Glasnik hemičara i tehnologa Bosne i Hercegovine

Bulletin of the Chemists and Technologists of Bosnia and Herzegovina

2015

44

Print ISSN: 0367-4444
Online ISSN: 2232-7266

UDC: _____

Original Scientific Article

43-52

Antioxidant and prooxidant activities of phenolic compounds of the extracts of *Echinacea purpurea* (L.)

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Article info
Received: 11/5/2015
Accepted:8/6/2015

Keywords:
antioxidants,
cytotoxicity,
poliphenolics,
Echinacea purpurea

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Abstract: Background and objectives: In recent years, there is a *growing interest* on natural and safer *antioxidants*. So far, little is known about the cytotoxic and (anti) oxidative potential of echinacea (*Echinacea purpurea*) extracts.

Methods: In order to evaluate the antioxidant activity of extracts, total phenolics content and the scavenging capacity on DPPH˙ radicals was determined. The ability of extracts to scavenge superoxide and hydroxyl radicals was tested using electron paramagnetic resonance (EPR) techniques. Also, the extracts were screened for cytotoxicity and antioxidative/prooxidative potential by neutral red and DCFDA assay respectively, using human colon cancer cell line SW480. *The cells were* exposed to *various concentrations* of extracts (range: 0,008; 0,08; 0,8; 1; 10 i 20 mg/mL) and *different treatment* times (2, 3, 4 and 24 h).

Results: The content of total phenolic compounds of extracts of *E. purpurea* was 10.57 % GAE. The scavenging activity of radicals was found to exhibit 50% of the inhibition value (IC50 value) at the concentration of 15.67 μg/ml for the investigated echinacea extract. Also, the calculated value of 210 mg/ml for hydroxyl and 76.7 mg/ml for superoxide anion radical indicates that the Echinacea extract is rich in antioxidant compounds that neutralize investigated radical species. In *in vitro* experiments, echinacea extract showed prooxidant effect at lower concentrations and shorter incubation period when SW480 cell line was used as test system. The highest concentration was also the most toxic which is particularly evident after 24 hours of treatment.

Conclusions: Echinacea extracts are shown to possess the strong antioxidant potential.

INTRODUCTION

Phenolic compounds are widely present *in almost all plants* and *food* products of *plant origin*. Polyphenols in plants can act as signaling molecules involved in the hormonal regulation of plant growth, and protect them from infection by microorganisms (antimicrobial activity). They also act as protective agents against UV radiation, attract pollinators and contributors in plants pigmentation (Naczk et al., 2004). In food, *phenolic compounds* contribute bitterness, clarity, colour, taste, flavour and oxidative stability. With multiple biological activities, extracts rich in phenols are of particular importance for the food industry because they slow down the oxidative degradation of lipids and thus improve the quality and

nutritional value of food (Mathew et al., 2006.). Also, consumption of phenols from plant based foods is associated with reduced risk of a variety of diseases.
In recent years, a growing interest in the food industry for the use of antioxidants from natural sources (e.g., polyphenolic compounds from plants, fruits and vegetables, whole grains) has been noticed. There is a growing body of evidence that the most commonly used synthetic antioxidants have potential to damage health. Food producers, in order to protect consumers, their interests and their safety have drawn attention to production of natural antioxidants instead to synthetic one. Benefits of polyphenols intake are result of their antioxidant activity, the presence in the human diet and their influence in the prevention of various chronic diseases associated with oxidative stress (Zhou et al., 2006.; Vinson et al., 1998.; Teow et al., 2007.). In our research we were investigated chemical composition and antioxidant activity of *Echinacea purpurea* (L.) extracts (Echinacea extracts). *Echinacea purpurea* (L.) is a good example of species that contains a number of bioactive compounds with potential antioxidant properties. So far, little is known about the potential of those extracts.

MATERIALS AND METHODS

Chemicals

Ethanol, ethylenediaminetetraacetic acid (EDTA), pyrogallol, tannic acid (95%) were purchased from Kemika (Zagreb, Croatia). Chlorogenic acid, 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\bullet}$), rosmarinic acid (96%), 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%) and quercetin-3-rutinoside (rutin, ≥95%) were obtained from Fluka (Buchs, Switzerland). Folin–Ciocalteu's phenol reagent, 3-*tert*-butyl-4-hydroxyanisole (BHA) and were obtained from Merck (Darmstadt, Germany). Dulbecco's modified Eagle medium (DMEM), Fetal bovine serum (FBS) and antibiotics were obtained from (GIBCO, Grand Island, NY, USA). All used chemicals and reagents were of the highest analytical grade and obtained from "Kemika" Zagreb (Croatia).

