather than from synthetic sources [1].

Halacsya sendtneri is a species of the monotypic genus *Halacsya* of the family Boraginaceae.This perennial tufted plant with a stout and long rootstock is an obligatorz serpentinophzte and grows on open serpentine rocks, along stone blocks at the altitude between 190/1500m [2]. Characteristic species of the endemic communitz in West Serbia *Halacsya sendtneri-Potentilla mollis. H. sendtneri* is considered a Tertiary relict [3] and endemic species in the central Balkan, particularly in Serbia, Bosnia-Herzegovina, and Albania. It is qualified as a vulnerable species (V) in the European Red List (marked as +) [4].

MATERIAL AND METHODS

The plant material (aerial parts) of *H. sendtneri* was collected in april 2011 near Gornji Milanovac (Serbia).

The leaves of *H. sendtneri* was previously homogenized and then extracted with methanol (3 x 50 ml). The extracts were filtered through a filter paper and the filtrates were evaporated to dryness under vacuum.

The antioxidant activity of the *H. sendtneri* extract was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, 2,2-diphenyl-1-picryl hydrazil - DPPH. The decrease in absorbance at 517 nm after 30 min reaction in the dark was determined by UV-VIS spectrophotometer. The percentage of inhibition of the DPPH radical by the samples was calculated according to the equation:

DPPH radical scavenging (%) = $A_c - A_u / A_c \times 100$

where A_c is the absorbance of the control and A_u is the absorbance of the remaining DPPH radical after reaction with antioxidant for 30 min.

RESULTS AND DISCUSSION

The various extracts of many plants as a natural antioxidants have been attracted a big attention as food additives in order to replace synthetic antioxidants by natural ones. The previous investigation confirmed the antioxidant activities of acetone, chloroform, ethyl acetate and petroleum ether extracts of the Serbian plant *Halacsya sendtneri*. The results showed that the acetone extract of *Halacsya sendtneri* (Boiss.) Dörfl. possessed the highest antioxidant activity. The IC 50 values determined was 9.45 μg/mL for DPPH free radical scavenging activity. Chloroform and ethyl acetate extracts showed lower activity, while petroleum ether extract possessed the lowest activity [5]. There are no data of the possible antioxidant activity of the methanol extract of *Halacsya sendtneri* by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. In the present study free radical scavenging activity (expressed as IC_{50} - concentration which decrease absorption of DPPH for 50%) of methanol extract was IC_{50} - 0,0480 mg/ml (Fig.1).

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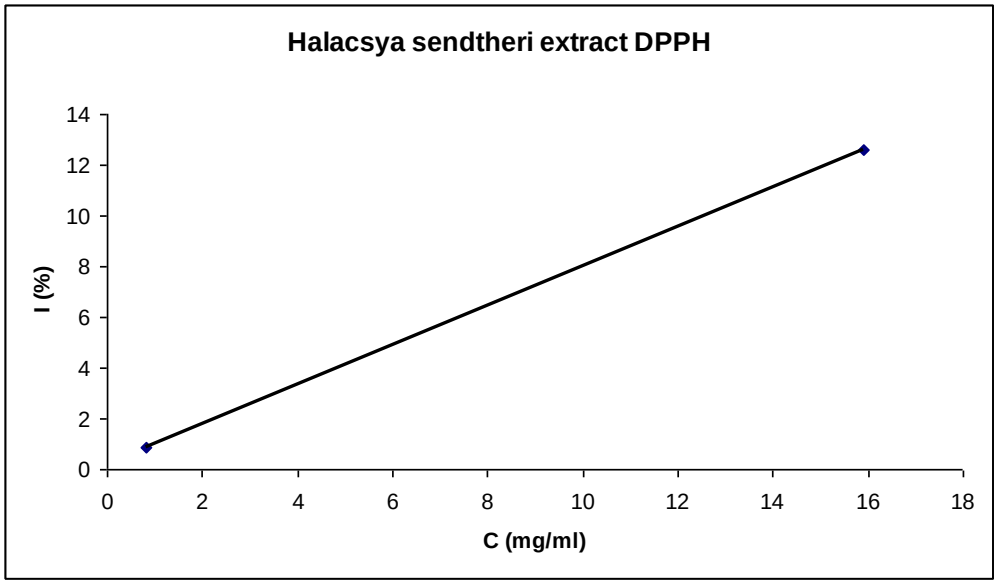

Figure 1. Methanol extract DPPH of *Halacsya sendtneri*

CONCLUSION

The present investigation showed that the methanol extract of *Halacsya sendtneri* have significant antioxidant activity, and may provide protection on free radicals, so it could be use as a new source of natural antioxidant.

