RADICAL SCAVENGING, ANTIMICROBIAL ACTIVITY AND PHENOLIC CONTENT OF CASTANEA SATIVA EXTRACTS

UKLANJANJE RADIKALA, ANTIMIKROBNA AKTIVNOST I SADRŽAJ FENOLNIH MATERIJA CASTANEA SATIVA EKSTRAKATA

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ABSTRACT

Radical Scavenging and antimicrobial activity of extracts of three cultivar of Castanea Sativa Mill. have been examined. Leaf and catkins have been extracted under the same conditions by 50% ethanol as extragens, afterwards dry extracts of examined samples have been gained. Total phenolic content was determined by HPLC, subsequent to preliminary acid hydrolysis of samples. For determination of extract influence to creating and transformation of hydroxide (OH) radicals and (DPPH) radicals, extracts have been dissolved in distillated water and added to Fenton's model system in concentration of 0.2 mg/ml. High value antioxidant activity (AA) in relation to hydroxyl radicals has turned out catkins from Lovran chestnut (59.09%). The greatest antiradical activity in relation to DPPH radicals occurred with extracts of catkins from domestic sweet chestnut (37.50). The antimicrobial tests have showed that ethanol extracts of leaf and catkins inhibited growth of Gram positive bacteria.

Key words: antioxidant activity, antimicrobial activity, Castanea sativa, extract, HPLC, EPR spectrometry

SAŽETAK

Ispitivano je uklanjanje radikala i antimikrobna aktivnost ekstrakata, tri kultivara Castanea Sativa Mill. Lišće i rese su ekstrahirane pod istim uvjetima 50%-tnim etanolom kao ekstragensom, a zatim su dobiveni suhi ekstrakti ispitivanih uzoraka. Nakon izvršene kisele hidrolize uzoraka ukupne fenolne tvari su određene HPLC-om. Radi određivanja efekta ekstrakata na stvaranje i transformiranje hidroksil (OH) radikala i (DPPH) radikala, ekstrakti su rastvoreni u destiliranoj vodi i dodani u Fenton-ov model sistem, do finalne koncentracije od 0,2 mg/ml. Visoka vrijednost antioksidativne aktivnosti (AA) u odnosu na hidroksil radikale određena je za resu Lovranskog kestena (59,09%). Najveća antiradikalna aktivnost u odnosu na DPPH radikale je utvrđena kod ekstrakta rese domaćeg pitomog kestena (37,50). Antimikrobni test je pokazao da etanolni ekstrakti rese i lista inhibiraju rast Gram pozitivnih bakterija.

Ključne riječi: antioksidativna aktivnost, antimikrobna aktivnost, Castanea sativa, ekstrakti, HPLC, EPR spektrometrija

INTRODUCTION

Oxidative stress may be involved in processes such as mutagenesis, carcionogenesis, lipid peroxidation, oxidation and fragmentation of proteins, as well as carbohydrate damage [16]. Protection of organisms against oxidative stress relies not only on endogenous antioxidants but also on exogenous compounds taken in as food [10]. Antioxidants delay or inhibit oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions [25]. The antioxidant effect is mainly due to phenolic components in food, such as flavonoids phenolic acids, and phenolic diterpenes [6].

Flavonoids are natural products present that are in fruits, vegetables, tea, and wine long recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities [13]. The flavonoids are typical phenolic compounds, and therefore, act as potent metal chelators and free radical scavengers [8]. They contain phenolic and polyphenolic groups (easily extractable H-atoms), which form a relatively stable phenoxy radical [3].

By investigating bioactive substances of plant origin, we have concentrated on chestnut (Castanea sativa Miller, Fagaceae) the edible fruit of which has become an important part of local diet. Chestnuts are an extraordinary horticultural opportunity for the edible nuts production and is also a source of timber and other forest products.

Among the 12 world chestnuts species, C. sativa is the most consumed [5]. In Europe, Turkey is the second main producer of chestnut. Monitoring agriculture in this country we can observed that 39% of total agricultural import refer to edible fruits and nuts. Most of these products have been exported from EU countries [21].

From a nutritional point of view, chestnuts are an important source of starch (up to 70%) but contain low amounts of protein (2–4%) and fat (2–5%) [23], some minerals and vitamins together with appreciable amounts of fibre [24]. Chestnuts can be processed and used for manufacturing a range of products including snacks, flakes, confectionery, pasta, purees and creams [19].

Romussi et al. (1981) reported that the cough suppressing activity of a leaf decoction of C.sativa was due to codeine. C. sativa leaf extract possesses a pronounced in vitro antibacterial effect [1]. So far, little is known about the antioxidant potential of chestnut fruit or other plant organs and tissue. Although phenolic compounds and flavonoids are considered to be nonnutritive agents generally, interest in these substances has arisen because of their possible effects on human health. This project investigated sweet chestnut extracts as potential new and valuable source of dietetic and pharmacological compounds.