Plant material

We used air dried aerial parts of purple Echinacea, taken from the Jan-Spider (Pitomača, HR). Samples were collected during the year of 2010.

Sample preparation

Sample preparation for antioxidant analysis

In the previously milled plant material (20.00 g) was added 200 mL of 70% ethanol and left to stand overnight at room temperature. Extraction of the ethanol dissolving compounds was then continued by applying ultrasound (30 min), using the ultrasonic bathroom Branson model b-220 Smith Kline Co., Shelton, USA (50/60 Hz, 125 W). The sample was filtrated and in plant material was added the new amount of 200 mL of 70% ethanol, and then previously described procedure of ultrasonic extraction was repeated. After filtration, the obtained filtrate by the first and second filtration were combined and dried by evaporation under vacuum at 313 K.

Sample preparation for electron paramagnetic resonance (EPR) techniques and for determining prooxidant and antioxidant effects of extracts on human cell line of colon carcinoma (SW 480)

The sample was milled for further analysis. After the addition of 200 mL distilled water to 20 g of sample, extraction of the water dissolving compounds was performed by ultrasound (30 min) at 303 K, using the ultrasonic bathroom Branson model b-220 Smith Kline Co., Shelton, USA (50/60 Hz, 125 W). The water extract were filtrated and dried by lyophilization. The obtain extract was used for electron paramagnetic resonance (EPR) techniques and to determine prooxidant and antioxidant effects of extracts on human colon carcinoma cell line (SW 480).

Determination of polyphenols

Determination of total polyphenols of the overground parts of the tested plant species was carried out by spectrophotometric method according to the method by Schneider (Schneider, 1976). Blue solution absorbance was measured at 720 nm with distilled water as a blank test. To calculate the concentration of total polyphenols, calibration line was prepared. For this purpose 10 mg of tannic acid was dried at 80 °C and dissolved in 100 mL of distilled water. Blue solution absorbance was measured at 720 nm with distilled water as a blank test. From the linear equation ($y = 9.7768\ x + 0.0099$) the proportion of polyphenols is calculated by first calculating the value of x corresponding to mg of polyphenols in the sample which is according to the procedure diluted 1,000 times. Then the polyphenols on the amount of plant material taken into the procedure are calculated (0.25 g) and finally expressed as a percentage. Share of polyphenols (%) = $x \bullet 100 / m$, x = value in mg (calculated from the calibration line) x 10-3, m = plant material in g.

Determination of total flavonoids

The total flavonoid contents of tested plant extract were determined using the spectrophotometric method of Christ et al., (1960). Briefly, 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 100.0 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 50.0 mL. To 10.0 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 25.0 mL with 5% methanolic solution of acetic acid. 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 × 0.772/b*, where *A* is the absorbance of the test solution at 425 nm and *b* is the mass of the sample, in grams.

Antioxidant activity of *E. purpurea* extract

2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH•) radical scavenging assay

The free radical scavenging activities of the samples were measured using the stable DPPH• radical, according to the method of Blois (1958). Briefly, 0.1 mM solution of DPPH• 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• radical was calculated using the following equation: $(\%) = [(A_0 - A_1)/A_0] \times 100$, 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.

ESR measurements

Hydroxyl radical scavenging activity

The influence of *E. purpurea* extract on the formation and stabilization of hydroxyl radicals was determined by adding investigated extracts in the Fenton reaction system at the range of concentrations 25-1500 μg/ml. 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 (112mM), 200 μL of DMF, 200 μL of H_2O_2 (2mM) and 200 μL of $FeCl_2$ (0.3 mM) (control). 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 3,440.00 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: $SA^{\bullet}_{OH} = 100 \times (h_0 - h_x) / h_0$ [%]; 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: $SAo_2^{\bullet-} = 100 \times (h_0 - h_x) / h_0$ [%]; 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.

