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PREVENTION OF GENOTOXIC EFFECT OF OCHRATOXIN A AND AFLATOXIN B1 BY ECHINACEA PURPUREA EXTRACTS

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Summary

The aim of this work was to examine the polyphenol content in *Echinacea purpurea* (L.) Moench extracts and to determine their antioxidant and antigenotoxic effect. The antioxidant activity of extracts and main components is determined by the application of electronic spin resonance and spectophotometric methods of capturing DPPH, superoxide and hydroxyl radical. The antigenotoxic effect was evaluated with genotoxic mycotoxins – aflatoxin B_1 (AFB_1) and ochratoxin A (OTA). Herbe ehinacea extract contained 13.31% of total polyphenols. Ehinacea extract contained a sufficient amount of phenolic acids (primarily chicoric and caftaric acid). Ehinacea extract showed the ability to neutralize DPPH radicals (EC_{50} = 15.67 µg/mL). Similar results were also identified by testing the capture of hydroxyl and superoxide radicals. The strong antioxidant activity of the dominant components that indicate phenolic acids as the ingredients that contribute the most to the antioxidant and antigenotoxic effect of echinacea extract was also found. Genotoxic suppression of AFB_1 and OTA was established by a comet test, establishing a significant reduction in tail, tail intensity and tail torque in leukocytes co-treated with mycotoxins and different concentrations of extracts.

Keywords: antioxidant activity, comet assay, *Echinacea purpurea*, genotoxicity, polyphenols

Introduction

Numerous epidemiological studies have shown that there is a strong association between fruit and vegetable intake and reduced mortality from heart disease, tumors and other degenerative diseases, and that intake of these foods reduces or delays the development of mentioned diseases and aging (Block et al., 1992; Scalbert et al., 2005). This effect is today attributed to the presence of various antioxidants, especially polyphenols. Among phytochemicals, polyphenols form one of the most numerous and widespread groups of compounds in the plant world, with more than 8.000 currently known structures. Polyphenols, which are normally present in the daily diet, have recently gained great importance due to their redox properties and possible positive impact on health, as well as in the prevention and treatment of diseases associated with oxidative stress, such as cardiovascular diseases and cancer (Zhou et al., 2006; Vinson et al., 1998; Teow et al., 2007).
Many substances of plant origin have been shown to "capture" free radicals, which are known to cause oxidative stress. Purple coneflower - *Echinacea purpurea* (L.) Moench is a good example of such plant species that contains a number of bioactive substances with potential antioxidant properties.
Species of the *Echinacea* L. genus are known immunomodulators, whose phytochemical composition indicates the presence of substances with antioxidant and anti-inflammatory effects (Barnes et al., 2005). Polyphenols from the group of phenol-carboxylic acid derivatives are associated with the prevention of diseases caused by oxidative stress, such as malignant, cardiovascular and neurodegenerative diseases (Mikulášová et al., 2005). Previous research has shown the anti-genotoxic effects of aqueous and alcoholic extracts of aboveground parts of *E. purpurea* (Kopjar et al., 2007), as well as the antimutagenic effect of the most representative phenolic carboxylic acids in *E. purpurea* extracts using the Ames test (Mikulášová et al., 2005). It is assumed that polyphenols with their antioxidant action reduce the formation of free radicals that significantly contribute to the cytotoxic and genotoxic effect of certain mycotoxins (Costa et al., 2007; Kopjar et al., 2007).
The aim of this study was to investigate whether extracts of *Echinacea purpurea* (L.) Moench extract are antigenotoxic *in vitro*. The results determined the concrete effect of the extract, as well as the most

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dominant components of *E. purpurea* after treatment of cells with mycotoxins ochratoxin A and aflatoxin B_1.

Materials and methods

Plant material and chemicals

Plant material

The leaves of the plant (belonging to the aboveground parts of the leaf, flower, stem) of the species Echinacea purpurea (L.) Moench, taken from the company Jan-Spider (Pitomača, Croatia), were used as material. Air-dried and pulverized plant material (20.00 g) was extracted with 200 mL of 70% ethanol using an ultrasonic bath for 30 minutes. The extract was then filtered and the residue was then re-extracted with 200 mL of the same solvent as described above. Obtained extracts were combined and then concentrated to dryness under vacuum at 50 °C using a rotary evaporator.

Chemicals

Acetic acid, aluminium chloride, formic acid, disodium hydrogen phosphate, ethanol, ethylenediaminetetraacetic acid (EDTA), hexamethylenetetramine, methanol, pyrogallol, sodium carbonate, sodium citrate, sodium dihydrogen phosphate, sodium hydroxide, sodium nitrite, sodium phosphate, sulphuric acid, tannic acid (95%), thiourea were purchased from Kemika (Zagreb, Croatia). Acetonitrile (HPLC grade) was purchased from J.T.Baker (Deventer, Netherlands). Ammonium molybdate, chlorogenic acid, caffeic acid, cynarin, echinacoside, chicoric acid, caftaric acid, casein, 2-deoxy-D-ribose, 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (ferrozine), 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^•$), hydrogen peroxide, potassium ferricyanide, rosmarinic acid (96%), sodium acetate, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), dimethylsulfoxide (DMSO) and sodium molybdate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Butylated hydroxytoluene (BHT, ≥ 99%), iron (II) chloride and quercetin-3-rutinoside (rutin, ≥ 95%) were obtained from Fluka (Buchs, Switzerland). Ascorbic acid (99%) and trichloroacetic acid (TCA) were purchased from Acros Organics (Geel, Belgium), Folin–Ciocalteu's phenol reagent, 3-*tert*-butyl-4-hydroxyanisole (BHA) and 2-thiobarbituric acid (TBA) were obtained from Merck (Darmstadt, Germany). Iron(III) chloride and hydrochloric acid were obtained from Riedel-de Haën (Seelze, Germany) and POCh (Gliwice, Poland), respectively.

Qualitative and quantitative analysis of E. purpurea extract

Determination of total polyphenols, tannins and flavonoids, phenolic acid

Determination of total tannin as well as total polyphenol contents was performed following the method described in European Pharmacopoeia (EDQM, 2004). Briefly, the extract (0.5 g) was boiled for 30 min in a water bath with water (150 mL), then the filtrate was made up to 250 mL with water and the obtained solution served as stock solution. An aliquot of stock solution was mixed with Folin–Ciocalteu's phenol reagent and sodium carbonate solution. After 30 min, the absorbance was read at 720 nm (A_1), and the quantification of total phenols was done with respect to the standard calibration curve of pyrogallol (6.25-50.00 μg). For the determination of tannins content, stock solution was vigorously shaken with hide powder for 60 min. Since the hide powder adsorbed tannins, phenols unadsorbed on hide powder were measured in filtrate, after addition of Folin–Ciocalteu's phenol reagent in a sodium carbonate medium (A_2). The percentage content of tannins, expressed as pyrogallol, was calculated from the following equation:

$$(\%) = 3.125\times(A_1-A_2)/(A_3\times m) \qquad (1)$$

where A_3 is the absorbance of the test solution containing 0.05 g of pyrogallol, and m the mass of the extract (g).