MATERIALS AND METHODS

Plant material

Chestnut samples of grafted cultivar, were collected in the area of the Una in the Sana canton of Bosnia and Herzegovina from four chestnut-producing areas (Bužim, Velika Kladuša, Cazin and Bosanska Krupa) while another chestnut sample, the Istrian cultivar, was collected from a producing farm located in Lovran (region of Istria), in NW Croatia. The Istrian cultivar belongs to the commercial nuts of France and Italy and is called “marrone”; it is probably a hybrid of European and Asian lines. The “marrone” type of chestnut is larger and heavier than the “castagna” or “chestnut” type: the marroni are round, easily peeled, sweet, with good flavour and consistent texture [14]. Samples were dried in air (25^0 C in the dark) and analyzed within 3 months of collection. For further analysis samples were milled in a laboratory homogenizer to the mean particle size from (0.178±0.008mm) to (0.340±0.007 mm) (Table 1), determined (according to Ph. Jug. IV 1984.). After milling leaves and catkins were extracted using 50% ethanol. The leaf and catkin of sweet chestnut and grafted Italian ″marrone″ were examined, while for Lovran’s marrone cultivar leaf was included.

Chemical reagents

Folin-Ciocalteu reagent was purchased from Sigma Chemical Co. (St. Louis, MO, USA), and gallic acid and (+) catechin hydrate from Fluka A.G., grad, Germany). 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) of analytical reagent grade were obtained from Sigma. All other chemicals and solvents that were used were of the highest analytical grade, and obtained from commercial providers.

Sample preparation

The extraction (method A) and acid hydrolysys (method B) used for dried samples of leaf and catkin were as it follows: Method A - to 50.0 g of sample 250 ml of 50% ethanol was added (ratio sample: solvent was 1:5; w/v). The mixture was exposed to ultrasound for 30 min. After mass checking for eventually evaporation and possible solvent addition, the liquid extract has been obtained by filtration through Whatman

No. 4 filter paper. The solvent was removed by evaporation under the reduced pressure, at maximum temperature of 40 ºC. Hydrolysis of all glycosides to aglycons offers a practical method for the quantitative determination of

flavonoids in foods. The principle of acidic hydrolisys in method B: Fifty milliliters of sulfuric acid (pH = 3.0) were added to 5 g of dried sample and the extraction mixture was refluxed in a water bath at 90^0 C for 2 h. Extracts were filtered, and then 20 ml of filtrate was extracted with 20 ml (2 x 10 ml) ethyl acetate. The organic layer was collected and the solvent was completely removed by evaporation under vacuum at 37°C. The yield of dry extract was calculated and dried samples were kept at 4°C.

Determination of total phenolics compounds

The content of total phenolic compounds was determined in dry extracts by the Folin-Ciocalteu procedure [21, 7]. Absorbance was measured at 765 nm. Content of total phenolics compounds in the methanol extracts was expressed as g of gallic acid equivalents (GAE) per 100 g of dried sample (%; w/w), i.e. %GAE.

Analysis of total flavonoids

Total flavonoids content was measured by the aluminium chloride colorimetric assay [11]. Total flavonoids were expressed as g of catechin equivalents (CE) per 100 g of dried sample (%; w/w), i.e. %CE. Analyses were performed on a Hewlett Packard 8452 spectrophotometer.

DPPH radicals-scavenging assay

Reduction of the stable DPPH radical was used as a marker of antioxidant capacity of chestnut extracts. A blank probe was prepared by mixing 400 µl 0.4 mM methanolic solution of DPPH and 200 µl water. A volume of x µl of 1% aqueous extract solution was added to a mixture of (200−x) µl of methanol and 400 µl of 0.4 mM methanolic solution of the DPPH radical (probe).

The preparation of the DPPH radical was done in methanol to the final concentration of 1.8×10^{-4} mM. Extracts were added to the DPPH solution at the final concentration of 0.2 mg/ml. Then 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 an EPR Bruker 300E spectrometer (Rheinstetten, Germany) under the following conditions: field modulation, 100 kHz; modulation amplitude, 0.256 G; centre field, 3440 G; sweep width, 100 G; power 20mW. The antioxidant activity (AA) of the extract was defined as: $AA(\%) = 100 (h_0 - h_x)/ h_0$, where h_o 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

Hydroxyl radicals were obtained during a Fenton reaction using: 0.2 ml 10 mM H_2O_2, 0.2 ml 10 mM Fe^{2+}, and 0.2 ml 0.8 M DMPO. The influence of different types of extracts on the formation of a DMPO adduct of hydroxyl radical was investigated by adding the extracts, diluted in water in the Fenton reaction system, at a final concentration of 0.2 mg/ml. EPR spectra were recorded after a 5 min incubation, with the following spectrometer settings: field modulation, 100 kHz; modulation amplitude, 0.521 G; centre field, 3440 G; sweep width, 100 G; power 20mW; at room temperature. The antioxidant activity AA of extracts was defined as: $AA(\%) = 100 \cdot (h_o - h_x)/h_o$, where h_o and h_x are the height of the second peak in the EPR spectrum of DMPO-OH spin adduct of the blank and the probe, respectively.

