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Antioxidant Properties of Phenolics in *Castanea sativa* Mill. Extracts

Jelena Živković[1], Zoran Zeković[2], Ibrahim Mujić[3], Vesna Tumbas[2], Dragoljub Cvetković[2] and Ivan Spasojević[4]*

[1]Department of Pharmacy, Medical Faculty, University of Niš, Bulevar Zorana Đinđića 81, RS-18000 Niš, Serbia

[2]Faculty of Technology, University of Novi Sad, Bulevar Cara Lazara 1, RS-21000 Novi Sad, Serbia

[3]Collegium Fluminense Polytechnic of Rijeka, Trpimirova 2/V, HR-51000 Rijeka, Croatia

[4]Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1a, RS-11030 Belgrade, Serbia

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Summary

Using electron paramagnetic resonance (EPR) spectroscopy, antioxidant properties of chestnut extracts have been investigated as a source of phenolic compounds. In addition to their high antioxidant activities against hydroxyl and organic free (DPPH) radicals, phenolics showed to be potent protectors of membranes from lipid peroxidation. To the best of our knowledge, the ability of an antioxidant to overcome body's refractory response towards antioxidant supplementation has been examined for the first time. The water soluble extracts obtained from leaves, catkins, and outer brown peel of *Castanea sativa* Mill. showed high antioxidant activity in scavenging $^{\cdot}$OH and DPPH radical. All extracts, except for sweet chestnut catkins, showed the ability to protect liposomes from peroxidation. Phenolic compounds, as active antioxidants, have the ability to enter and protect cell membranes from lipid peroxidation, thus overcoming the body's refractory response to the antioxidant supplements in the diet. It is shown that phenolics are easily accessible natural antioxidants that can be used as food supplements or for the treatment of pathophysiological conditions related to oxidative stress.

Key words: *Castanea sativa* Mill., antioxidant activity, phenolic compounds, lipid peroxidation, refractory response

Introduction

Free radicals can provoke damage to proteins, nucleic acids, and membrane lipids. Elevated cellular levels of reactive oxygen species have been associated with many age-related health problems including neurodegenerative diseases, malignancy, arthrosclerosis, and heart diseases (*1*,*2*) as well as other pathophysiological conditions such as diabetes, ischemia/reperfusion injury (*1*), sepsis (*3*) and some complications during pregnancy (*4*).

While antioxidant supplements may compensate for dietary deficiencies, they have little impact on the underlying rate of aging and disease in people with well-balanced diet (*5*–*7*). Even more, the application of excessive amounts of antioxidants in everyday diet could lead to increased susceptibility to age-related diseases (*8*). On the other hand, the application of antioxidants in the treatment of pathophysiological conditions related with oxidative stress could have beneficial effects and prolong life (*8*). However, the difficulty is that the body acts to maintain flexible and responsive intracellular re-

*Corresponding author; Phone: ++381 18 511 359; Fax: ++381 18 226 644; E-mail: jelenazi2003@yahoo.com, jelenazi@med.fak.ni.ac.yu

dox poise, enabling a swift genetic response to stress. In other words, the body as a whole prevents the intracellular redox state from being 'swamped' by antioxidant supplements (*8,9*). For example, plasma vitamin C levels are tightly controlled by intestinal absorption and renal excretion, rendering megadose of oral vitamin C futile (*10*). The levels of other antioxidants, such as vitamin E, are also tightly regulated (*11*). Therefore, antioxidants able to overcome the refractory response of the organism have to be supplemented in order to equilibrate intracellular redox status under pathophysiological conditions related with increased generation of oxidizing reactive species. Such exigency led to the development of synthetic antioxidants with high antioxidant potential. For example, fullerene C_{60} was recognized as efficient free radical scavenger almost 20 years ago (*12*) and since then different amphiphilic derivatives have been developed (*13*). Their antioxidant properties and the ability to enter and protect cell membranes from lipid peroxidation were employed in the treatment of oxidative stress related conditions, such as neurodegenerative diseases (*14*). However, the application of fullerene derivatives seems to be limited due to their affinity to certain nucleic acids and proteins (*15*). Therefore, some more biocompatible amphiphilic antioxidants should be searched for in plants and other natural resources in order to meet the demands of application in the diet and treatment of oxidative stress-mediated conditions.

