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Original Article

Co-extracted bioactive compounds in *Capsicum* fruit extracts prevents the cytotoxic effects of capsaicin on B104 neuroblastoma cells

Viktorija Maksimova[a,*], Liljana K. Gudeva[b], Rubin Gulaboski[a], Karen Nieber[c]

[a] *Faculty of Medical Sciences, Goce Delcev University, Stip, Macedonia*
[b] *Faculty of Agriculture, Goce Delcev University, Stip, Macedonia*
[c] *Faculty of Pharmacy, Biology and Psychology, Institute of Pharmacy University of Leipzig, Leipzig, Germany*

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ABSTRACT

The aim of this study was to investigate the effect of capsaicin and ethanolic *Capsicum* extracts on B104 neuroblastoma cells as a potential anticancer agent. Additionally, this study also aims to examine the influence of co-extracted bioactive compounds (vitamin E, vitamin C and quercetin) in *Capsicum* fruit extracts on the cytotoxic effects of capsaicin in neuroblastoma cells. MTT and LDH assays were used to determine viability and cell death in B104 neuroblastoma cells. Antioxidative properties of capsaicin, vitamin E, vitamin C and quercetin were estimated by means of cyclic and square wave voltammetry. There was a significant cytotoxicity of capsaicin (100 μmol/L) after 24 h incubation and for capsaicin (250 μmol/L), even when cells are treated for 1 h. On the other hand, ethanolic *Capsicum* extracts which contained capsaicin (0.5–2.1 mmol/L) did not show any cytotoxic effect. We suggest therefore, that other co-extracted compounds within the ethanolic extracts interact antagonistic with the cytotoxic effect of capsaicin and their interactions should be further investigated. Our results indicate that capsaicin in high concentration induces cytotoxic effects in a dose dependent manner, but other bioactive compounds present in *Capsicum* fruits prevent the cytotoxic effects of the extracts on neuroblastoma cells.

Introduction

Capsaicin (*N*-vanillyl-8-methyl-6-nonenamide, **1**) is the major component of capsaicinoids. This alkaloid is a secondary metabolite in different species of the genus *Capsicum* (Buczkowska et al., 2013). It gives the pungency of hot peppers and is responsible for many physiological and pharmacological properties of this plant. Although topical creams with capsaicin are used to treat peripheral neuropathic pain conflicting epidemiologic data, many basic research studies results suggest that capsaicin can act as a cytotoxic or as a cytoprotective agent (Bode and Dong, 2011). The majority of research studies suggest that capsaicin induces cell-cycle arrest or apoptosis or inhibits proliferation in different malignant cells including lung cancer, adeno carcinoma, pancreatic cancer, breast cancer Díaz-Laviada and Rodríguez-Henche (2014) hepatocellular carcinoma Baek et al. (2008), osteosarcoma and many others Cho et al. (2013). Various mechanisms for capsaicin-induced apoptosis have been proposed for different cell systems. Physiological processes linked to the intracellular calcium increase, reactive oxygen species generation, disruption of mitochondrial membrane transition potential and activation of some transcription factors are involved Clark and Ho-Lee (2016) are closely related to the capsaicin activity.

According to Sanchez et al. (2006), capsaicin can act as cytotoxic agent through evoking apoptosis in prostate cancer cells through mechanism which includes increased production of reactive oxygen species (ROS), disruption of inner mitochondrial membrane potential and activation of caspase-3. Pramanik et al. (2011) and Zhang et al. (2008) showed that apoptosis provoked by capsaicin in pancreatic cells is accompanied by 4–6 fold increase of the concentration of free radicals and consequently disruption of the mitochondrial membrane potential. Therefore capsaicin has provoked an inhibition of cell proliferation and induced apoptosis in a dose dependent manner.

In contrast, hot peppers fruits are widely used in everyday nutrition and have shown many benefits for human health. As known from the literature, beside capsaicin, these extracts represent a complex mixture of many other bioactive compounds as vitamin C (**2**), vitamin E (**3**), carotens, quercetin (**4**) and luteolin (Asnin and Park, 2015). These molecules are reported as compounds which have shown high antioxidative potential and protective role in carcinogenesis (Materska and Perucka, 2005). Acting as antioxidants,

* Corresponding author.
E-mail: viktorija.maksimova@ugd.edu.mk (V. Maksimova).