Cell line

Human colon cancer cell line (SW 480) was used in all *in vitro* experiments. The cell line was obtained from the Ruđer Bošković Institute, Zagreb. Monolayer culture of human cells grown in DMEM medium with 10% calf serum at 37 °C in a humid atmosphere with 5% CO_2 was used. During the transplant of the cells, 0.25% solution of trypsin was used for detachment of cell monolayers.

Determination of prooxidant and antioxidant effects of the echinacea extracts on human colon carcinoma cell line (SW 480)

Non-toxic concentrations of hydrogen peroxide is used, which will cause oxidative stress in the cells. This is done in a way that 10 mL 6% H_2O_2 is added to 990 mL of PBS. In this way, a solution which will be used to treat cells is obtained, 0.06% concentrations of H_2O_2 (conc. 17.6 mM). After trypsinization and cell counting, cell suspension is prepared at a concentration of 105 cells/mL and 180 mL of cell suspension is inoculated in a black microtiter plate with 96 wells. The next day, after the cells tied to the bottom of the wells, the cells are treated for half an hour with 100 mL of 0.06%-percent H_2O_2. Thereafter, hydrogen peroxide is removed, and various concentrations of plant extracts (0.008, 0.08, 0.8, 1, 10 and 20 mg, / ml) are placed on the cells for 2, 3, 4 and 24 hours with and without recovery. After treatment, the medium is removed and cells are washed with PBS. Solution of DCFH-DA is prepared in PBS containing 1% bovine serum albumin (BSA) and the cells are treated with solution of DCFH-DA for half an hour. After that the intensity of fluorescence is determined in the fluorimeter at excitation wavelength of 485 nm and emission wavelength of 520 nm. To express the measured fluorescence as the percentage of surviving cells, neutral red test is performed again as well as calculation of the quotient of survival. Results are expressed as the ratio of fluorescence and survival quotient which is calculated relative to the negative control. Each experiment was repeated three times, and each concentration was tested in triplicate.

$$\text{DCF} = \frac{\text{fluorescence (arbitrary unit)}}{Q_{survival}}$$

Negative controls show the basic level of ROS measured in the cells that are not treated, and positive control represents the level of ROS in the cells treated with hydrogen peroxide.

''Neutral red'' test

Treatment of cells was performed during the exponential phase of cell growth at 2, 3, 4 and 24 hours, corresponding to the exposure of cells of the investigated compounds during one cell division. Therefore, 105 cells / mL was seeded in the 96-well plate. The next day, after the cells

had adhered to the surface and after the division started, cells were treated with plant extracts at different concentrations (0.008 , 0.08, 0.8 , 1, 10 and 20 mg/mL). As a control, cells grown in growth medium were used. After the treatment, medium was removed and cells were washed for twice with 100 μL PBS. The "neutral red" solution was added in the wells and cellswere incubated for 90 min at 37°C. "Neutral red" was then removed and the cells were again washed twice with 100 μL of PBS. Color remains accumulated in the lysosomes of viable cells. In order to extract the color, 100 μL of mixture of ethanol/water/glacial acetic acid (50:49/1) was added on the washed cells (Costa et al., 2005). The intensity of color separation was determined spectrophotometrically at 430 nm. Dead or damaged cells do not retain their color after washing and fixation processes (Babich et al., 1990.).
Percentage of survival is determined in relation to the negative control by the formula set out below: % Survival = (A430 nm of the studied compound / A430 nm of control) x 100 Each experiment was repeated 3 times, and each concentration was investigated in 4 replicas.

Statistical Analysis

One-way analysis of variance (ANOVA) and multiple comparisons (Duncan's *post-hoc* test) were used to evaluate the significant difference of the data at $p < 0.05$. All experiments were performed at least in triplicate. Results are presented as mean values ± SD.