The focus of our study is on phenolics found in chestnut water soluble extracts, which could vanquish refractory response of the body by intercalating into cell membranes due to their amphiphilic character. Phenolic compounds in various plant products are well recognized as dietary antioxidants (*16–18*). It has been shown that phenolics possess a radical scavenging and metal chelating activity, as well as anti-carcinogenic properties (*19*). Plant phenols and polyphenols have been shown to inhibit lipid peroxidation and lipoxygenases *in vitro* (*20*). It had been shown previously that *Castanea sativa* Mill. leaves contain phenolic compounds (*21*), and that these compounds could represent efficient natural antioxidants (*22*).

In the present study, electron paramagnetic resonance (EPR) spectroscopy was used to determine the capacity of nine selected chestnut extracts to scavenge $^{\cdot}OH$, an exclusively damaging reactive species (*9*), and DPPH radical, a model compound for organic radicals in biosystems (*23*). Antioxidant capacity was correlated with total phenolics. The ability of extracts to prevent and/or remove lipid peroxidation was tested on liposomes exposed to $^{\cdot}OH$, which efficiently provokes lipid peroxidation. EPR spin-probing technique and the 7-doxyl stearate spin probe were used to assess membrane fluidity, the decrement of which is known to correlate with the increased lipid peroxidation (*24*). To the best of our knowledge, the ability of an antioxidant to overcome body's refractory response towards supplementation of antioxidants has been investigated for the first time.

Materials and Methods

Chemical reagents

Folin-Ciocalteu reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Gallic acid and (+)-catechin hydrate (Fluka A.G., Buchs, Switzerland) were used. Peroxylamine disulphonate (Fremy's salt), butylated hydroxyanisole (BHA), stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), and spin-trap 5,5-dimethyl-1-pyroline-*N*-oxide (DMPO) were obtained from Sigma, while 7-DS (2-(5-carboxypentyl)-2-undecyl--4,4-dimethyloxazolidine-3-oxyl) was purchased from Molecular Probes (Junction City, OR, USA). All other chemicals and solvents used were of the high analytical grade and obtained from commercial providers.

Plant material

Chestnut samples were collected from the area of the Una-Sana canton (Bosnia and Herzegovina) in 2006. Leaves, catkins, and outer brown peel were obtained from three chestnut cultivars: sweet chestnut, Lovran's Marrone cultivar, and grafted Italian Marrone cultivar. The samples were milled in laboratory homogenizer for further analysis. Table 1 shows the mean particle diameter of the investigated samples.

Table 1. Mean particle size and yield of dry extract of *Castanea sativa* Mill. cultivars

Sample	Mean particle size*	Yield of dry extract
	mm	%
Sweet chestnut		
Seed	0.27±0.01	7.09±0.09
Peeled seed	0.26±0.01	12.79±0.09
Outer brown peel	0.40±0.02	3.30±0.08
Leaf	0.18±0.01	4.94±0.04
Catkin	0.24±0.02	10.04±0.05
Lovran's Marrone cultivar		
Outer brown peel	0.31±0.01	7.13±0.03
Leaf	0.31±0.01	7.03±0.06
Grafted Italian Marrone cultivar		
Leaf	0.23±0.01	6.18±0.05
Catkin	0.34±0.01	10.58±0.08

*Each value in the table is the mean of three replications±standard deviation

Sample preparation

A volume of 250 mL of 50 % ethanol was added (the sample/solvent ratio was 1:5; by mass per volume) to 50 g of the sample. The extraction by ultrasound was performed (30 min at 50 Hz and 125 W). After the determination of mass and the addition of solvent, the liquid extract was obtained by filtration through Whatman Grade no. 4 filter paper. The aliquot of liquid extract was taken and the solvent was completely removed by evaporation under vacuum, at 40 °C. In this way, the dry extract of the investigated chestnut samples was obtained. The yield of dry extract was calculated (Table 1), and the samples were kept at 4 °C.