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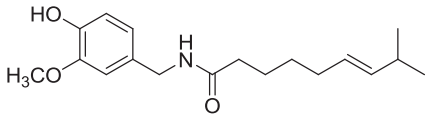
these molecules are capable to neutralize or scavenge the free radicals which are responsible for many degenerative diseases as well as progression of cancer (Uttara et al., 2009). Therefore, the total anitoxidative capacity of pepper extracts can modulate the cytotoxicity of capsaicin present in the extracts.



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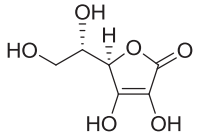

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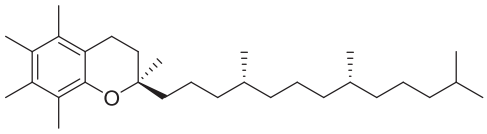

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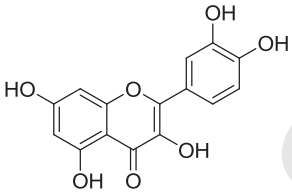

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The aim of the present work was to study the cytotoxic properties of capsaicin on B104 neuroblastoma cells, and also to examine the toxicity of the *Capsicum* fruit extracts obtained from a several different varieties of hot peppers. The current study also aims to address the possible interactions and the synergistic antioxidant effects of the co-extracted compounds with capsaicin. To the best of our knowledge, the influence of the other co-extracted bioactive compounds in *Capsicum* fruit extracts on the cytotoxic effects of capsaicin on B104 neuroblastoma cells, have not been evaluated so far.

Materials and methods

Plant materials

Capsicum fruits from four different genotypes of *Capsicum annuum* L. Solanaceae, (hot peppers) were taken for this experiment. Different plant seeds were stored in the gen bank at Goce Delcev University, Faculty of Agriculture, at the campus of Strumica, Macedonia. Plant name has been checked on the web: www.theplantlist.org on February 10, 2015. These seeds have been taken for cultivation and their fruits were collected from the field in the phase of botanical maturity. The fruits from four genotypes of hot peppers with local names: *Bombona*, *Feferona*, *Vezena*, and *Sivrija*, were dried on room temperature for about two weeks. Afterward, they were grounded and the powder was used for extraction.

Cell line

Cells of the rat neuroblastoma line B104 (ATCC, Manassas, VA, Schubert et al. 1974) were maintained in DMEM/Ham's with L-glutamine (Dulbecco's modified Eagle's medium) (PAA GmbH) supplemented with 15% fetal bovine serum (FBS), and antibiotics 1% penicillin/streptomycin solution. The medium was changed every 2–3 days. Cells were incubated at 37 °C in an atmosphere containing 5% CO_2 and saturating humidity. Cells were allowed to be adherent for 24 h before treatment with capsaicin or *Capsicum* extracts.

Reagents

Stock solutions of capsaicin (**1**) (>95%, natural capsaicin), vitamin E (**2**), quercetin and ascorbic acid were freshly prepared by using standard substances obtained from Sigma-Aldrich and 96% ethanol (reagent grade) (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) (MTT), sodium dodecylsulfate (SDS), dimethylformamide (DMF), lactate, NAD, diaphorase, HCl, SDS lysis buffer, DMEM, phosphate buffer solution) PBS, phosphate buffer (pH 7.4), fetal bovine serum, penicillin, streptomycin (PAA GmbH), triton X, ethanol 96% (Sigma–Aldrich). All solutions were stored at 4 °C. In all solutions used for electrochemical measurements, KCl was added as an additional electrolyte at concentration of 0.010 M.

Methods

Extraction method

Extraction was performed by maceration using ethanol 96% (v/v) as a solvent (Rafajlovska et al., 2007). Maceration was performed for 5 h, on 60 °C, and afterward the extracts were filtered by gouch filter, using a vacuum pump. 0.2 g of the pulverized plant material was macerated with 25 mL solvent. Stock solution was prepared by using capsaicin, with 96% ethanol and then diluted to appropriate concentrations.

Spectrophotometric method

UV/VIS spectrophotometry was used for quantification of capsaicin in ethanolic extracts and standards solutions (Perucka and Oleszek, 2000). The concentration of capsaicin was measured by using a Cary 100 spectrometer, instrument version no. 9.00, on a specific maximum wavelength of 280 nm. A serial of standard dilutions of capsaicin (0.25, 0.125, 0.0625, 0.0312, 0.0156 mg/mL) were prepared for obtaining the regression curve. The ethanolic extracts were measured and according to the absorbance obtained for them, regression analysis was performed for calculation of concentration of capsaicin in the extracts.