The total flavonoid contents of tested plant extract were determined using the spectrophotometric method of Christ and Müller (1960). Each powdered plant sample (0.2 g) was mixed with 20 mL of acetone, 2 mL of 25% hydrochloric acid and 1 mL of 0.5% hexamethylenetetramine solution and heated under reflux in a water bath for 30 min. The extract was filtered and re-extracted twice with 20 mL of acetone for 10 min. Filtrates were combined and made up to 100 mL with acetone. An aliquot of 20 mL of the acetone extract was mixed with 20 mL of water and then extracted with three quantities, each of 15 mL, of ethyl acetate. Combined ethyl acetate layers were washed twice with water then filtered and diluted to 50 mL. To 10 mL of this solution 0.5 mL of 0.5% solution of sodium citrate and 2 mL of 2% aluminium chloride solution (in 5% methanolic solution of acetic acid) was added and then diluted to 25 mL with 5% methanolic solution of acetic acid. The mixture was allowed to stand for 45 min and the absorbance was measured at 425 nm. A sample solution prepared in the

same manner but without addition of aluminium chloride solution served as a blank. All determinations were performed in triplicate. The percentage content of flavonoids, expressed as quercetin, was calculated from the equation:

$$(\%) = A \times 0.772/b \qquad (2)$$

where A is the absorbance of the test solution at 425 nm and b is the mass of the sample, in grams.

Determination of total phenolic acids

Determination of hydroxycinnamic acid derivatives was performed according to procedure described in European Pharmacopoeia (EDQM, 2004). Briefly, 0.2 g of the powdered plant material was extracted with 80 mL of 50% ethanol under a reflux condenser in a boiling water bath for 30 min. The cooled extract was filtered, the filter rinsed with ethanol, and then combined filtrate and rinsing was diluted to 100 mL with 50% ethanol. An aliquot of 1 mL of the extract was mixed with 2 mL of 0.5 M hydrochloric acid, 2 mL of Arnow reagent (10% aqueous solution of sodium nitrite and sodium molybdate), and 2 mL of 8.5% sodium hydroxide and diluted to 10 mL with water. The absorbance of the test solution was measured immediately at 505 nm against sample blank. The percent of total hydroxycinnamic acid content was calculated and expressed as rosmarinic acid, according to the following expression:

$$(\%) = A \times 5.3/m \qquad (3)$$

where A is the absorbance of the test solution at 525 nm and m is the mass of the sample, in grams. Analysis of each sample was performed in triplicate.

HPLC analysis of E. purpurea extract

Herbal extracts were analyzed according to the method described by Belščak-Cvitanović et al. (2011). The samples were filtered through a 0.45 µm filter (Nylon Membranes, Supelco, Bellefonte, USA) before HPLC analysis. 20 µL of each sample was injected for HPLC analysis using a Varian Pro Star Solvent Delivery System 230 (Varian, Walnut Creek, USA) and a Photodiode Array detector Varian Pro Star 330 (Varian, Walnut Creek, USA) by using a reversed-phase column Gemini-NX C-18 column (Phenomenex, USA) (150×4.6 mm, 2.6 µm i.d.). The solvents consisted of 3% formic acid in acetonitrile (solvent A) and 3% formic acid in water (solvent B) at a flow rate of 0.9 mL/min. The elution was performed with a gradient starting at 10% A, rising to 40% A after 25 min, then to 70% A after 30 min and becoming isocratic for 5 min. Chromatograms were recorded at 278 nm. Detection was performed with a Photodiode Array Detector by scanning between 200 and 400 nm, with a resolution of 1.2 nm. Phenolic compounds were identified by comparing the retention times and spectral data with those of standards. The data acquisition and treatment were conducted using Star Chromatography Workstation Version 5 software. All analyses were repeated three times.

Antioxidant activity of E. purpurea extract

2, 2-Diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) radical scavenging assay

The free radical scavenging activities of the samples were measured using the stable $DPPH^{\bullet}$ radical, according to the method of Blois (1958). Briefly, 0.1 mM solution of $DPPH^{\bullet}$ in ethanol was prepared and 1 mL of this solution was added to 3 mL of sample solution in ethanol at different concentrations (0.39-200 µg/mL). The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm. The capability to scavenge the $DPPH^{\bullet}$ radical was calculated using the following equation:

$$(\%) = [(A_0 - A_1)/A_0] \times 100 \qquad (4)$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of sample, corrected for the absorbance of sample itself. Butylated hydroxytoluene (BHT) was used for comparison. All determinations were done in triplicate.

Hydroxyl radical ($OH^{\bullet}$) scavenging assay

Hydroxyl radicals were generated by a Fenton reaction (Fe^{3+}-ascorbate-EDTA-H_2O_2 system), and the scavenging capacity towards the hydroxyl radicals was measured by using a deoxyribose method as described by Halliwell et al. (1987) with a slight modification. The reaction mixture contained, in a final volume of 1 mL, 2-deoxy-2-ribose (2.8 mM), phosphate buffer (0.1 mM, pH 7.4), iron (III) chloride (20 µM), EDTA (100 µM), hydrogen peroxide (500 µM), ascorbic acid (100 µM) and various concentrations (12.5-1600 µg/mL) of the test sample or reference compound. After incubation for 1 h at 37 °C, an aliquot of the reaction mixture (0.5 mL) was

added to 1 mL of 2.8% TCA solution, followed by 1 mL of TBA solution (1% in 50 mM sodium hydroxide) and then the mixture was heated 20 min at 90 °C to develop the colour. After cooling, the absorbance was measured at 532 nm against an appropriate blank solution. All experiments were performed in triplicate. Hydroxyl radical scavenging activity was evaluated with the inhibition percentage of 2-deoxyribose oxidation by hydroxyl radicals, according to the following equation:

$$(\%) = [A_0 - (A_1 - A_2)]/A_0 \times 100 \qquad (5)$$

where: A_0 is the absorbance of the control without a sample, A_1 is the absorbance in the presence of the sample and deoxyribose and A_2 is the absorbance of the sample without deoxyribose. Thiourea was used as a positive control.

Reducing power assay

The reducing power of samples was determined by the method of Oyaizu (1986). An aliquot of 1 mL of the sample at various concentrations (0.20-50 µg/mL) was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After adding 2.5 mL of 10% trichloroacetic acid, the mixture was centrifuged at 650 rpm for 10 min. The supernatant (2.5 mL) was mixed with distilled water (2.5 mL) and 0.1% iron (III) chloride (0.5 mL), and the absorbance was measured at 700 nm using appropriate blank. Assays were carried out in triplicate. BHT was used as a reference.

Metal ion chelating assay

The ability of samples to chelate iron (II) ions was estimated using the method reported by Gülçin (2006) and compared with that of reference chelator agent EDTA. Different concentrations of the sample (final concentration 0.78-800 µg/mL) were added to a solution of 2 mM iron (II) chloride (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was finally quantified to 4 mL with ethanol, shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm. All assays were done in triplicate. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the formula given below:

$$(\%) = [A_0 - (A_1 - A_2)]/A_0 \times 100 \qquad (6)$$

where A_0 is the absorbance of the control, containing iron (II) chloride and ferrozine only, A_1 is the absorbance in the presence of the tested sample and A_2 is the absorbance of the sample under identical conditions as A_1 with water instead of iron (II) chloride solution.