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Additional Paper

EFFECT OF DIRECT SELECTION ON PRODUCTIVE TRAITS OF MARSHMALLOW (ALTHAEA OFFICINALIS L.)*

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ABSTRACT

Breeding goal was to create an improved and homogenous genotype, which will contribute to the stability and higher production, as well as conservation of natural resources. As a source of variability we used selection material which consisted of the collection (*ex situ*) of marshmallow germplasm. Using the method of direct selection, we created 28 promising clones. Following traits were observed at the selected offspring and standard ("vojvođanski"): plant height (cm), yield of fresh roots per plant (g), plant fats, starch, sucrose and number of swellings. Clones marked with numbers 20, 25 and 26 have achieved reliably higher root yields of 20-27% compared to the standard. Application of direct selection resulted in a high selection gain for yield of roots per plant. The selection gain was greater at the selection intensity of 5% than of 10%.

***Key words**: clones, direct selection, marshmallow, yield, quality*

INTRODUCTION

It is thought that marshmallow originates from the countries around Caspian and Black Sea, as well as eastern parts of Mediterranean Sea. The *Althaea* genus includes fifteen species within *Malvaceae* family. In our country seven species are represented. From economic aspect the most important species is *Althaea officinalis* L. (2n=42) [1].

Marshmallow grows on moist terrains, near rivers, streams, roads, on saline soils and within various plant communities (phytocenosis). Most of it is found in Vojvodina. Variability and diversity of this species determines its usefulness [2, 3]. In our country there is no assortment of this plant species, because only one population ("vojvođanski") is being produced. It is created on the basis of indigenous material and exhibits wide adaptability and generaly lower yield comparing to homogeneous varieties [4, 5].

Basic requirement for creating improved or new variety is presence of variability in starting material of natural and local population, the commercial variety. Beside polyploidy, studies of native marshmallow (*in situ*) showed presence of variability in plant height, number of outgrowths, yield and number of swellings, which can represent a source of germplasm for creating improved cultivars [5, 6].

In marshmallow breeding the programs of direct and indirect selection can be applied, whereby it can be used generative and vegetative reproduction. Breeding goal was to create an improved and homogenous genotype, wich will contribute to the stability and higher production, as well as conservation of natural resources.

*(Since legislators excluded medicinal plants from the process for registration of varieties, Ministry of Education, Science and Technology Development of the Republic of Serbia has recognized the newly created clone variety as a technical solution).

MATERIAL AND METHODS

As a source of variability we used selection material which consisted of the collection (*ex situ*) of marshmallow germplasm, that has been collected during tha inventory of the habitat. Using the method of direct selection (by vegetative multiplication), we created 28 promising clones. Seven of 28 clones were chosen. They were homogeneous and had a higher mean values for important traits, so they were additionaly studied in comparative experiments. Selected clones were observed in the Nova Pazova experimental field in the course of 2008-2011. The experiment was based over nursery [7]. Planting was done at a distance 70 x 30 cm, which corresponds to the density of 48.000 plants/ha. Selected clonal offspring were denoted by numbers and monitored in the course of three years.

Following traits were observed at the selected offspring (seven) and standard (''vojvođanski''): plant height (cm) and yield of fresh roots per plant (g). Via adequate analytical methods we determined corresponding values for important root quality parameters (plant fats, starch, sucrose and number of swellings).