Quantification of the phenolics

Total phenolics were determined in dry extracts by Folin-Ciocalteu method (*25*). For the preparation of cali-

bration curve, 0.1-mL aliquots of 0.037, 0.072, 0.108, 0.144 and 0.180 mg/mL of gallic acid solutions in ethanol were mixed with 7.9 mL of H_2O, 0.5 mL of Folin-Ciocalteu reagent and 1.5 mL of 20 % sodium carbonate. Blind probe was the distilled water. The absorption was read after 2 h at 20 °C and 765 nm, and the calibration curve was drawn. A volume of 0.1 mL of methanol plant extract (1 mg/mL) was mixed with the same reagents as described above, and after 2 h the absorption was measured for the determination of plant phenolics. The content of total phenolic compounds in the investigated plant methanol extracts was expressed as g of gallic acid equivalents (GAE) per 100 g of dry extract sample (by mass).

Total flavonoid content was measured by the aluminium chloride colorimetric assay (*26*). An aliquot (1 mL) of 0.037, 0.074, 0.112, 0.149 or 0.186 mg/mL of methanol catechin solutions or methanol plant extracts (1 mg/mL) was added to a 10-mL volumetric flask containing 1 mL of H_2O. A volume of 0.3 mL of 5 % $NaNO_2$ was added to the flask. After 5 min, 0.3 mL of 10 % $AlCl_3$ were added. After 6 min, 2 mL of 1 M NaOH were added and total volume was made up to 10 mL with H_2O. The solution was mixed thoroughly and the absorbance was measured against the prepared blank at 510 nm. Total flavonoids were expressed as g of catechin equivalents (CE) per 100 g of dry extract sample (by mass).

Determination of antioxidant activity using EPR spectroscopy

DPPH radical scavenging assay

Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of chestnut extracts. DPPH was dissolved in methanol at a concentration of 0.4 mM. The solution was further dissolved in water to obtain the final concentration of DPPH of $11.8 \cdot 10^{-4}$ mM, which was used as a blank probe. The investigated extracts were added to the DPPH solution at the final concentration of 0.2 mg/mL. After that, the mixture was stirred for 2 min and transferred to a quartz flat cell ER-160FT. The EPR spectra were recorded at room temperature using EPR spectrometer Bruker 300E (Rheinstetten, Germany) using the following settings: field modulation 100 kHz, modulation amplitude 0.256 G, centre field 3440 Gauss (G), sweep width 100 G, power 20 mW. The antioxidant activity (AA) of the extract is defined in Eq. 1:

$$AA = 100 \cdot (h_0 - h_x)/h_0 \qquad /1/$$

where h_0 and h_x are the height of the second peak in the EPR spectrum of DPPH radicals of the blank and the probe, respectively.

Hydroxyl radical scavenging assay

Since hydroxyl free radical is highly reactive, with very short half-life (10^{-9} s), its concentrations found in natural systems can only be determined using EPR spin probe technique (*27*). In the present study, spin-trap DMPO was used to determine the effect of chestnut extract on the level of ·OH production through the Fenton system (*22*). Fenton reaction was performed using 0.2 mL of 10 mM H_2O_2, 0.2 mL of 10 mM Fe^{2+} and 0.2 mL of 0.8 M DMPO. The influence of different types of extracts on the formation of DMPO adduct of hydroxyl radical was investigated by adding the extracts diluted in water to the Fenton reaction system, at the final concentration of 0.2 mg/mL. EPR spectra were recorded after 5 min of incubation, with the following settings: field modulation 100 kHz, modulation amplitude 0.521 G, centre field 3440 G, sweep width 100 G, power 20 mW, at room temperature. The AA value of the extract was defined using Eq. 1.