Total antioxidant capacity assay

The total antioxidant capacity of tested plant extract and their active constituents were evaluated by the phosphomolybdenum method, according to the procedure of Prieto et al. (1999). An aliquot of 0.4 mL of the sample solution in ethanol was combined in a vial with 4 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The effective concentrations of the sample in the reaction mixtures were in the range of 12.5-100 µg/mL. The vials were capped and incubated in a water bath at 95 °C for 90 min. After cooling the mixture to room temperature, the absorbance was measured at 695 nm against a blank. The antioxidant capacity of the sample was expressed as equivalents of ascorbic acid (AAE), utilizing a calibration curve of ascorbic acid in the concentration range from 1.56 to 100 µg/mL. All assays were run in triplicate.

ESR measurements

Hydroxyl radical scavenging activity

As hydroxyl free radicals (•OH) are highly reactive, with relatively short half-lives, the concentrations found in natural systems are usually inadequate for direct detection by ESR spectroscopy. Spin-trapping is a chemical reaction that provides an approach to help overcome this problem. Hydroxyl radicals are identified because of their ability to form nitroxide adducts (stable free radicals form) from the commonly used DMPO as the spin trap (Buettner, 1985). The Fenton reaction was conducted by mixing 200 µL of DMPO (112 mM), 200 µL of DMF, 200 µL of H_2O_2 (2 mM) and 200 µL of $FeCl_2$ (0.3 mM) (control). The influence of *E. purpurea* extract on the formation and stabilization of hydroxyl radicals was investigated by adding investigated extracts in the Fenton reaction system at the range of concentrations 25-1500 µg/mL. ESR spectra were recorded after 5 minutes, with the following spectrometer settings: field modulation 100 kHz, modulation amplitude 0.226 G, receiver gain 5 x10^5, time constant 80.72 ms, conversion time 327.68 ms, center field 3440.00 G, sweep width 100.00 G, x-

band frequency 9.64 GHz, power 20 mW, temperature 23 °C.
The SA_{OH} value of the extract was defined as:

$$SA^{\bullet}_{OH} = 100 \times (h_0 - h_x) / h_0 \, [\%] \quad (7)$$

where h_0 and h_x are the hight of the second peak in the ESR spectrum of DMPO-OH spin adduct of the control and the probe, respectively.

Superoxide anion radical scavenging activity

Superoxide anion radicals ($O_2^{\bullet -}$) were generated in the reaction system obtained by mixing 500 µL of dry dimethylsulfoxide (DMSO), 5 µL of KO_2/crown ether (10 mM / 20 mM) prepared in dry DMSO and 5 µL of 2 M DMSO solution of DMPO as spin trap. The influence of extracts on the formation and transformation of superoxide anion radicals was obtained by adding the DMF solution of *E. purpurea* extract to the superoxide anion reaction system at the range of concentrations 5-100 µg/mL. After that the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT. The ESR spectra were recorded on an EMX spectrometer from Bruker (Rheinstetten, Germany) under the following conditions: field modulation 100 kHz, modulation amplitude 4.00 G, receiver gain 1×10^4, time constant 327.68 ms, conversion time 40.96 ms, center field 3440.00 G, sweep width 100.00 G, x-band frequency 9.64 GHz, power 20 mW, temperature 23 °C.

The $SAo_2^{\bullet -}$ value of the extract was defined as:

$$SAo_2^{\bullet -} = 100 \times (h_0 - h_x) / h_0 \, [\%] \quad (8)$$

where h_0 and h_x are the height of the second peak in the ESR spectrum of DMPO-OOH spin adduct of the control and the probe, respectively.

Comet assay

This test was performed using whole human blood in the volume of 5 mL that was taken by venipuncture from a forty-year-old male volunteer (non-smoker). Blood was mixed with cell culture media RPMI 1640 (1:1) and transferred into 96-well plates. Prior to cell treatment water extract of *E. purpurea* was sterilised by filtration trough millipore filter (0.2 nm). Aflatoxin B_1 (AFB_1) was dissolved in dimethyl sulfoxide (DMSO), while absolute ethanol was used as solvent for OTA. Leukocytes were exposed for 2 hours to plant extracts at concentrations 1 mg/mL (E1), 10 mg/mL (E2) and 20 mg/mL (E3), single AFB_1 (3 µM) and OTA (10 µM), as well as combination of each mycotoxin with each concentration of plant extract. Water (10%), DMSO (0.03%) and ethanol (0.3%) were used as controls.
The comet assay was carried out according to Singh et al. (1988). After cell treatment Aliquots of 20 µL of cell suspension were mixed with 80 µL 0.5% low melting point agarose (LMP), and 100 µL of agarose-cell suspension was spread onto a fully frosted slide (Surgipath, Richmond, Il, USA) pre-coated with 1.5% normal melting point agarose, NMP (in Ca- and Mg-free PBS buffer). The slides were allowed to solidify on ice for 10 minutes. After overnight lysis at 4 °C in a mixture of 2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (pH 10) supplemented with 1% Triton-X, the slides were placed in denaturation and electrophoresis buffer (10 mM NaOH, 200 mM Na_2EDTA, pH 13), incubated for 20 min, and electrophoresed for 20 min at 25 V and 300 mA. DNA was neutralised with a neutralisation solution (0.4 M Tris/HCl, pH 7.5) three times 5 min each. The slides were kept in a humid atmosphere in a dark box at 4 °C until further analysis. For image analysis, DNA was stained with 100-250 µL ethidium bromide solution per slide for 10 min. Slides were scored using an image analysis system (Comet assay II, Perceptive instruments Ltd., U.K.) connected to a fluorescence microscope (Zeiss, Germany). Images of 100 randomly selected cells were measured. Only comets with a defined head were scored. Comet parameters considered in this study were the tail length, the proportion of DNA in the comet tail (tail DNA or tail intensity), and tail moment, which was calculated as the product of the fraction of DNA in the comet tail and the tail length.

Results and discussion

The total amount of polyphenols in Echinacea purpurea

Echinacea species were investigated regarding their polyphenolic content and separation. The presence of flavonoids, phenolic acids and tannins in ethanolic plant extracts was detected by spectrophotometrical method. The results of spectrophotometric determination of the total amount of polyphenols (prepared as shown in chapter 2.2) are presented in the Table 1.
The determination of the amount of polyphenols in the species *E. purpurea* was performed with the spectrophotometrical method.
Table 1 shows the values of the content of the specimens of the ethanol extract in *E. purpurea* and the estimated content of the total polyphenols, tannin,

phenolic acid, flavonoids. It was determined that the overhead parts of the examined species contain ranging between 12.98% and 13.80% of polyphenols, tannin between 0.85% and 0.92; 3.23% to 3.72% hydroxycinnamic derivatives and portions of flavonoid between 0.123% to 0.131%.

Table 1. Contents of phenolic acids, flavonoids, tannins and total polyphenols in *E. purpurea* selected *Echinacea* species

Plant extracts	Contens (%)			
	Total polyphenols	Flavonoids	Phenolics acids	Tanins
Echinacea purpurea	13.31±0.43	0.126±0.004	3.47±0.25	0.863±0.003

Each value is the mean ± SD of three indenpendent measurements.

Phytochemical analysis of polyphenolic compounds

A large number of HPLC methods for determining phenolic compounds in plant extracts have been published. Essentially, they are adapted to determine the content of the most dominant polyphenols in one plant species or a certain number of compounds of that class in a variety of extracts (Harnly et al., 2006). Given the large number of compounds belonging to the flavonoid group, as well as the fact that most are related in the form of glycosides, it is difficult to find the ideal method for determining their total content. Flavons and flavonoids (quercetin, luteoline, apigenin, routine) in such biological substrates most often appear in glycosylated form, so a specific glycoside is also needed to identify them There are a large number of works relating to the examination of the composition of polyphenol *E. purpurea* (Cech et al., 2006; Lin et al., 2011).