Cytotoxicity methods

The viability of B104 cells after treatment with capsaicin or *Capsicum* extracts was assessed using MTT method. For determination of the cell death, the LDH method was used.

MTT assay

The MTT assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to an insoluble formazan. The formazan is then solubilized and its concentration was determined by optical density at 570 nm (Mosmann, 1983). MTT cell proliferation assay was performed

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according to the protocol given by the manufacturer Roche Diagnostics GmbH for Cell Proliferation Kit I. In brief, B104 cells were seeded in 96-well cell culture plates (2×10^4 cells/well) and subsequently treated with capsaicin (0.5, 1, 10, 100 and 250 μmol/L) or different ethanolic *Capsicum* extracts at 37 °C for 1, 6 and 24 h, respectively.

After the incubation period, 10 μL of the MTT labeling reagent (final concentration 0.5 mg/mL) was added to each well. The plate was incubated for 4 h in a humidified atmosphere (*e.g.* 37 °C, 6.5% CO_2). Subsequently, 100 μL of the solubilized micture was added into each well. Finally, the plate was allowed to stand overnight in the incubator in a humidified atmosphere. After checking the complete dissolving of the purple formazan crystals formed into the cells, absorbance of the samples was measured spectrophotometricaly using a microplate reader (Antho, 2010) at a test wavelength of 570 nm and reference wavelength of 630 nm. A 0.1% Triton X solution was used as positive control and DMEM (assay medium) as negative control.

LDH assay

The LDH assay offers a simple way to measure plasma membrane damage, based on the release of lactate dehydrogenase (LDH), a stable cytoplasmic enzyme present in many cells (Chou et al., 2009). Assay was performed according to the manufacturers protocol (Roche Diagnostics GmbH) given for the LDH cytotoxicity detection kit. In brief, after adherence of the cells to the wells, test substances (capsaicin in different concentrations and ethanolic extracts) diluted in an appropriate assay medium (DMEM), were titrated in a separate micro plate by several dilutions (final volume up to 200 μL/well). Then, the assay medium was removed and 100 μL fresh assay medium was added to each well. 100 μL of the test substance dilutions were transferred into corresponding wells containing the adherent cells. Thereafter, 100 μL of the supernatant were removed from the wells carefully and transferred into corresponding wells of an optically clear 96-well flat bottom microplate. To determine the LDH activity in the supernatants, 100 μL of the reaction mixture (freshly prepared) was added to each well and incubated for 30 min at +15 to +25 °C in a dark atmosphere. The reaction mixture was prepared by mixing the Catalyst (Diaphorase/NAD^+mixture) and Dye solution (INT and sodium lactate).

Absorbance of the samples was measured at 492 nm by an ELISA plate reader. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. A 0.1% Triton X solution was used as positive control and DMEM (assay medium) as negative control.

Voltammetric methods: cyclic voltammetry and square wave voltammetry

Electrochemical determination of the antioxidative potential of the extracts and a mixture of standard solution was performed by means of square-wave voltammetry (SWV), after short electrochemical characterization of the electrochemical features by cyclic voltammetry (CV), at a glassy carbon working electrode. Experiments in cyclic voltammetry were conducted over a potential range from −0.200 to 1.000 V, with a scan rate of $\nu = 10$ mV/s. Experimental conditions for SWV were: potential step $dE = 0.001$ V, pulse height (SW amplitude) $Esw = 0.050$ V and frequency of $f = 10$ Hz. Prior to each electrochemical experiment the working electrode was polished by using $AlCl_3$ on a polishing cloth, followed by rinsing of the electrode with water and acetone and drying in air.

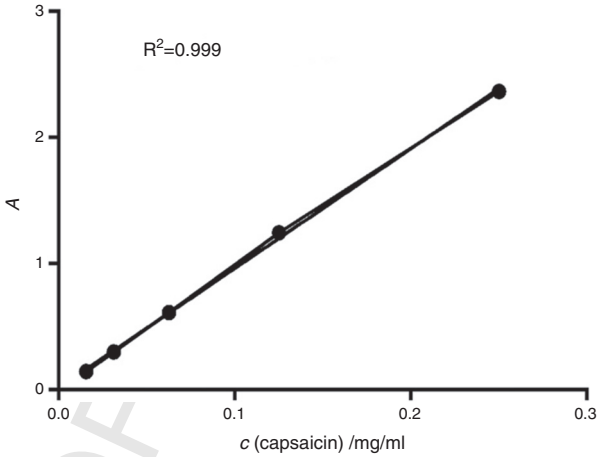

Fig. 1. Calibration curve constructed for standard solutions of capsaicin by UV VIS.