EPR spin probe study of lipid peroxidation induced by Fenton reaction

For the preparation of liposomes, a chloroform solution of L-α-phosphatidylcholine (purchased from Sigma-Aldrich, USA) was evaporated under vacuum to dryness. After that, phosphate buffer (Na_2HPO_4 1.2 g/L, NaH_2PO_4 0.43 g/L, pH=7.4) was added to obtain the concentration of 125 mM of lipids, and the suspension was vortexed for 5 min. Extracts dissolved in buffer were added to a final concentration of 0.2 mg/mL (final concentration of lipids was 100 mM). In the control sample, an aliquot of the buffer was added. Samples were exposed to radical-generating system consisting of 0.5 mM H_2O_2 and 0.075 mM $FeSO_4$ for 20 min, after which 10 aliquots of the buffer were added in order to stop the reaction. Samples were centrifuged for 10 min at 10 000 rpm and the supernatant was discarded. Liposomes were again dissolved in the buffer, and the solution was applied to dried 7-DS (previously dissolved in methanol), and vortexed. Control samples were treated in the same manner, but without exposure to the radical-generating system. The ratio of spin probe/membrane lipid of approx. 1:200 was used (*28*). Order parameter (S) was calculated from spectral parameters as described in Fig. 1, which shows 7-DS spectra of untreated liposomes and liposomes exposed to Fenton system. S is reciprocally proportional to the membrane fluidity (*29*).

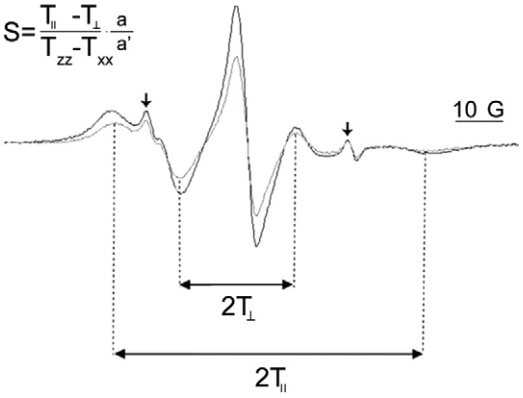

Fig. 1. EPR spectra of liposomes labelled with 7-DS (black line= liposomes treated with ·OH radicals, gray line=untreated liposomes); S=order parameter; $2T_{II}$=outer hyperfine splitting; $2T_{\perp}$= inner hyperfine splitting; a=isotropic hyperfine coupling constant in the crystal [$a=1/3(T_{xx}+T_{yy}+T_{zz})$]; a'=isotropic hyperfine coupling constant in the membrane [$a'=1/3(T_{II}+2T_{\perp})$]; Txx, Tyy, Tzz=hyperfine constants (for 7-DS, they were taken to be $T_{xx}=T_{yy}=6.1$ G, $T_{zz}=32.4$ G). Figure shows how $2T_{II}$ and $2T_{\perp}$ for untreated liposomes were measured. Two narrow lines (vertical arrows) originate from the 7-DS in the solution

EPR spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at X--band (9.51 GHz) with the following settings: modulation amplitude 2 G, modulation frequency 100 kHz, microwave power 10 mW, time constant 0.25 s, field centre 3390 G, scan range 100 G, scan time 8 min. Spectra were recorded using EW software (Scientific Software, Bloomington, IL, USA). Samples were drawn into 10-cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and internal diameter of 0.6 mm, Zeus industries, Raritan, USA). Measurements were performed using quartz capillaries in which Teflon tubes were placed.

Statistical analysis

Statistical analysis was carried out using Statistica v. 6.0 (StatSoft Inc, Tulsa, OK, USA). Pearson correlation test was conducted to determine the correlation among variables. Significant levels were defined at $p \leq 0.05$. All experiments were performed at least in triplicate. The results are presented as mean values±S.D.