RESULTS AND DISCUSSION

The total amount of polyphenols in Echinacea extracts
The content of total phenolics compounds in plant material was (10,57 ± 0,35)%, expressed as g of gallic acid per 100 g of the dry sample (%; w/w). Total flavonoids content was (0,13 ± 0,004)%, expressed as g of quercetin per 100 g of the sample.
The reviewed scientific literature states that large differences in the values of total polyphenols can be observed, which are determined by spectrophotometry, with appliance of the Folin-Ciocalteuova reagent. The method according to the Folin-Ciocalteu is fast and widely used, however, it is not quite specific and it can detect phenols with different sensitivity (Kahkonen et al., 1999.). Hence, the results do not provide a complete quantitative and qualitative picture of the polyphenolic compounds in the extracts, due to the possible presence of interfering compounds (Singleton et al., 1999.). A number of environmental factors that contribute to the variability of polyphenols in plant material should be added to the fact. The plant world recognizes more than 4,000 flavonoids, which are used in the traditional and Eastern medicine over a thousand years (Hertog et al., In 1992. Peterson et al., 1998.). Flavonoids are attributed with positive effects on human health, which are manifested through its anticarcinogenic, antibacterial, immune-stimulating, anti-virus and the anti-inflammatory properties (Havseen, 2002.). The benefit of fruits and vegetables consumption is largely attributable to the positive effects of flavonoids (Howard et al., 1997.).

Antioxidant activities of E. purpurea ethanolic extracts
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.). 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. The antiradical activity of the ethanol extract of the overhead part of the species *Echinacea purpurea*, chlorogenic acid, rutin, tannic acid, was copared with the synthetic antioxidant butyl-hydroxy anisole (BHA). After measuring absorptions at 517 nm, the percentage of the inhibition capacity of DPPH$^{\bullet}$ radicals were calculated. The plant extract in lower amounts had quite a weaker effect then the synthetic antioxidant. 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%. 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$^{\bullet}$. 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. In the Picture 1 it is visible that concentrations higher than 15 μg/ml achieve a 50% inhibition of free radicals. Concentrations higher than 50 μg/ml approaches the effect of pure substances and BHA. These results show that flavonoids, phenolic acid and tannins, present in the examined species, equally contribute to the antiradical effect of the extract. The research of Yokozawa et al., (1998.) has shown that tannins and some flavonoids show an activity in relation to DPPH$^{\bullet}$ 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 as a "quenchers" of 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$^{\bullet}$ 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$^{\bullet}$ radicals in plants, and that its 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$^{\bullet}$ 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.).

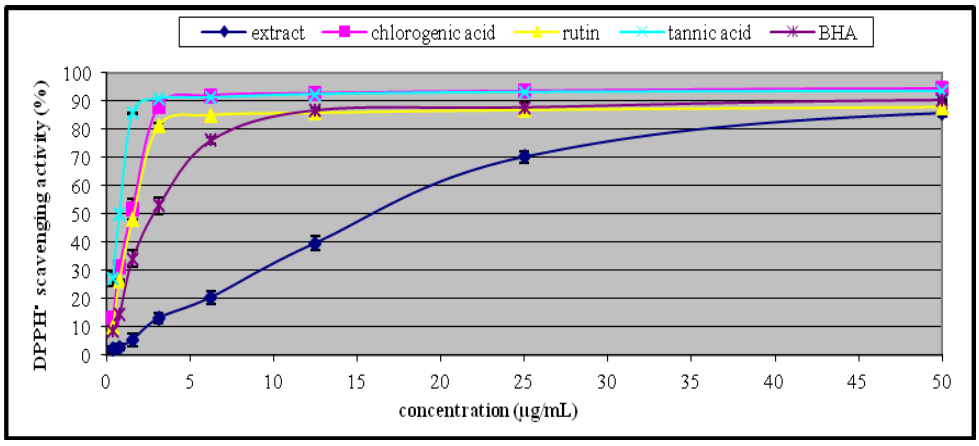

Figure 1. Antioxidant activities of *E. purpurea* ethanolic extracts

Results ESR

Other part of our investigation on antioxidant activity of *E. purpurea* extract was the measurement of the scavenging activities on hydroxyl and superoxid anion radicals 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 1500 µg/ml of extract, which indicates that this applied concentration completely inhibits the production of hydroxyl radicals. The investigated extract showed dose-dependent radical scavenging activity. 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.