Statistics

Variance analyses were performed by using a Graph Pad Prism 6.0. Processing of the results was done by one way ANOVA, for three groups of results (different time of exposition), in which each treatment of the cells was compared by the treatment with negative control and/or positive control. All graphs represent means and standard deviations for triplicate samples from each of three independent experiments ($n = 9$). Results which are statistically significant are showed on the graphs, $p < 0.01$. The regression analysis was performed using the program Graph Pad Prism 6.0, XY analyses, linear regression.

Results

Determination of the capsaicin concentration

Spectrophotometric measurement of the concentration of capsaicin in the ethanolic oleoresins was determined on the basis of standard solutions of capsaicin. The concentration of capsaicin in the samples were calculated using the regression curve and linearity equation (Fig. 1). Because the extracts were obtained with 96% ethanol, we diluted the extracts with DMEM in ratio 1:100, in order to escape any cytotoxic effects of ethanol on the cells. Additionally, the dilution affected the color of the extracts. The color comes from the high concentration of pigments, and it vanished after the dilution. Therefore, to obviate the probability of giving false results, extracts were diluted to appropriate concentrations for treatment of the cells given in Table 1. *Bombona* genotype contained the highest concentration of capsaicin with 2.10 mM, for this

Table 1
Concentration of capsaicin in standard solution and extracts of *Capsicum* used for treatment of B104 cells in MTT and LDH assays, (extracts were diluted with DMEM in ratio 1/100).

Standard solutions	Concentration of capsaicin [μM]	Diluted extracts for treament of cells	Concentration of capsaicin in extracts [mM]
St. solution 1	0.5	Solution of Vezena	0.51
St. solution 2	1	Solution of Feferona	0.78
St. solution 3	10	Solution of Bombona BBBombona	2.1
St. solution 4	100	Solution of Sivrija	0.65
St. solution 5	250		

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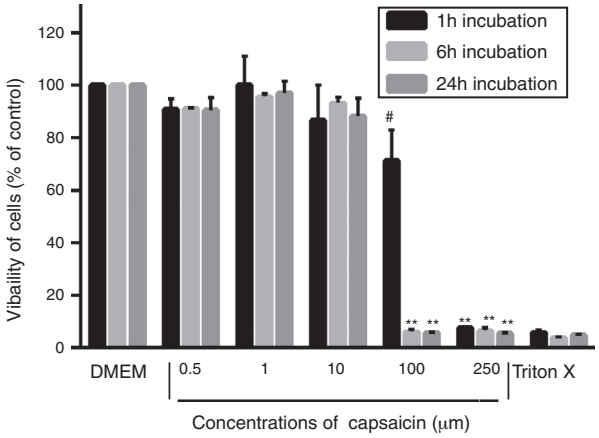

Fig. 2. Effect of different capsaicin concentrations on cell viability (MTT assay) on B104 neuroblastoma cells incubated for 1, 6 or 24 h. There is a significant difference in the results for the effect of 100 μM capsaicin for 1 h compared to the effect for 6 h. Significance: # ($p < 0.05$), ** ($p < 0.01$) *vs.* DMEM.

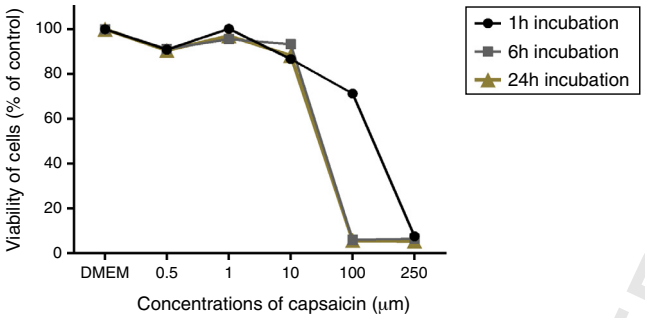

Fig. 3. Capsaicin suppresses cell viability in a time and dose dependent manner. There was a significant cytotoxicity of higher concentrations of capsaicin (100 and 250 μM), after 6 and 24 h incubation. IC_{50} 61.9 μM for 6 h and IC_{50} 61.6 μM for 24 h.

extract. Concentrations of capsaicin calculated as mg/g DW are in line with the results found in the literature (Tilahun et al., 2013).