Results

The yield of the dry extract of sweet chestnut (Table 1), expressed in % (by mass) was from (3.30±0.08) % for outer brown peel to (12.79±0.09) % for peeled seed.

Total phenolic content of chestnut extracts

Table 2 shows total phenolic (TP) and total flavonoid (TF) content of chestnut extracts. The highest content of total phenolic compounds ((4.24±0.02) % of GAE) and flavonoids ((2.41±0.06) % of GAE) were determined in dry extract of outer brown peel of Lovran's Marrone cultivar. The TF/TP ratio was from 11.86 for the extract of peeled chestnut to 56.83 % determined in the outer brown peel of Lovran's Marrone cultivar.

Polyphenols and flavonoids were found in the samples in the following order: outer brown peel>catkin> leaf>seed>peeled seed. In the investigation of extracts from chestnut flower, leaf, skin and fruit (*30*), similar order of polyphenolic content was also determinated.

Antioxidant capacity

DPPH radical scavenging activity

Reduction of stable nitrogen-centred free radical (DPPH) has been frequently used to evaluate natural antioxidants for their radical quenching capacities, since it represents a relatively fast assay when compared to other methods (*31*). The EPR spectrum of stable DPPH radical is easily recognized in blank probe by its five lines of relative intensities (1:2:3:2:1) and hyperfine coupling constant (a_N=9.03 G) (Fig. 2a).

In all the examined cases, there was no change in the shape of EPR spectra, but the relative intensity of EPR signals was reduced after the addition of *Castanea sativa* Mill. extracts. Catkin of sweet chestnut showed the highest AA values (Fig. 2b). The antioxidant activity (AA) of 0.2 mg/mL of water extracts of various cultivars and parts of *Castanea sativa* Mill. on DPPH radical is presented in Table 2.

Hydroxyl radical scavenging activity

The capacity of various extracts of *Castanea sativa* Mill. to inhibit hydroxyl radical generated by the Fenton reaction (Fe^{2+}/H_2O_2) was determined (Table 2). As shown in Fig. 3a, the reaction of Fe^{2+} and H_2O_2 in the presence of spin trapping agent DMPO generated a 1:2:2:1 quartet of lines in the EPR spectrum with the hyperfine coupling parameters (a_N and a_H=14.9 G).

The EPR spectra of DMPO/OH adducts formed in the presence of 0.2 mg/mL of water extract of the catkin of grafted Italian Marrone cultivar is shown in Fig. 3b. This extract showed the highest hydroxyl radical scavenging activity (AA=(59±2) %). Higher activity in scav-

Table 2. Total phenolic and total flavonoid contents (expressed as GAE) and antioxidant activity of extracts of *Castanea sativa* Mill. cultivars

Extract	TP	TF	$\left(\frac{TF}{TP}\right)\cdot 100$	AA(DPPH)	AA($^{\cdot}$OH)
	%	%	%	%	%
Sweet chestnut					
Seed	0.42±0.07	0.17±0.01	40.48	0	5.5±0.3
Peeled seed	0.59±0.03	0.070±0.003	11.86	0	0
Outer brown peel	1.2±0.1	0.65±0.02	54.62	21.4±0.2	21.8±0.9
Leaf	1.4±0.01	0.33±0.01	23.57	21.4±0.8	0
Catkin	3.3±0.2	0.60±0.03	18.30	38±1	43.6±1.7
Lovran's Marrone cultivar					
Outer brown peel	4.24±0.02	2.41±0.06	56.83	37±1	56±3
Leaf	2.42±0.06	0.61±0.02	25.52	15.5±0.1	5.5±0.1
Grafted Italian Marrone cultivar					
Leaf	1.71±0.07	0.42±0.04	24.56	30.0±0.8	48±1
Catkin	3.96±0.01	0.83±0.06	20.95	9.6±0.1	59±2

TP=total phenolic content, TF=total flavonoid content, AA=antioxidant activity against DPPH or $^{\cdot}$OH radical
Values are presented as mean±S.D.

enging ·OH relative to the removal of DPPH radical could be explained by iron chelating properties of tannins (*32*), which were also present in these extracts (data not shown).