In the extract of echinacea (Fig. 1) the presence of chicoric, caftaric, chlorogenic, and caffeic acids and echinacoside were identified the quantified. The chromatogram (Fig. 1) of the ethanol extract of the echinacea herb clearly shows pronounced peaks of chicoric and caftaric acid.

Chicoric acid derivative is a caffeic acid and is the most represented phenolic component in *E. purpurea*. Similar results were obtained from research and other authors (Kuštrak, 2005; HMPC, 2008; Stanisavljević et al., 2009; Lin et al., 2011). Cynarine was not detected, similar to the work of Lin and associates (2011), who did not detect it in any part of the plant (overhead parts, stems and roots).

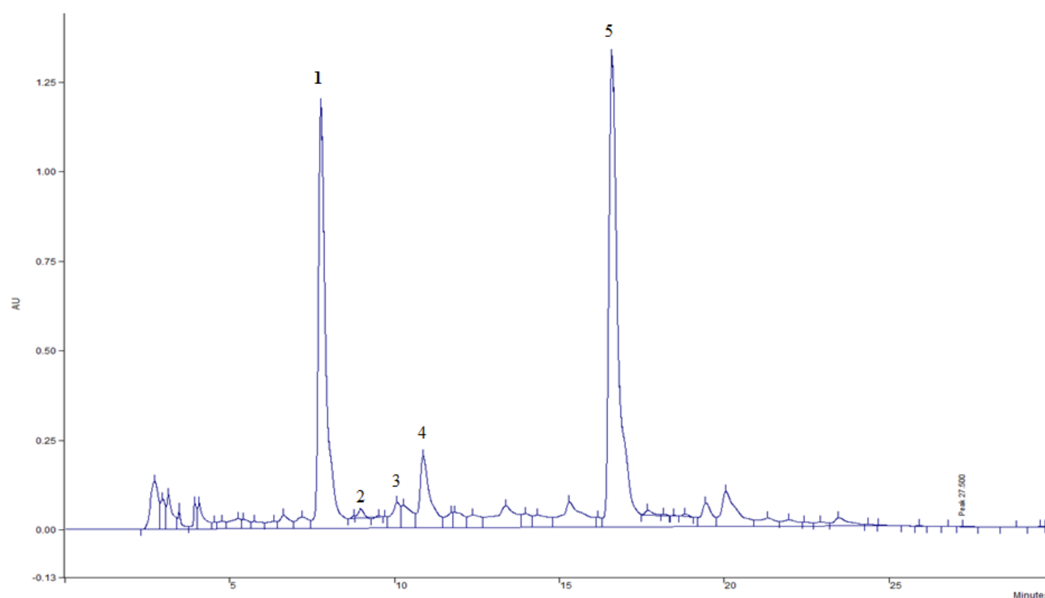

Fig. 1. Chromatogram of extract *Ehinacea purpurea* : 1- caftaric acid, 2- chlorogenic acid, 3- caffeic acid, 4- echinacoside, 5- chicoric acid

Table 2 shows the data on the content of the extract of the plant *E. purpurea*, where the largest share of chicoric and caftaric acid. This agrees with Pellati et al. (2004) that the main phenolic components of the species *E. purpurea* are chicoric and caftaric acid. According to literature sources, the main components

of the overhead part of the plant are alkylamides, polyacetylenes and caffeic acid derivatives, polysaccharides and glycoproteins (Stanisavljević et al., 2009). Established quantities of chicoric acid (Table 2) are in accordance with literature data, where it is stated that it is the dominant component and that *E. purpurea* extracts contain this acid in a very wide range (Lee and Scagel, 2010).

Table 2. Polyphenol components in purple ehinacea extract

Substance	Extract	
	mg/L	*mg/g plants*
Caftaric acid	2447.56	12.23
Chlorogenic acid	34.69	0.17
Caffeic acid	36.19	0.18
Echinacoside	400.04	2.00
Cynarine	nd	nd
Chicoric acid	3144.52	15.72

*nd - not detected

For example, the established share chicoric acid of the extract of overhead parts of ehinacea was 13 mg/g (Becker and Hsieh, 1985) or ranged from 1.4-38.3 mg/g (Bauer and Remiger, 1989; Stuart and Wills, 2000; Wills and Stuart, 1999). Differences in the content of chicoric acid are somewhat expected, as a large number of factors affect the content of bioactive components. Differences in concentrations of chicoric acid and other caffeic acid derivatives in plant material and preparations occurring by a particular analogue method may be affected by genetic variations and many environmental factors, light, temperature, extraction process, formulations and storage conditions (Pellati et al., 2004). Chicoric acid shows immunomodulating properties (Lee and Scagel, 2010; Stanisavljević et al., 2009), stimulates phagocytosis *in vivo* and *in vitro*, shows antihyaluronidase activity and protects collagen from degradation with free radicals. Chicoric acid showed antiviral action and was found to inhibit HIV-1 hiv integration and replication (Lee and Scagel, 2010; Stanisavljević et al., 2009; Lou et al., 2003). The content of chicoric acid, also correlated well when assessing antioxidant activity using different methods: the ability to capture DPPH radicals and the effect of peroxide lipid emulsion on oxygen consumption (Thygesen et al., 2007). Therefore, a significant influx of this component on the antioxidant effect of the extract of overhead parts of ehinacea can be expected.
Caftaric acid may inhibit the mutagenicity of heterocyclic aromatic amines in the Ames and micronucleus assays (Zhang et al., 2011).
It has also been shown to be effective in inhibiting phase I enzymes (cytochrome P450 1A1 and 1A2) and enhancing phase II enzyme activity (UDP-glucuronosyl transferase and GST-glutathione S-transferase) (Zhang et al., 2011). Phenolic acids can also inhibit the formation of mutagenic and carcinogenic N-nitroso components because they inhibit the reactions of their bioactivation *in vitro* (Kono et al., 1995). According to Kosalec (2006), laboratory studies have identified the immunostimulation effect of caftaric acid, similar to other derivatives of caffeic acid from purple echinacea (chicoric, chlorogenic, ferrulic, *p*-coumaric, *p*-hydroxybenzoic and vanillic acid). Caftaric acid is found predominantly in overhead parts of the plant (Perry et al., 2001).

Antioxidant activities of Echinacea ethanolic extracts

The antioxidant activities of polyphenols were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, as well as their metal chelating abilities. Polyphenolic compounds such as flavonoids, phenolic acids and tannins are considered to be the major contributors to the antioxidant activity of medicinal plants, fruits and vegetables (Pereira et al., 2009; Rice-Evans et al., 1996). The ability to remove free radicals mostly depends on the structural properties of phenol compounds such as the energy of dissociation of O-H bound, the delocalisation of phenol radicals (PheO*) and steric disturbances caused by the substitutes on the aromatic ring (Sanchez-Moreno et al., 1998). Therefore, in the present study five different assays were employed in order to determine and compare the antioxidant properties of selected *Echinacea* species, as well as to elucidate their mode of action.
Unlike free radicals generated in a lab, such as superoxide and hydroxyl radicals, the use of free stable radicals is an advantage, since it is not influenced by the secondary reactions, such as chelate with metals and enzyme inhibition, caused by additives (Yamaguchi et al., 1998).
After measuring absorptions at 517, the percentage of the inhibition capacity of $DPPH^{\bullet}$ radicals were calculated. The plant extract in lower amounts has quite a weaker effect than the synthetic andtioxidant. Although it lags continually after the effect of BHA, the difference is significantly lowered in the amounts above 50 μg/mL. It was also revealed that the chlorogenic acid, rutin and tannic acid are better catchers of $DPPH^{\bullet}$ than the referent antioxidant. The effect of BHA is equalised with the effect of rutin only at the amount of 12.5 μg/mL when it accomplished the inhibition above 85%.