Effect of capsaicin on cell viability and cell death

As shown in Fig. 2, capsaicin present in low concentrations (0.5–10 μM) did not significantly influence the cell viability. The concentration required to inhibit 50% of the cells viability was found to be 61.9 μM, which is in accordance with the previous findings for capsaicin (Richeux et al., 1999). In concentration of 100 and 250 μM it inhibited the viability of B104 cells compared to the effects of 0.1% Triton X solution, used as positive control and DMEM (assay medium) as negative control. The incubation of 1 h resulted in a moderate inhibition for 100 μM (29 ± 11.5% inhibition, $p > 0.05$), whereas the effects were more pronounced after a longer incubation period or at a higher concentration. Capsaicin (100 μM) incubated for a period of 6 h resulted in 94 ± 1% inhibition, while after an incubation period of 24 h, the inhibition was 95 ± 0.1% ($p < 0.01$). Higher concentration of capsaicin (250 μM) resulted in much higher inhibition of cell viability even for 1 h incubation (93 ± 0.5%, $p < 0.01$) and for 6 h and 24 h (94 ± 1% and 95 ± 0.2%, $p < 0.01$, respectively).

The results from the MTT assay (Fig. 3) showed that there is significant difference in the viability of cultured cells when they were treated with capsaicin for 1 h, and 6 or 24 h. The calculated IC_{50} value for 6 h (IC_{50} 61.9 μM) was almost the same as found after 24 h incubation period (IC_{50} 61.6 μM) indicating that the effect of capsaicin was not pronounced within a longer incubation period.

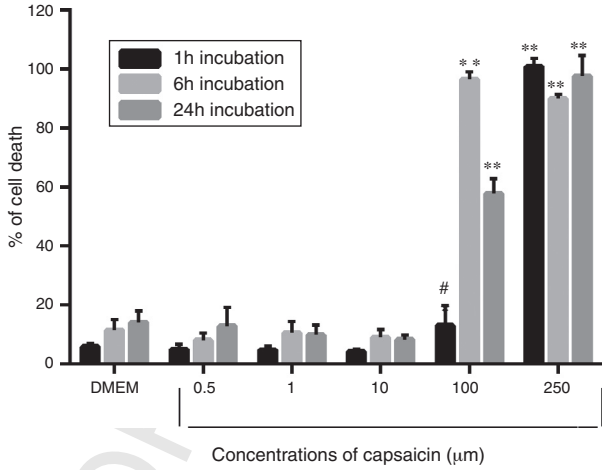

Fig. 4. Effect of capsaicin in different concentration (0.5, 1, 10, 100, 250 μM), examined by LDH assay on B104 neuroblastoma cells for 1, 6 or 24 h of incubation. Treating of the neuroblastoma cells with capsaicin (100 and 250 μM) for 6 or 24 h resulted in high increase in LDH release indicating cell death. (# $p < 0.05$; * $p < 0.01$). Values are means ± SE compared with DMEM as negative control. $n = 9$ for each group.

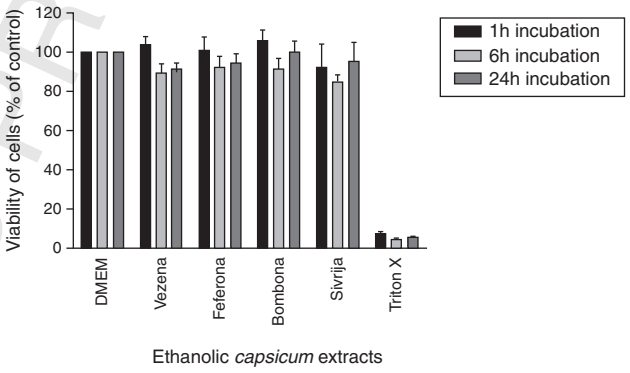

Fig. 5. Effect of ethanolic *Capsicum* extracts (obtained from *Capsicum* genotypes: *Vezena*, *Feferona*, *Bombona*, *Sivrija*) on cell viability (MTT assay) treated for 1, 6 or 24 h. Viability of the cells was compared to negative control (DMEM).