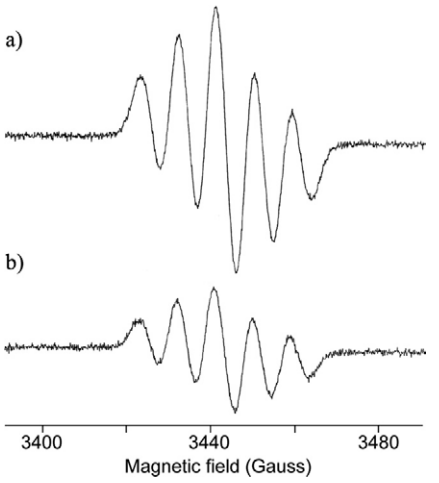

Fig. 2. EPR spectra of DPPH radical: (a) DPPH ($11.8 \cdot 10^{-4}$ mM) without the addition of extracts (blank), (b) DPPH+catkin extract of sweet chestnut (0.2 mg/mL) after 2-minute incubation period

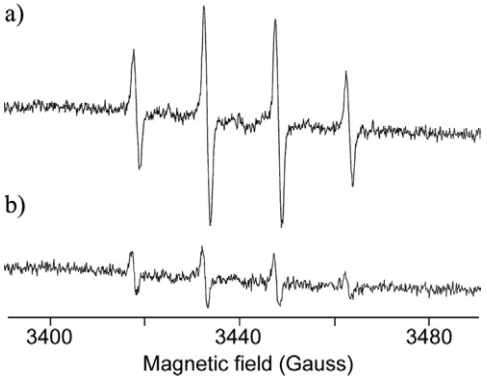

Fig. 3. EPR spectra of DMPO/OH spin adduct: (a) Fenton reaction (3.3 mM H_2O_2, 3.3 mM Fe^{2+}), (b) Fenton reaction+catkin extract of grafted Italian Marrone cultivar (0.2 mg/mL) after 5-minute incubation

Correlation between phenolic content and antioxidant activity

Significant linear correlations ($p<0.05$) were determined between AA(·OH) and total phenolic content (R= 0.80), AA(DPPH) and total phenolic content (R=0.60), AA(·OH) and total flavonoid content (R=0.63), as well as AA(·OH) and AA(DPPH) (R=0.67). The presented correlations show that phenolic compounds represent the principal basis for high antioxidant capacity of the examined chestnut extracts.

Capacity of chestnut extracts to remove lipid peroxidation

Reactive oxygen species can induce lipid peroxidation which can break down membrane integrity. The ability of chestnut extracts to prevent and/or remove lipid peroxidation of liposomes was tested using liposomes exposed to the Fenton reaction as producing system of ·OH, which efficiently provokes peroxidation. EPR spin probe technique and the membrane spin probe 7-DS were used to assess membrane fluidity, the decrement of which is known to correlate with the increased lipid peroxidation (*24*).

Table 3 shows the order parameters (S) of: (*i*) pure liposomes, (*ii*) liposomes exposed to the Fenton system, (*iii*) liposomes mixed with chestnut extracts, and (*iv*) liposomes mixed with chestnut extracts exposed to the Fenton system.