Following main biometric parameters have been calculated: mean value, variance and coefficient of variation. The results were processed by variance analysis application, and significance by LSD test. The effects of selection of superior clones for the yield and the number of swellings were evaluated. Other parameters haven't been evaluated due to equalization of their values. The gain of the selection was expressed in absolute and relative values, as a difference between mean values obtained from selected clones and mean values gained from the entire population of clones.

RESULTS AND DISCUSSION

The mean values for the plant height obtained from selected clones were approximately equal, Table 1. Variational width was low, as well as variance and coefficient of variation, Table 2. Variation of this trait in a wild marsmallow (starting) population was much higher and reached 26% [6].

Yields of a fresh root were different. They amounted from 75g (clone 8) to 251g (clone 25). Average yield of selected clones was 193 g per plant. The clones 20, 25 and 26 reliably achieved higher fresh root yields compared to standard. There are no significant statistical differences among these clones. The yield increased, compared to standard, of clones 20, 25 and 26 were 60g or 25%, 69g or 27% and 44g or 20%, respectivelly, Table 1. The variance value as well as yield variance were higher (29%), Table 2. However, based on earlier researches on native marhmallow population, it is observed that the variation of this trait was much higher (44%) [6].

The number of swellings as an indicator of mucus presence had different values [8]. In the case of clone 25 the swelling number was much higher than standard, Table 1. The value of this parameter was on standard level in other clones, Table 1. Relatively high values of variation width, variance and variation coefficient of this property are ascertained, Table 2.

The contents of plant fats, starch and sucrose were very uniform for all clones. The lowest variation width was for vegetable fat and sucrose content, as indicated by low values of variation coefficients, Table 2. Based on these data, it can be adduced that stability of these properties is higher compared to yield and number of swellings.

Acomplished selection gain varied depending on the properties and selection intensity. The gain varied 37,5% and 43,8% for selection intensity of 5% and 10%, respectively, Table 3. It was the same with the number of swelling 32%. With selection intensity of 10% the number of swellings was 37,7% and with selection intensity of 5% it was 32%. Variations of fresh root yields and number of swellings had no effect on changing other root ingredients.

Table 1. Mean values of traits to select clones

Nº	Clones	Height (cm)	Yield (g)	Plant fats (%)	Starch (%)	Sucrose (%)	Number of swellings
1.	3	147	201	2,00	33,18	9,14	16
2.	8	136	75	2,04	32,58	8,91	20
3.	12	140	171	2,o2	32.59	8.68	20
4.	18	130	188	2,o2	32,77	8,90	13
5.	20	122	242**	2,17	34,33	9,14	10
6.	25	133	251**	1,95	33,72	9,12	24**
7.	26	120	226*	2,03	33,70	8,92	15
8.	Standard	117	182	2,05	33,90	9.05	20
LSD	5%	-	40	-	-	-	2
	1%	-	55	-	-	-	4

*, ** Significant at the 0,05 and 0,01 probability level, respectively

Table 2. Parameters of variability code to seven selected clones

Trait	Mean	Min.	Max.	Variance	CV
Height (cm)	132	120	147	79.14	7
Yield of fresh roots per plant (g)	193	75	251	3.050.143	29
Plant fats (%)	2,04	1,95	2,17	0.02	0,8
Starch (%)	33,35	32,57	34,33	4.08	6,5
Sucrose (%)	8,98	8,68	9,14	0.0064	1
Number of swellings	17	10	24	19.57	26

Table 3. Selection gain

Selection intensity	Selection for a trait			
	Roots yield (g)		Number of swellings	
	Absolute	Relative	Absolute	Relative
10%	84	43,8	6,5	37,7
5%	72	37,5	5,5	32,0

CONCLUSION

Out of 28 marhmallow clones, only the offspring of 20, 25 and 26 had a significantly higher mean values of fresh root compared to standard. The yield increase expressed in relative term amounted 20-27%. In addition to more yield, clone 25 exhibited reliably higher number of swellings. The content of plant fats, starch and sucrose was very uniform for all clones. By applying the method of direct selection a high selection gain for root yield and the number of swellings was achieved. With selection intensity of 10% the selection gain was higher than the one obtained with intensity of 5%. Selection gain varied depending on the traits.

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