The result obtained from the LDH assay is presented in Fig. 4. and it confirmed the results obtained from the MTT assay. Capsaicin in low concentrations (0.5–10 μM) did not significanly influence the LDH release. In concentration of 100 and 250 μM it resulted in high LDH release from B104 cells, indicating cell death. Results were compared to the effects of 0.1% Triton X solution, used as positive control and DMEM (assay medium) as negative control. Concentration of capsaicin of 100 μM incubated for 1 h resulted in a moderate LDH release (13 ± 6%, $p > 0.05$), whereas the effects were more pronounced after a longer incubation period or at a higher concentration. The same concentration of capsaicin (100 μM) for a period of 6 h incubation resulted in 96 ± 2.3% cell death, while for 24 h treatment, the cell death was incresed to 58 ± 5.1%, ($p < 0.01$). Higher concentration of capsaicin (250 μM) resulted in much higher cytotoxicity of cells even for 1 h incubation (100 ± 2.7%, $p < 0.01$), as well as for 6 h and 24 h consequently (90 ± 1.38% and 98 ± 6.9%, $p < 0.01$).

Effect of ethanolic Capsicum *extracts on cell viability and cell death*

In contrast to capsaicin, the ethanolic *Capsicum* extracts did not influence significantly neither the cell viability nor the cell death. In the MTT assay (Fig. 5) there was no significant inhibition of the cell viability after treatment of the cells with the extracts. The

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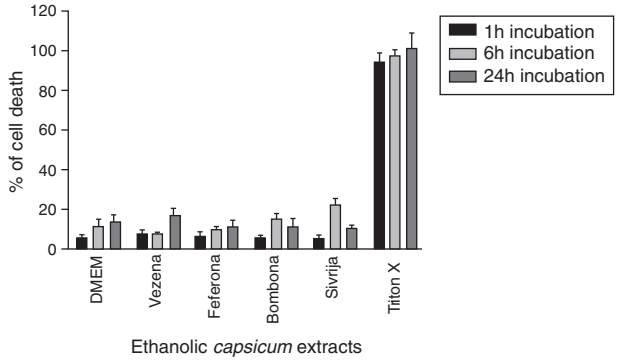

Fig. 6. Effect of ethanolic *Capsicum* extracts on cell death (LDH assay) treated for 1, 6 or 24 h. There was no significant difference with ethanolic *Capsicum* extracts compared to the negative control (DMEM).

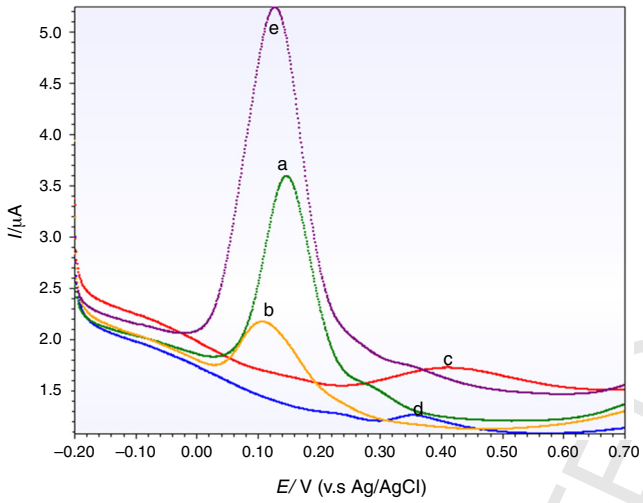

Fig. 7. Square-wave voltammogram of: (A) vitamin E; (B) quercetin, (C) ascorbic acid and (D) capsaicin (10 μM), (E) equimolar mixture containing vitamin E, quercetin, ascorbic acid and capsaicin at concentration of 10 μM, recorded at a glassy carbon electrode in a buffer solution at pH = 7.1. Instrumental parameters were: step potential dE = 0.001 V, square-wave amplitude E_{sw} = 0.05 V and frequency of 10 Hz.

incubation time period had not effect on the cell viability. The LDH assay (Fig. 6) confirmed the results of MTT assay. No effects on the LDH release was found, indicating that the extract did not have any cytotoxic effects.