Table 3. Order parameter (S) of pure liposomes and liposomes mixed with extracts of *Castanea sativa* Mill. cultivars, evaluated using EPR and spin probe 7-DS. In the treated samples Fenton reaction was applied as a radical-generating system

	Sample	S
Control	Untreated liposomes	0.590±0.002
	Treated liposomes	0.609±0.004
Sweet chestnut		
Seed	Untreated	0.621±0.002
	Treated	0.619±0.010
Peeled seed	Untreated	0.595±0.004
	Treated	0.587±0.005
Outer brown peel	Untreated	0.613±0.003
	Treated	0.611±0.003
Leaf	Untreated	0.610±0.004
	Treated	0.604±0.003
Catkin	Untreated	0.590±0.004
	Treated	0.601±0.002
Lovran's Marrone cultivar		
Outer brown peel	Untreated	0.604±0.007
	Treated	0.603±0.009
Leaf	Untreated	0.592±0.003
	Treated	0.588±0.003
Grafted Italian Marrone cultivar		
Leaf	Untreated	0.593±0.002
	Treated	0.597±0.002
Catkin	Untreated	0.598±0.005
	Treated	0.606±0.003

Values are presented as mean±S.D.

The order parameter of liposomes without the added extract, exposed to ·OH generated in the Fenton system, was significantly increased. This shows that ·OH provoked lipid peroxidation (see also Fig. 1), which is known to lead to the decrease of membrane fluidity (which is reciprocal to S). In the presence of chestnut extracts, the order parameter of liposomes exposed to ·OH were not significantly different from the S of untreated liposomes mixed with the same extract. Only one extract showed no ability to prevent or remove lipid peroxidation (extract of catkin of sweet chestnut). Leaf extracts of

sweet chestnut and outer brown peel induced the decrease of the membrane fluidity in the untreated samples.

Discussion

The water soluble extracts obtained from leaves, catkin, and outer brown peel of *Castanea sativa* Mill. showed high antioxidant activity in scavenging ·OH and DPPH radical. Superoxide, hydrogen peroxide and other reactive species show damaging effects when present in excess, but also constructive biological functions, *e.g.* by participating in various signaling pathways, while hydroxyl radical is exclusively damaging due to its high reactivity (*1*). Thus, the activity of the chestnut extracts against ·OH could be beneficial in systems exposed to oxidative stress, such as cells and tissues under various pathophysiological conditions. Besides provoking direct damage, hydroxyl radical can oxidize biomolecules to produce large organic radicals. DPPH radical is one of the available model compounds for such radicals. Chestnut extracts showed high ability to scavenge DPPH radical. It is well accepted that the DPPH radical scavenging by antioxidants is attributable to their hydrogen donating ability (*33*), so active compounds from chestnut extracts should be able to reduce organic radicals generated during oxidative stress in biosystems. Antioxidant activities against ·OH and DPPH showed high positive correlation, which indicates that a specific group of compounds is involved in both variants of antioxidant activity.

Total content of phenolics in the chestnut extracts showed high correlation with determined antioxidant potential. In other words, antioxidant activities of the extracts were dependent on the phenolic content. Phenolics are well known for their antioxidant activity (*34*), so it seems that this group of biocompatible compounds (present in everyday diet) represents active antioxidant substances in chestnut extracts.

All extracts, except for sweet chestnut catkin, showed the ability to protect liposomes from peroxidation. The capacity of the extracts to protect liposomes from lipid peroxidation and their antioxidant activity against ·OH showed no correlation (R=0.04). This indicates that phenolics do not prevent membrane damage by removing ·OH from the solution, but most likely intercalate into the membranes to remove lipid peroxides. Fluidity of the cell membrane represents an important biological parameter; thereby one of the central biological activities of phenolics from the extracts could be to protect cell membranes from lipid peroxidation, which is related to the decrease of the fluidity.

Conclusions

To conclude, phenolics from the extracts of *Castanea sativa* Mill. showed high antioxidant capacity, as well as the ability to protect cellular membranes from oxidative damage by entering the lipid bilayer and removing lipid peroxidation. Intercalation of phenolics, as active antioxidants, into the cellular membrane could overcome body's refractory response known to occur towards supplementation of 'usual' antioxidants. These characteristics indicate that selected phenolic compounds of chestnut extracts could be used as easily accessible, natural antioxidants in the diet and treatment of pathophysiological conditions related to oxidative stress.

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