Abilities of the tested samples to scavenge DPPH˙ were assessed on the basis of their IC_{50} values which were inversely related to their antioxidant capacities, as they express the amount of the antioxidant needed to decrease the radical concentration by 50%. The IC_{50} values obtained in this study are listed in Table 3. The strongest antiradical activity was determined for the tannic acid which already in the amount of 0.78 µg/mL accomplishes a 50% exhibition of DPPH˙. The chlorogenic acid shows the same effect in the amount of 1.56 µg/mL and is equalised with the tannic acid in the concentration of 6.25 µg/mL. The extract of the species also shows a significant scavenging capacity of DPPH˙. In the concentration higher than 50 µg/mL the effect of the extract approaches the effect of clear substances and BHA. The results show that flavonoids, phenolic acid and tannins, present in the examined species, equally contribute to the antiradical effect of the extract.

The DPPH assay has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances. Nitrogen centered radicals such as DPPH˙ react with phenols via two different mechanisms: direct abstraction of phenol H-atoms and electron transfer processes. The contribution of one or the other pathway depends on the nature of solvent and/or the redox potentials of the species involved. DPPH˙ is a stable free radical compound with a characteristic absorption at a wavelength of 517 nm. Antioxidants upon interaction with DPPH˙ either transfer an electron or hydrogen atom to DPPH˙, thus neutralizing its free radical character. The colour of the reaction mixture changes from purple to yellow with a decrease of the 517 nm absorbance. The degree of discolouration indicates the scavenging potential of the antioxidants (Villaño et al., 2007; Foti et al., 2004). The research of Yokozawa et al. (1998) has shown that tannins and some flavonoids show an activity in relation to DPPH˙ radicals and that the activity is closely related to their chemical structure. With the increase in galiol groups, the molecular mass and ortho-hydroxy groups in the structure, the activity of tanine increases, and the number and position of hydroxyl groups represent an important characteristic of flavonoids for "quenchers" free radicals.

Fenglin et al. (2004) released the results of the study of the 'scavangers' activity on DPPH radicals of water-methanol extracts of more then 300 medicinal herbs. For 56 of the examined specimens they got EC_{50} values under 0.500 mg of the specimen/mL of the extragent. The same authors attribute the activity of DPPH˙ radicals of plants to the present flavonoids and tannins in the extract. Chen et al. (2004) discovered that the chlorogenic acid most actively removes DPPH˙ radicals in plants, and that their activity in the same test is the same and larger than the activity of tocopherol.

Orhan et al. (2009) got similar results when they studied antioxidant activities of the species *E. purpurea* and *E. pallida* by determining the catching capacity of DPPH˙ of free radicals and chelate ions of iron. A chrloroform extract in air of dry plant material *E. purpurea* showed the greatest capacity of chelate iron ions (Orhan et al., 2009).

Table 3. Comparative overview of IC50 values as well as total antioxidant capacities of *Echinacea* ethanolic extracts, polyphenolic compounds and reference antioxidants

	IC_{50}* (µg/mL)				**Total antioxidant capacity (mg AAE/g)****
	DPPH scavenging activity	**OH scavenging activity**	**Reducing power**	**Iron chelating activity**	
***E. purpurea* extract**	15.7	1071.9	47.69± 2.96	125.86±11.33	2.44±0.23
Rosmarinic acid	-	-	-	-	-
Rutin	1.7	62.0	7.89±0.66	nd	3.19±0.16
Tannic acid	0.78	11.9	2.58±2.96	nd	8.72±0.50
BHT	-	-	4.95±0.36	nd	7.79±0.13
Thiourea	-	81.3	-	-	-
Chlorogenic acid	1.56	260.8	3.48±0.33	nd	6.13±0.05
BHA	2.8	37.6	3.40±0.08	nd	8.70±0.39
EDTA	-	-	-	0.94±0.04	-

*IC_{50} value: concentration at which the DPPH and OH radicals were scavenged by 50%, absorbance was 0.5 for reducing power, iron(II) ions were chelated by 50%, respectively;
**Results are calculated for sample concentrations of 12.5 µg/mL. Each value is expressed as mean ± SD. (n = 3); nd - not determined at tested concentrations; − not tested.

The method based on the determination of the catching ability of OH˙ free radical was used, according to the principle of oxidising deoxyribose when exposed to the hydroxyl radicals which appear with the Fenton's

reaction. In the reaction the hydroxyl radicals appear with the decomposure of H_2O_2, whereby the high potential of EDTA-Fe^{2+} causes the decomposure of deoxyribose. The oxydative decomposure can be perceived by heating up products with 2-thiobarbituric acid (TBA) in acidic conditions, whereby the pink chromogene (TBARS, thiobarbituric acid reactive species), which has the absorption maximum at 532 nm. The added antioxidants compete with the deoxyribose for the hydroxyl radicals and reduce the amount of chromogenes (Cheng et al., 2003).

The study results showed that the plant extract has a lower scavenging capacity of the free radical $OH^•$ in comparison to the pure polyphenol components and standard antioxidants. The highest tested concentration of the extract 1600 μg/mL achieved a 57% inhibition of $OH^•$. BHA constantly shows a stronger antiradical activity than thiourea, so that it achieves a 57% inhibition in the concentration of 50 μg/mL, and thiourea in the concentration of 100 μg/mL. In comparison to the tested standard antioxidants, polyphenol compounds achieved an effect according to the following order: tannic acid > BHA > rutin > thiourea > chlorogenic acid. In this case too, tannic acid showed the strongest antiradical activity and performed a 52% inhibition already in the concentration of 12.5 μg/mL.

The extract constantly lags in its scavenging capacity of $OH^•$, whereas the chlorogenic acid approaches in the highest tested concentration 1600 μg/mL. The results point out that flavonoids, phenolic acid and tannins, present in the examined plant species, are responsible for the antiradical effect of the extract and that favonoids contributes more to the effect.

Reduction ability

The reduction ability of the extract was studied, along with chlorogenic acid, rutin and tannic acid, in comparison to the BHA and BHT referent antioxidants. The yellow colour of the examined solutions is changed into varios nuances of green and blue depending on the reduction ability of the studied antioxidant specimens. The reduction component can serve as an important indicator of its potential antioxidative ability.