Antioxidative potential of capsaicin and other co-extracted bioactive compounds present in the ethanolic Capsicum *extracts*

A brief electrochemical characterization of capsaicin, vitamin E, ascorbic acid and quercetin has been performed by means of cyclic voltammetry (results not shown), while their potential synergistic antioxidative effect has been analyzed by means of square-wave voltammetry. Typical net SW voltammograms of capsaicin, vitamin E, quercetin, and ascorbic acid (each at concentration of 10 μmol/L) recorded at a glassy carbon electrode in a buffer solution at pH = 7.0 are given in Fig. 7. The net SW peaks of vitamin E and quercetin are closely positioned at potentials of $E_{p,net}$ = 0.146 V and $E_{p,net}$ = 0.108 V, respectively, while the peak of ascorbic acid is located nearby the capsaicin potential (at $E_{p,net}$ = 0.409 V for ascorbic acid and $E_{p,net}$ = 0.352 V for capsaicin). Vitamin E elevates to the highest net peak current ($I_{p,net}$ = 1.894 μA) compared to the maximal peak currents of quercetin, ascorbic acid and capsaicin (0.580 μA for quercetin, 0.193 μA for ascorbic acid and 0.086 μA for capsaicin). The obtained square-wave voltammograms for the

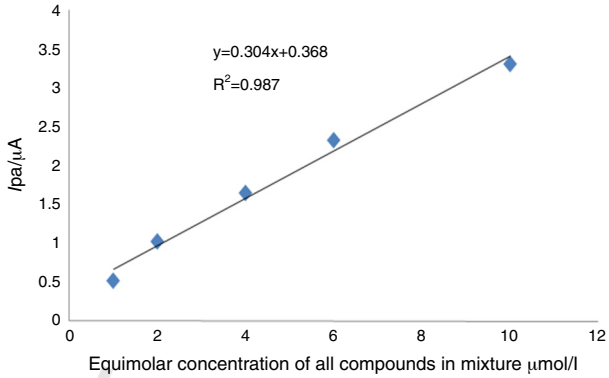

Fig. 8. Dependence on the anodic peak current and the concentration of equimolar mixtures containing vitamin E, quercetin, ascorbic acid and capsaicin at increasing concentration from 1 to 10 μM, in a phosphate buffer at pH = 7.1 (dE = 0.001 V, E_{sw} = 0.050 V and frequency of 10 Hz).

equimolar mixture of 10 μmol/L of each compound show that the voltammetric response consists of a single SWV peak at the potential about 0.128 V, which is between the typical peak of Vitamin E and quercetin. The measured net peak current obtained for the mixture of all four compounds was $I_{p,net}$ = 3.313 μA, Figs. 7(e) and 8. Q5

Discussion

It is well known that capsaicin has different carcinogenic effects on neuronal and non-neuronal cells (Chou et al., 2009). However, capsaicin induced cytotoxicity on pancreatic neuroendocrine tumor cells Skrzypski et al. (2014), human skin fibroblasts Kim et al. (2004), human gastric adenocarcinoma cell line (Yi-Ching et al., 2005). Ethanolic extracts of several spices, in which chilli pepper was included, inhibited cell growth at concentrations of 0.2–1 mg/mL *in vitro* (Unnikrishnan and Ramadasan, 1988). Other authors confirmed that ethanolic *Capsicum* extracts (0.01–1000 μg/mL) did not alter endothelial cell survival (Chularojmontri et al., 2010).

Our results showed that the cytotoxic activity of capsaicin on neuroblastoma B104 cells was pronounced at concentrations of 100 and 200 μM. The IC_{50} values were found to be 61.9 μM for incubation time period of 6 h and 61.6 μM for 24 h, respectively, indicating that the efficiency under the given conditions did not change when the incubation time period was extended form 6 h to 12 h. Previous findings of capsaicin cytotoxicity have shown a similar IC_{50} value but it was obtained for 5 days treatment of the cells with capsaicin (Richeux et al., 1999). Lower concentrations of capsaicin (Figs. 2 and 4) did not show any cytotoxic activity. Interestingly, the extracts obtained from different genotypes (Figs. 5 and 6) which contained even higher concentration of capsaicin did not induce the expected cytotoxicity.