Determination of oxidative and antioxidant effect of extracts of cultured human colon cancer cells (SW 480)

In order to determine potential antioxidant effect of Echinacea extracts against free radicals produced by hydrogen peroxide which was added onto cells, colon cancer cells (SW-480) were pretreated with hydrogen peroxide and subsequnently they were exposed to different concentrations of Echinacea extracts. Figures 2-4 show the changes made to the contents of ROS in the cells treated only with Echinacea extracts and in the cells pre-treated with H_2O_2 respectively. As it can be seen on Figure 2, there is a lower intensity of the positive control fluorescence, i.e. cells treated with a two-hour treatment with H_2O_2 in the presence of a medium (a medium in which the cells were growing and in which dilutions were made - DMEM + 10 % serum) as compared to untreated cells (negative control). In fact, the level of oxidative stress is more or less stable in the positive control cells despite various times of incubation, as opposed to the basic oxidative stress in the negative control cells, which decreases progressively (Figure 2-5). Oxidative stress in the positive control was lower as compared to the half-hour treatment with hydrogen peroxide without treatment. Obviously, treatment with H_2O_2 leads to formation of ROS 's, but also leads to the simultaneous induction of detoxifying enzymes in the cells that neutralize ROS and the volume of oxidative stress in the induced cells decreased. It is unclear why a longer incubation reduces oxidative stress in the negative controls (Figures 3 and 4), although the formation of hydrogen peroxide and rapid degradation of polyphenolic compounds in similar cell cultures has been observed (Long et al., 2010.). Addition of low concentrations (up to 0.8 mg/mL) of the Echinacea extract acted as a prooxidant after 2 h of incubation. After 3 and more hours of incubation this effect had faded, and the addition of the extract generally did not raise the amount of ROS's in comparison to the negative control (Figure 3-5). Similarly to the effect of the positive control, in the cells pretreated with hydrogen peroxide, and than with the extract, significant reduction in oxidative stress occured. Antioxidant activity was strong and generally strengthens with the amplification of concentration of added extract, regardless of the time of incubation (Figures 2-5). Only for two hours and three hours incubation, decline of oxidative stress at low concentrations of extract and increase at higher concentrations has been observed. This discrepancy could be explained by the time required for the formation of a critical amount of ROS`s due to the interaction of H_2O_2 and polyphenol extract, while longer incubation time leads to the suppression of oxidative stress by induction of cellular antioxidant protection. Prooxidant effect of many phenolic compounds was observed in diclorofluoresceine and other *in vitro* tests. For example dopamine, in concentrations of less than 500 µM, decreases the fluorescence intensity thus showing his

antioxidant properties, while at a concentration of 1 μM increases the fluorescence intensity, acting as a prooxidant (Wang and Joseph, 1999.). It was found that lower concentrations of flavonoids (nM to low values μM) can stimulate antioxidant genetic response including the detoxification enzymes of phase II. On the other hand, higher concentrations of the flavonoids may support the activation of mitogen-activated protein kinases which can lead to apoptosis (Chen et al., 2000.; Kong et al., 2000.). Many reports describe the negative effects of flavonoids on the cellular level. For example, due to the toxic effects of flavonoids at high concentrations, several studies have suggested a DNA strand breakage when flavonoids such as epigallocatechin-3-gallate are used (Tian et al., 2007.), quercetin (Beatty et al., 2000.), and kaempherol (Niering et al., 2005.). SW480 are cancer cells, and they have higher levels of ROS's, so the extracts could by prooxidant activity induce apoptosis in such an environment (Hail et al., 2008.). The results confirm the prooxidant effects of extracts at lower doses which is consistent with the hypothesis on the control of transformed cells by apoptosis induced by phytochemicals (Hail et al., 2008.). Numerous other mechanisms of anticarcinogenic polyphenols activities have been proposed, some of which are unrelated to the redox capacity (Finley, 2005.). Of course, one must not forget the fact that the tests were carried out in vitro or ex situ. To determine the full effect of the extracts and their components, it will be necessary to take into account the processes of absorption and metabolism under the action of digestive enzymes and intestinal microflora, distribution, degradation in the intestinal mucosa and liver, conjugation and excretion. Intestinal microbial enzymes hydrolyze flavonoid glycosides to their aglycone and sugar. Most aglycones are then metabolized by the microorganisms, while a negligible part is absorbed (Gugler et al., In 1975. Hollman et al., 1995.). The low doses of polyphenols are more relevant for modeling of their effect in the organism with respect to the concentration of these compounds in the plasma after the average daily intake (50 mg) ranging up to 0,4 microns, i.e., about $0{,}03 \cdot 10^{-6}$ mg/mL of quercetin (Manach et al., 2005.).