Fe^{3+} from $FeCl_3$ forms Fe^{3+}-ferricyanide in a reaction with the solution $K_3F_3(CN)_6$. The presence of reducents such as a specimen of antioxidants causes the reduction of Fe^{3+}-ferricyanide into the Fe^{2+} form. Fe^{2+} with ferricyanide produces a blue solution, whose colouring intensity can be observed by measuring the absorption at 700 nm (Fe^{2+} +$[Fe(CN)6]^{3-}$ Fe^{3+} + $[Fe(CN)_6]^{4-}$) (Akand and Gülçin, 2008; Prasad et al., 2009). The reduction ability increases with an increase in the concentration of the specimen. Solutions with greater concentration are prominently blue due to the presence of a greater amount of Fe^{2+} and show greater absorption, i.e. a greater reduction ability, thus acting as stronger antioxidants. With a decrease in concentration, the studied specimens become weaker reducents. That is the reason why a greater amount of Fe^{3+}-ferricyanide is present in solutions, making the specimens greener. Without antioxidants the solution of the Fe^{3+}-ferricyanide complex is yellow.

The herbal extract has a visibly lower reduction ability than the synthetic referent antioxidants BHA and BHT and the usual substances such as phenolic acid, flavonoids and tannins. Chlorogenic acid also proved to have a better reduction ability than BHT, showing a better ability even than BHA in concentrations above 25 μg/mL. Rutin has a lower reduction ability than BHA, but shows a greater ability than BHT in concentrations above 25 μg/mL. Tannic acid shows the best reduction ability. In comparison to the tested standard antioxidants, polyphenol compounds achieved the following performance in concentrations above 25 μg/mL: tannic acid > chlorogenic acid > BHA > rutin > BHT.

The 0.5 absorption value can be clearly noticed, i.e. a 50% reduction ability, which is achieved by tannic acid already in the concentration 2.58 ± 0.21 μg/mL and by the *E. purpurea* extract only in the concentration 47.69 ± 2.96 μg/mL. Chlorogenic acid reached a 50% reduction ability in the concentration 3.48±0.36 μg/mL, rutin in 7.89 ± 0.66 μg/mL, BHA in 3.40 ± 0.08 μg/mL and BHT in 4.95 ± 0.36 μg/mL. In higher concentrations chlorogenic acid is a better antioxidant than BHA. In concentrations under 25 μg/mL, the reduction ability of rutin lags a little bit behind BHT; in higher concentrations rutin is a better antioxidant than BHT. By far the best reduction ability is that of tannic acid, that is, it acts as the best antioxitant. On the basis of the examined pohyphenol compounds and referent antioxidants, the extract of *E. purpurea* constantly lags in reduction ability.

The results indicate that flavonoids, phenolic acid and tannins, present in the studied species, contribute to the antioxidant effect of the extract together, tannins contributing the most, followed by phenolic acid and, lastly, flavonoids.

Iron ions chelating capacity (II) was studied for the extract of the *E. purpurea* species, chlorogenic acid, rutin and tannic acid, in comparison to the synthetic referent antioxidants with BHA and BHT. EDTA was used as the standard chelator.

The appearance of reactive oxygen species such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical was catalysed with free iron in the Haber-Weiss reaction (O_2^- + H_2O_2 →O_2+ OH^- +

$OH^{\bullet}$). In the transition metals family, iron is known as the most important pro-oxidant of lipid oxidation. The Fe^{2+} iron state accelerates lipid oxidation, transforming hydrogen and lipid peroxides into free reactive radicals in the Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} also creates radicals from peroxide but ten times weaker than Fe^{2+}. Fe^{2+} is the strongest oxidising agent among metal ions. Ferrozine forms quantitatively pink complexes with Fe^{2+}. The presence of chelating agents in a reactional compound inhibits the formation of ferrozine and Fe^{2+} complexes, resulting in the reduction of the pink colour of the solution. Colourimetric measuring of a colour intensity enables the estimation of metal ion chelating ability for the studied element. A lower absorbing value indicates a greater chelating ability. The structures which have one or more functional groups (-OH, -SH, -COOH, $-PO_3H_2$, C=O, $-NR_2$, -S-, -O-) are known to show a good metal ion chelating ability in a convenient configuration (Gülçin et. al., 2010).

As the standard chelator, EDTA was shown to have a great iron ion chelating ability even in low concentrations, that is, a 100% chelating ability in a concentration range of 6.25 – 800 μg/mL. It is only in a concentration at 3.13 that its chelating ability begins to decrease. In lower concentrations, the extract of *E. purpurea* has a noticably lower effect than the standard metal chelator EDTA. Although it continually lags behind the effect of EDTA, the difference is considerably lowered in concentrations above 200 μg/mL. The study also showed that the extract is a better iron ion chelator than the BHA and BHT referent synthetic antioxidants, which did not show metal ion chelating ability at all in the tested concentrations. Unlike chlorogenic acid and rutin, tannic acid showed a certain iron ion chelating ability, although it was weak if compared to the extract of *E. purpurea*.

Unlike the extract of *E. purpurea* and tannic acid, EDTA approaches the maximum iron ion chelating ability in concentrations above 6.25 μg/mL. The value of IC_{50} is noticeable also, i.e. the concentration of the examined specimen which causes a 50% iron ions chelation. With interpolation from a linear regression analysis the concentration of 125.86 ± 11.33 μg/mL is obtained, i.e. the EDTA concentration 0.94 ± 0.04 μg/mL which causes a 50% iron ions chelation. The results showed that out of the studied bioactive substances of *E. purpurea* only tannic acid possessed iron ion chelating ability, and was only slightly responsible for the chelating ability of the studied extract. It was shown that iron ion chelating ability was dependent on the extract concentration and tannic acid. With an increase in concentration, the Fe^{2+}-ferrozine complex absorption is decreased in a linear manner, i. e. iron ion chelating ability is increased. Metal ion chelating ability is a significant trait of antioxidants, since it lowers the concentration of a catalysing transition metal in lipid peroxidation. Chelating agents are effective as secondary antioxidants as they lower the redox potential, thus stabilising the oxidised form of the metal ion.

The total antioxidant capacity of the extract of *E. purpurea*, chlorogenic acid, rutin and tannic acid, in comparison to the synthetic referent antioxidants BHA and BHT was tested. The formation of the complex also depends on the antioxidant concentration. While a greater amount of complexes is formed in higher concentrations and the examined solution shows a more prominent green colour, the solution becomes less green when the concentration is lowered. When there is a lack of antioxidants, the solution is colourless as there is no complex formation.

Antioxidants act as reducents whose ability is based on breaking the chain reaction of creating free radicals by donating a hydrogen atom, i.e. by donating electons they react with free radicals and transform them into more stable products. They also react with peroxide precursors, thus stopping peroxide formation. The antioxidant capacity depends on the number of free –OH groups, so polyphenols exhibit a greater antioxidant ability than monophenol. The greatest antioxidant capacity among phenolic acid is exhibited by gallic acid with three –OH groups on the aromatic ring, i.e. epigallocathechin with three –OH groups in the B ring among flavonoids. Glycoside flavonoids have a weaker antioxidative capacity than aglycones alone (Kim et al., 2004).

The total antioxidative capacity was calculated at 695 nm as a ascorbic acid equivalent from the estimated absorptions. The results showed that the extract of *E. purpurea* had a lower total antioxidative capacity expressed as a ascorbic acid equivalent in relation to pure polyphenol components and standard antioxidants. In comparison to the standard tested antioxidants, polyphenol compounds have a lower total antioxidative capacity. Rutin has the lowest capacity, whereas chlorogenic acid and tannic acid have an equal total antioxidative capacity. BHT in higher concentrations has a clearly weaker effect than BHA. Although it continually lags behind the effect of BHA, the difference decreases considerably in greater dilutions.