It is already known that these extracts contain many other phytochemicals which can also possess different pharmacological properties. Vitamin C, vitamin E, (Palevitch and Craker, 1995; Daood et al., 1996), provitamin A and carotenoids, as compounds with well-known antioxidant properties (Krinsky, 1994, 2001; Matsufuji et al., 1998) can be also found in pepper fruits. The presence of flavonoids (quercetin and luteolin) and phenolic compounds and derivatives of cinnamic acid have been also found in pepper fruits (Sukrasno and Yeoman, 1993). These chemical compounds may interact with capsaicin in the ethanolic extracts and significantly change its pharmacological effect. Taking into account that all these compounds are present in the extracts of *Capsicum* fruit, it is particularly intriguing to study the voltammetric response in a mixture containing some of these compounds (vitamin E, vitamin C, quercetin and capsaicin) at equimolar level

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(Fig. 7). The current produced in the course of the voltammetric experiment is commonly proportional to the concentration of the redox compounds studied (Dobes et al., 2013; Pohanka et al., 2012). The net peak current obtained for the mixture of four compounds ($I_{p,net}$ = 3.313 μA) is exceeding the sum of the individual peak currents recorded separately, which implies that these compounds, being present simultaneously in the electrolyte solution, can exhibit a synergistic antioxidative effect. The antioxidant compounds in a solution can act as reduction agents, therefore they have a tendency to be oxidized on the working electrode. Hence, the correlation between electrochemical behavior of a compound with the antioxidant activity is plausible, as the "low oxidation potential" corresponds to the "high antioxidant power" (Barros et al., 2008). Considering that vitamin E and quercetin are yielding their peaks on potentials ($E_{p,net}$ = 0.146 V and $E_{p,net}$ = 0.108 V) which are much lower than the potential of capsaicin ($E_{p,net}$ = 0.352 V), we could report that vitamin E and quercetin can eventually neutralize the ROS produced by capsaicin at the mitochondrial membrane.

Hu et al. (2008) have shown that TRPV1 receptor is included in capsaicin induced Ca^{2+} influx by generation of reactive oxygen species (ROS), depolarization of the mitochondrial membrane, and ultimately cell death on the synovial fibroblasts in rats. Huang et al. (2009) have demonstrated that the apoptotic process on hepatocellular cancer was also accompanied by increasing of the intracellular Ca^{2+} level, increased production of ROS, and disruption of mitochondrial membrane potential. This apoptotic mechanism was also confirmed for many other types of cancer.

Therefore, we assume that high antioxidative potential of these co-extracted compounds present in the ethanolic extracts could have an antagonistic effect to capsaicin cytotoxic mechanism. This hypothesis enforced us to consider that the synergistic antioxidative effect of the complex composition of hot pepper fruits is responsible that ethanolic *Capsicum* extracts have not shown cytotoxic activity, beside its high concentration of capsaicin.

Conclusions

This study exhibited that capsaicin can act as cytotoxic agent in neruoblastoma cells in a dose dependent manner. Knowing that capsaicin can be easily extracted and isolated from *Capsicum* fruits offers the chance for discovering a phytochemical agent which possesses a strong pharmacological activity in antitumor therapy. In contrast, *Capsicum* extracts did not show any anti-proliferative activity. Therefore, additional electrochemical experiments were performed to explain the synergistic effects between capsaicin and vitamin E, quercetin and ascorbic acid, present together in a complex mixture. As shown in the literature, a common mechanism of capsaicin cytotoxicity is achieved through production of reactive oxygen species on cellular level. This leads to disruption of mitochondrial membrane potential, activation of caspase-3 activity and successive apoptosis. We assumed that this phenomenon of synergism between the studied compounds could be a possible reason for antagonistic effects of the other co-extracted phytochemicals from the hot pepper fruits on the cytotoxicity of capsaicin. In order to ensure in this hypothesis further experiments are needed to obtain more detailed results of the mechanism of cytotoxicity of capsaicin when it is in a mixture with other bioactive compounds found in peppers.

Author contributions

VM (PhD student) contributed in collection and identification of the pepper fruits, preparation of the extracts, running the laboratory experiments in (spectrophotometry, cytotoxic analyses and voltammetry), analyses of the data and preparation of the manuscript. LKG contributed in the UV analyses and supervised the extraction procedures. RG designed the study of the antioxidative analyses in voltammetry and supervised all the voltammetric experiment and results. KN designed the study of cytotoxicity analyses and supervised all the experiments performed on the cell cultures. All coauthors contributed with their critical reading of the manuscript. All the authors have read the final manuscript and approved it for publication.

Ethical disclosures

Protection of human subjects and animals in research. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare no conflicts of interest.

Uncited references

Q6

The Plan List (n.d.) and Won et al. (2013).

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