Ehinacea purpurea

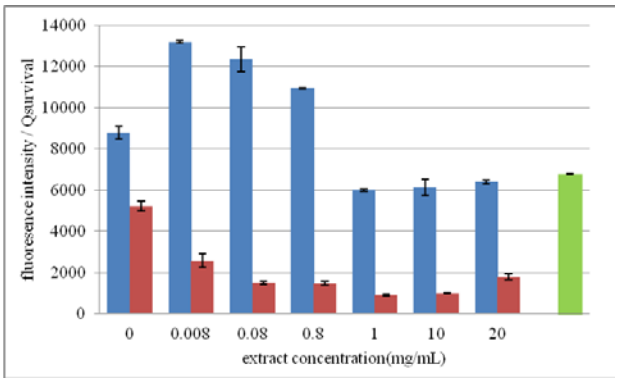

Figure 2. Oxidative stress determined by diclorofluoresceine test after treatment of SW480 cells with H_2O_2 and/or extract of purple Ehinacea after two-hour incubation

blue - cells treated with Echinacea extract (at concentrations of extract 0 only the medium was added) red - cells treated with H_2O_2 (at concentrations of extract 0 only the medium was added), green - cells exposed to a half-hour treatment with H2O2; * height of the column represents the mean of three determinations ± SD.

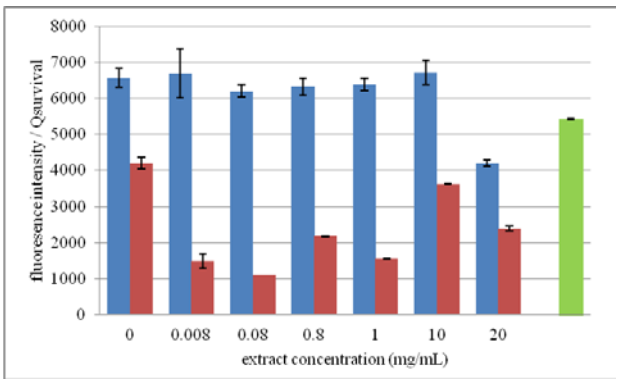

Figure 3. Oxidative stress determined by diclorofluoresceine test after treatment of SW480 cells with H_2O_2 and/or extract of *E. purpurea* after three hour incubation

blue - cells treated with Echinacea extract (at concentrations of extract 0 only the medium was added) red - cells treated with H_2O_2 (at concentrations of extract 0 only the medium was added), green - cells exposed to a half-hour treatment with H_2O_2; * height of the column represents the mean of three determinations ± SD.

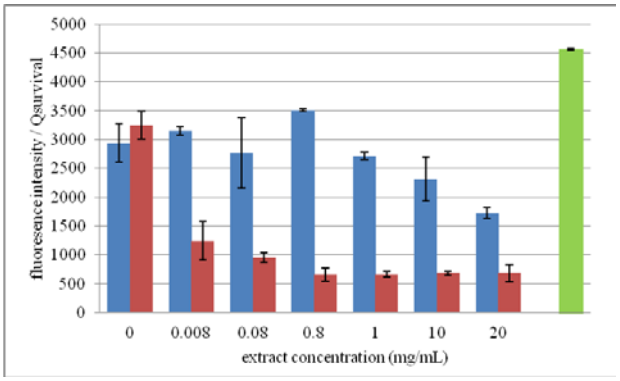

Figure 4. Oxidative stress determined by diclorofluoresceine test after treatment of SW480 cells with H_2O_2 and/or extract of *E. purpurea* after four hour of incubation

blue - cells treated with Echinacea extract (at concentrations of extract 0 only the medium was added) red - cells treated with H_2O_2 (at concentrations of extract 0 only the medium was added), green - cells exposed to a half-hour treatment with H_2O_2; * height of the column represents the mean of three determinations ± SD.

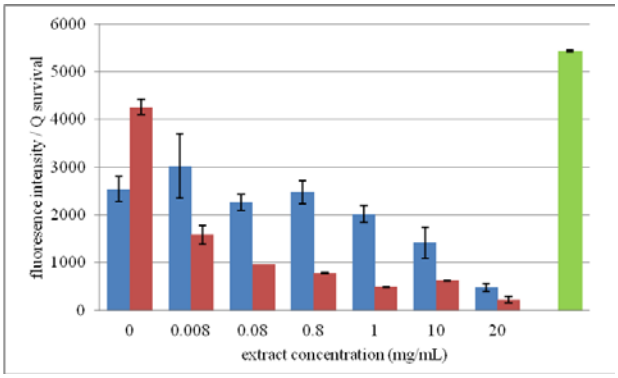

Figure 5. Oxidative stress determined by diclorofluoresceine test after treatment of SW480 cells with H_2O_2 and/or extract of *E. purpurea* with twenty four hours of incubation

blue - cells treated with Echinacea extract (at concentrations of extract 0 only the medium was added) red - cells treated with H_2O_2 (at concentrations of extract 0 only the medium was added), green - cells exposed to a half-hour treatment with H_2O_2; * height of the column represents the mean of three determinations ± SD.