One can see that the total antioxidative capacity of standard polyphenol substances visibly lags behind the total antioxidative capacity of standard polyphenol antioxidants. However, the difference is decreased so that in 12.5 μg/mL tannic acid and BHA almost have an equal total antioxidative capacity. It is aslo visible that the total antioxidative capacity of the extract lags behind the standard polyphenol substances. But this

difference also decreases in greater dilutions, so that in 12.5 μg/mL, the extract of *E. purpurea* and rutin almost have an identical total antioxidative capacity. The results indicate that flavonoids, phenolic acid and tannins, present in *E. purpurea*, contribute to the total antioxidative capacity of the extract together, with phenolic acid and tannins contributing the most, followed by flavonoids.

Results ESR

One part of our investigation on antioxidant activity of *E. purpurea* extract was the scavenging activities on hydroxyl and superoxid anion radicals measured by ESR method. Using a spin trap, such as DMPO, it is possible to convert reactive hydroxyl radicals to stable nitroxide radicals (DMPO-OH adducts) with spectral hyperfine splitting that reflects the nature and structure of these radicals. The reaction of Fe_2^+ with H_2O_2 in the presence of the spin trapping agent DMPO generated a 1:2:2:1 quartet of lines with hyperfine coupling parameters (aN=aH= 14.9 G) (Čanadanović-Brunet, et al., 2005). The intensity of the ESR signal, corresponding to the concentration of free radicals formed, was decreased in the presence of different amounts of *E. purpurea* extract. The total elimination of hydroxyl radical (SA_{OH} = 100%) was obtained in the presence of 1250 μg/mL of extract, which indicates that this applied concentration inhibits the creation of hydroxyl radicals completely. This was confirmed by the calculated EC_{50} values of 210 μg/mL.
Comparing the intensity of the ESR signal of the DMPO-OOH spin adduct blank test and the sample, it was determined that aqueous solutions of Echinacea extract lyophilisates in the range of tested concentrations have an inhibitory effect on $O_2^{\bullet-}$ formation or participate in their transformation. The addition of the extract at a mass concentration of 10 μg/mL causes an inhibitory effect on the formation of $O_2^{\bullet-}$ radicals, and at the same concentration it reaches an antioxidant activity of 53.75%. Lee et al. (2009) using a spectrophotometric method at a concentration of 200 μg/mL of *E. purpurea* extract (whole plants) found 41.7%, and at 1600 μg/mL 91.1% inhibition of $O_2^{\bullet-}$. The investigated extract showed dose-dependent radical scavenging activities. The EC_{50} value, defined as the concentration of extract required for 50% scavenging of superoxid anion radicals under experimental condition employed, is a parameter widely used to measure the free radical scavenging activity (Cuvelier et al., 1992); a smaller EC_{50} value corresponds to a higher antioxidant activity. The EC_{50} value of *E. purpurea* extract (76.7 μg/mL) shows that extract is rich in antioxidant compounds and efficiently scavenge superoxide anion radicals.

Comet assay

Table 4 shows the results of alkaline comet assay following 2 hours exposure to various concentrations of *E. purpurea* extracts (E1, E2, E3), single AFB_1 and OTA as well as combination of extracts and mycotoxins. Plant extracts did not exert genotoxic activity taking into account values of tail intensity and tail moment. However, significant increase in tail length was observed upon exposure to highest concentration of plant extract (P<0.05), while lower concentrations did not provoked increase of this parameter.

Table 4. Evaluation of primary DNA damage measured in human leukocytes following 2-h exposure to *E. purpurea* extracts and single AFB_1 and OTA or combinations of each mycotoxin with plant extracts

Cell treatment	Tail length (μm)				Tail intensity (% DNA)				Tail moment			
	Mean±SD	M	25%-P	75%-P	Mean±SD	M	25%-P	75%-P	Mean±SD	M	25%-P	75%-P
C1 (10% water)	14.19±1.75	14.10	12.82	14.74	0.58±1.23	0.0	0.0	0.52	0.076±0.17	0.0	0.0	0.055
C2 (0.03% DMSO)	13.53±2.15	13.46	12.18	14.74	0.48±0.97	0.0	0.0	0.55	0.060±0.11	0.0	0.0	0.062
C3 (0.3% ethanol)	14.61±2.07	14.74	13.46	13.38	0.53±1.57	0.0	0.0	0.21	0.076±0.23	0.0	0.0	0.023
E1 (1 mg/mL)	13.49±1.35	13.46	12.82	14.10	0.50±1.05	0.13	0.0	0.63	0.063±0.13	0.018	0.0	0.077
E2 (10 mg/mL)	14.09±2.09	14.10	12.82	15.38	0.58±1.25	0.02	0.0	0.62	0.075±0.15	0.002	0.0	0.081
E3 (20 mg/mL)	15.34±2.11	15.38*	13.46	16.67	0.39±0.85	0.0	0.0	0.47	0.054±0.10	0.0	0.0	0.070
AFB1 (3 μM)	16.58±4.63	15.38*	13.46	19.71	1.73±2.49	0.54*	0.0	2.55	0.240±0.33	0.078*	0.0	0.364
E1+AFB_1	14.38±1.81	14.10**	12.98	15.38	0.52±1.46	0.0**	0.0	0.41	0.070±0.18	0.0**	0.0	0.059
E2+AFB_1	15.60±1.84	15.38	14.10	16.67	0.40±0.87	0.0**	0.0	0.44	0.060±0.12	0.0**	0.0	0.065
E3+AFB_1	14.82±2.49	14.74	13.46	15.87	0.96±2.13	0.06	0.0	0.83	0.130±0.27	0.004	0.0	0.125
OTA (10 μM)	17.30±4.85	16.03*	13.62	19.87	1.16±2.10	0.21*	0.0	1.25	0.170±0.30	0.030*	0.0	0.277
E1+OTA	14.48±1.86	14.10**	13.46	15.38	0.56±1.04	0.02	0.0	0.85	0.073±0.13	0.001	0.0	0.106
E2+OTA	14.26±2.35	14.10**	12.82	15.22	0.34±0.67	0.0**	0.0	0.30	0.045±0.08	0.0**	0.0	0.040
E3+OTA	13.68±2.08	13.46**	12.18	14.74	0.41±1.04	0.0**	0.0	0.47	0.052±0.12	0.0**	0.0	0.058

C1, C2, C3 – control solvent; SD- standard deviation; 25%- P- 25% percentile; M – median; 75% -P- 75% percentile; *- compared to control (P<0.05); **- compared to AFB_1 or OTA given alone (P<0.05)

As it was expected, exposure to AFB_1 at 3 μM and OTA at 10 μM significantly increased all three comet parameters comparing to control solvents ($P<0.05$). Leukocytes simultaneously exposed to AFB_1 and E1 or E2 had significantly lower tail intensity and tail moment, as compared to cells exposed to mycotoxin alone ($P<0.05$), showing antagonizing effect of plant extracts. Highest extract concentration (E3) also decreased this comet parameters but without significant difference comparing to AFB_1 given alone. At the same time, tail length was significantly lower only when AFB_1 was simultaneously applied with E1 ($P<0.05$). Higher concentrations of plant extracts also decreased tail length but without significant difference comparing to effect of single toxin.

E. purpurea extracts also antagonized genotoxicity of OTA. Tail intensity and tail moment were significantly lower in cells exposed to combination of OTA and E2 or E3 than in cells treated with OTA alone ($P<0.05$). All of three extract concentrations showed significant protective effect considering measurements of tail length.

Since both AFB_1 and OTA are food contaminants with genotoxic activity the purpose of this study was to see whether their genotoxic action could be antagonised if leukocytes are simultaneously exposed to this mycotoxins and water extracts of *E. purpurea.* Results of comet assay showed that plant extract did not induced DNA damage taking into account tail intensity and tail moment but significant increase of tail length was observed when highest concentration of extract was used. These results could be explained by the theory of comet tail formation. Tail length is the length of relaxed DNA loops, which migrated from the core during electrophoresis, while tail intensity is the number of DNA breaks in the loop (Colins et al., 2008) mining that high concentration of extract increases relaxation of DNA strands rather than causing DNA fragmentation. Genotoxicity of AFB_1 is well documented. It is known that AFB_1 is metabolized to AFB -8,9B epoxide by cytochrome *P*450. Epoxide could covalently bind to DNA and form 8,9- dihydro-8-(N(7)-guanyl)-9-hydroxy-AFB_1 (Wogan, 1992; Nakai et al., 2008). Results of our study support the findings of AFB_1 genotoxicity observed by comet assay in hepatocytes, whole blood (Miele et al., 1999; Anderson et al., 1999; Williams et al., 2004). Besides DNA adductation, AFB_1 can induce oxidative DNA damage contributing to AFB_1 genotoxicity (Halliwell et al., 1999). Extracts of *E. purpurea* given at 1 mg/mL and 10 mg/mL were able to prevent DNA strand breaks rather than increase of tail length, which could be attributed to antioxidant capacity of extract. Previously we demonstrated that OTA given at 5 μM for 1 h increases tail intensity and tail moment in human leukocytes (Šegvić Klarić et al., 2010) and this study confirms that short time exposure to OTA leads to DNA damage. The mechanism of OTA genotoxicity is still under debate. According to current literature, OTA genotoxicity may be assigned as direct (DNA adduct formation) and indirect (oxidative DNA damage) mechanisms of action. An Fpg-modified comet assay showed that oxidative DNA damage in rat kidney was significantly higher than damage observed by standard alkaline comet assay (Domijan et al., 2006). Taking into account antioxidative activity of *E. purpurea* extract, which showed concentration-dependent antagonizing activity to OTA, we could conclude that oxidative stress plays a key role in OTA genotoxicity.

Polyphenols could act as antioxidants and bind ROSs that is produced by mycotoxins such as OTA. Alternatively, there is evidence that polyphenols can act as prooxidative *in vivo* and promote antioxidant cell protection that removes ROSs (Hail et al., 2008.; Long et al. 2010). Flavonoids also have an inhibitory effect on the activity of many prooxidant enzymes, including lipoxygenase (Laughton et al., 1991; Schewe et al., 2002), cyclooxygenase (Laughton et al., 1991), inducible nitric monoxide synthase (Raso et al., 2001), monooxygenase (Siess et al., 1995), thyroid peroxidase (Doerge and Chang, 2002), xanthine oxidase (Sheu et al., 1998), NADH-oxidase (Hodnick et al., 1994), etc. They also inhibit β-glucuronidase (Kim et al., 1994), phosphodiesterase (Picq et al., 1989), phospholipase A_2 (Gil et al., 1994) and protein kinase (Cushman et al., 1991). Some of the best phenol chemoprevention studies have used green tea and its predominant polyphenol, epigallocatechin gallate. Inhibition of DNA adducts formation and induced chemical carcinogenesis in experimental animals (Xu et al., 1992), was observed, as well as inhibition of DNA methyl transferase and reactivation by methylation-silenced expression of genes important in the process of carcinogenesis (Fang et al., 2003), etc.

Polyphenols can reduce or inhibit the mutagenic potential of mutagens and carcinogens (Miadakova et al., 2008). Controlling cell mutation with natural antimutagens can result in a variety of ways to prevent mutations that are essential, both in the case of cancer and in diseases caused by genotoxic agents (Birt et al., 2001).

After many laboratory and epidemiological studies, it has been found that diet is responsible for approximately 35% of all human cases of cancer (Doll and Peto, 1981). Based on epidemiological research, Block et al (1992) and Steinmetz et al. (1996) have estimated that the incidence of cancer can be reduced by at least 20-30 % with a healthy diet. The central role

of diet in preventing carcinogens has also been confirmed by the World Cancer Research Foundation (World Cancer Research Fund, 2007). The interactions between diet and the biological processes that lead to cancer are very complex. Although a large number of carcinogenic substances have been found in food, the human body has its own defense mechanisms that are sufficient if the exposure is not quantitatively excessive and chronic.

Conclusion

The content of total polyphenols was 13.31% in herba purple ehinacea extract. The analysis of phenolic acids, flavonoids, tannins and proantocyanidine in the samples identified the multiple proportion of phenolic acids in herba ehinacea extract.

Qualitative and quantitative analysis of echinacea extract determined the presence of chicoric, caftaric, chlorogenic, caffeic and echinacoside acid. The most represented were chicoric and caftaric acid.

The echinacea extracts studied showed relatively poor ability to capture DPPH radicals (EC_{50} = 15.67 μg/mL) compared to reference antioxidant and ingredient standards. Efficiency order: tanic acid > chlorogenic acid > routines > BHA > ehinacea extract.

The use of another method of comparative testing of plant extract and reference substances has proven that ehinacea extract has a lower ability to capture $OH^{\bullet}$ compared to DPPH radical. The EC_{50} value for extract (1071.91 μg/mL) was thirteen times less than the value obtained for tioureu. According to the ability to capture radicals $OH^{\bullet}$ the following order has been established: tanic acid > BHA > routines > tiourea > chlorogenic acid > ehinacea extract.

The ESR spectral analysis, by comparing the intensity of the ESR signal of the DMPO-OH spin ducts, found that ehinacea extracts, in the range of studied concentrations, inhibit the formation of hydroxyl radical and/or affect its transformation.

The EC_{50} value established according to this radical was 210 μg/mL. The results obtained found that phenolcarbonic acids, hydroxycymetic acid derivatives present in the plant species tested contribute the most to the action of extract according to this radical.

The ESR analysis of the DMPO-OOH spin adduct identified the antioxidant activity of ehinacea extracts according to superoxide radical. The EC_{50} extract value was 76.7 μg/mL.

Ehinacea extract at applied concentrations mainly showed no genotoxic effect in the comet test according to established tail intensity and tail torque values compared to control. However, a significant increase in the length of the comet's tail was noticed at exposure to the highest concentration of extract.

Leukocytes that were simultaneously exposed to AFB_1 and ehinacea extract had significantly lower tail torque, tail intensity and tail length at lower tested extract concentrations, compared to cells that were exposed only to mycotoxin. This demonstrates the neutralizing effect of herba purple ehinacea extract in the specified range of concentrations.

Ehinacea extracts also neutralized the genotoxicity of OTA. Tail intensity and tail torque were significantly lower in cells exposed to the combination of OTA and the two highest concentrations than in cells treated only with OTA. All three concentrations of extracts showed a significant protective effect given the length of the comet's tail.

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