Cytotoxicity Test

Determination of the cytotoxic effect of plant extracts on human colon carcinoma cells (SW 480)

Cytotoxicity of extracts of Echinacea herb was determined on a human cell line of colon cancer SW 480 in six different concentrations of the extract with different duration of treatment. Being that in the studied extracts of species *E. purpurea*, according to qualitative analysis, the presence of polyphenolic compounds that may help prevent cancer, including flavonoids, was noted, the antiproliferative effects of extracts are expected. Kuntz et al. (1999) found that polyphenols may play a significant role in preventing colon cancer by blocking the hyperproliferative epithelium by inducing apoptosis. The ability of a compound to inhibit the growth of tumor cells in a culture is an indication of its potential value as a therapeutic agent in vivo (Lieberman et al., 2001.). According to **Figure 6**, the Echinacea extract treatment for 2 and 3 hours shows a slight increase in the number of cells at the highest tested concentration. When treated for 3 and 4 hours, the extract showed no cytotoxicity effect to cells SW 480. After 24 hours of treatment a slight decrease in surviving cells as regard to concentration is observed.

Induction of apoptosis is important issue and it was suggested that the *in vitro* apoptotic activity should be used in the evaluation of potential phenolic phytochemicals in cancer prevention (Hsu et al., 2003b). Among the most important findings in the field of biology and genetics of cancer is that the genes that control apoptosis have the greatest effect on malignancy in case of disruption of their function, which can cause duplication of damaged cells, tumor initiation, progression and metastasis. Therefore, one of the mechanisms that prevent tumor production by use of natural products which are rich on different phytochemicals, could be the induction of apoptosis, which can be the basis for the prevention and therapy of cancer with biologically active compounds (Colic and Pavelic, 2000.). An increasing list of chemopreventive agents, including many food ingredients, for example, polyphenols, and synthetic derivatives, which have been shown to activate apoptosis of tumor cells *in vivo* and/or *in vitro*. Moreover, the vast majority of these agents induce mitochondrial - mediated apoptosis due to their prooxidant effects on transformed cells. These mechanisms of elimination of cells are non-specific. For example, tumor suppressors such as p53 use similar mechanisms for the eradication of damaged cells in order to maintain tissue homeostasis (Fridman and Lowe, 2003.; Halliwell, 2007.). Other researchers suggest that prevention of cancer by means of food rich in polyphenols may be due to their indirect antioxidant action. Frei and Higdon (2003.) were reviewing studies on the antioxidant activity of green tea and suggest that polyphenols can act indirectly as antioxidants, namely: a) inhibiting redox-sensitive transcription factors such as nuclear factor-kappaB and activator protein-1, b) inhibiting prooxidant enzymes such as inducible synthase nitric monooxides, lipoxygenase, cyclooxygenase, and xanthine oxidase, or c) inducing phase II enzymes and antioxidant enzymes such as GST and SOD. Such indirect antioxidant activity, which almost certainly could not be detected by conventional in vitro tests, could slow or stop cancer cell proliferation (Jia et al., 2002.; Hanif et al., 1997.). In recent decades, researchers and food manufacturers have shown great interest in the natural phenolic compounds. The main reason for this interest is their antioxidant activity, their representation in the human diet and their potential role in the prevention of various chronic diseases associated with oxidative stress. Consuming foods rich in natural antioxidants, as well as processed foods enriched with the same, ensure the desired supply of antioxidants and help prevent diseases in which oxidative stress is the key cause (Hardy, 2000.). Thus, natural sources of antioxidant polyphenols such as *E. purpuree* could be particularly significant and effective. Polyphenols are intentionally added to functional foods (Wyk and Wink, 2004; Buncova et al., 2008.; Sakač et al., 2005.; Huang et al., 2010.), and are often also found naturally in foods in quantities that have active effect on human health. Purple Echinacea herb extracts could serve as antioxidants that are added to prevent autooxidant food spoilage, but also as functional ingredients that can act antimutagenic and anticancerogenic and thus reduce the potential damage to the body.

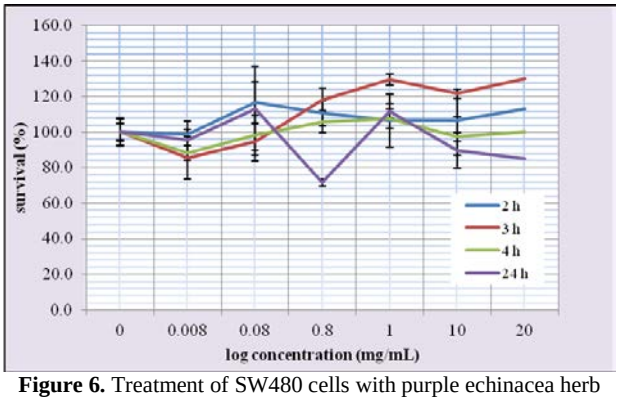

Figure 6. Treatment of SW480 cells with purple echinacea herb extract

CONCLUSIONS

The investigation of redox effect of Echinacea extract on cancer cells determined the prooxidant effect at lower concentrations and shorter duration of incubation. Higher concentrations and incubation of 4 hours or longer reduced the amount of ROS's in comparison to the control sample. Cells in which oxidative stress is fueled by hydrogen peroxide treatment showed a significant reduction in oxidative stress, which was particularly pronounced during longer incubation times. Based on the research of other authors, it can be assumed that with the time suppression of ROS`s had occured due to the induction of cellular antioxidant protection;

Treatment of cancer cells (SW480) with Echinacea extract for 2 and 3 hours showed a slight increase in the number of cells in the range of concentrations tested. After 24 hours of treatment a slight decrease in the number of surviving cells depending on a concentration was observed;

Due to a better understanding of the importance of polyphenols in the diet, it is necessary, beside bioavailability and mechanisms of action, to examine the possible synergism with other components present in food and the human body. Further investigations are required to identify a specific group of polyphenols or phenolic compounds that are most responsible for the positive effects on human health.

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Summary/Sažetak

U posljednjih nekoliko godina, postoji sve veći interes za prirodne i sigurnije antioksidanse. Do sada se malo zna o citotoksičnom i (anti) oksidativnom potencijalu echinacea (Echinacea purpurea) ekstrakata.

Metode: U cilju procijene antioksidativne aktivnosti ekstrakata, utvrđen je ukupan sadržaj fenola i ispiranje kapaciteta na DPPH radikalima. Sposobnost ekstrakata da izbace superoksid i hidroksilne radikale je testiran pomoću elektronske paramagnetske rezonancije (EPR) tehnike. Također, ekstrakti su prikazivani za citotoksičnost i antioksidativno/prooxidativni potencijal neutralnim crvenim i DCFDA testom, odnosno, koristeći liniju stanice ljudskog raka debelog crijeva SW480. Ćelije su izložene različitim koncentracijama ekstrakata (raspon: 0.008; 0.08; 0.8; 1, 10 i 20 mg/mL) i različito vrijeme tretmana (2, 3, 4 i 24 h).

Rezultati: Sadržaj ukupnih fenolnih jedinjenja ekstrakta E. purpurea je 10.57% GAE. U ispiranju aktivnosti radikala je utvrđeno da izlaže 50% od vrijednosti inhibicije (IC50 vrijednosti) u koncentraciji od 15.67 g/ml za ispitivane ekstrakt ehinaceae. Također, izračunata vrijednost 210 mg/ml za hidroksil i 76.7 mg/ml za superoksid anion radikale ukazuje na to da je ekstrakt Echinacea bogat antioksidans spojevima koji neutraliziraju radikale. U in vitro eksperimentima, ekstrakt ehinaceae pokazao prooxidativan učinak na nižim koncentracijama i kraći period inkubacije kada je korišten mobilni line SW480 kao test sistem. Najveća koncentracija bila je i najotrovnija što je posebno vidljivo nakon 24 sata tretmana.

Zaključci: Pokazalo se da Echinacea ekstrakt posjeduju jak antioksidans potencijal.