Antimicrobial assay

Presence of an inhibition zone indicated activity extracts against the following bacteria or yeasts: Sarcina lutea (ATCC 9341), Staphylococcus aureus (ATCC 25923), Bacillus cereus (ATCC 10876) (all Gram-positive), Proteus mirabilis (ATCC 35659), Escherichia coli (ATCC 25922) (all Gram-negative), Saccharomyces cerevisiae (112, Hefebank Weihenstephan) and Candida albicans (ATCC 10231). Penicillin (10 IU/disc), amoxicillin (25 µg/disc), ketoconazole (10 µg/disc) and nystatin (100 IU/disc) were used as reference standards and obtained from Bioanalyse Co., Ltd., (Ankara, Turkey). In parallel with antimicrobial investigation of C. sativa extracts, pure solvent was tested; it showed no antimicrobial activity (data are not shown). Bacteria and yeasts were supplied from the stock cultures of the Microbiology Laboratory, Faculty of Technology University of Novi Sad.

The disc diffusion method was applied in this study. From a primary isolation medium 2-3 colonies of investigated microorganisms were taken by flamed loop and suspended in Mueller-Hinton (Difco, Detroit, MI, USA) or Sabouraud dextrose broth (Merck, Darmstadt, Germany). Incubation was at 37^0C (bacteria) or 25^0C (yeasts). The suspension for inoculation was prepared from broth cultures. The number of cells in 1 ml of suspension for inoculation measured by a McFarland nephelometer was $1x10^7$ cfu ml^{-1}. 1 ml of this suspension was homogenized with 9 ml of melted (45^0C) Mueller-Hinton or Sabouraud dextrose agar and poured into Petri dishes. For screening, sterile, 6mm discs (HiMedia, Mumbai, India) were impregnated with 10 µl of 10 mg ml^{-1} C. sativa extracts diluted in 30% ethanol. After incubation for 48 hours at 37^0C for bacteria or 25^0C for yeasts, inhibition zone diameters (ZI, including disc) were measured and expressed in mm to an accuracy of 0.1mm and the effect was calculated as a mean of triplicate tests.

HPLC analysis

HPLC was performed with a liquid chromatograph HP1090 (Hewlett-Packard Company, Avondale, PA, USA) with diode-array detector (DAD). A reversed-

Table 1: Mean particle size, yield of dry extract, total phenolic and flavonoid contents of extracts obtained as described in method A. Values are means ± S.D. (from 3 separate experiments)
Tablica 1: Srednji promjer čestica, prinos suhog ekstrakta, sadržaj ukupnih fenola i flavonoida ekstrakata dobijenih na način kako je objašnjeno u metodi A. Vrijednosti su predstavljene kao srednja vrednost ± standardna devijacija (za 3 eksperimeta)

Extract	Mean particle size (mm)	Yield of dry extract % (w/w)	Total phenolic content (%; w/w, i.e. % GAE)	Total flavonoid content (%; w/w, i.e. % CE)
Sweet chestnut				
Leaf	0.178 ± 0.008	4.940 ± 0.038	1.396 ± 0.011	0.325 ± 0.021
Catkin	0.237 ±0.023	10.04±0.046	3.278 ± 0.154	0.599 ± 0.031
Lovran's marrone cultivar				
Leaf	0.306 ± 0.007	7.030 ± 0.063	2.425 ± 0.056	0.613 ± 0.024
Grafted Italian ″marrone″ cultivar				
Leaf	0.227± 0.012	6.176 ± 0.045	1.713± 0.065	0.422 ± 0.031
Catkin	0.340 ± 0.007	10.580 ± 0.078	3.959 ± 0.012	2.190 ± 0.032

Table 2: Antioxidant activity of extracts obtained as described in method A
Tablica 2: Antioksidativna aktivnost ekstrakata dobijenih na način opisan u metodi A

Extracts	DPPH radical scavenging activity (%)	Hydroxyl radical scavenging activity (%)
Sweet chestnut		
Leaf	21.43 ± 0.78	0
Catkin	37.50 ± 0.96	43.64 ± 1.72
Lovran's marrone cultivar		
Leaf	15.45 ± 0.08	5.45 ± 0.07
Grafted Italian ″Marrone″ cultivar		
Leaf	29.96 ± 0.78	48.18 ± 1.32
Catkin	9.56± 0.09	59.09 ±1.78

phase column (Zorbax SB-C18, 5 µm, 3.0x250 mm i.d.), protected by guard column (Zorbax SB-C18, 5 µm, 4.6x12.5 mm i.d., Agilent, USA) was used. The detection was performed at 277 nm and the absorption spectra of the compounds were recorded between 210 and 400 nm. The solvent gradient was formed by varying the proportion of the solvent A (1% acetic acid in water, v/v) to the solvent B (acetonitrile) [9].

HPLC separations were performed by the following linear gradient: 0-10 min, 80 %A; 10-40 min, 60 %A; 40-80 min, 40 %A; 80-130 min 50 %A. The column was equilibrated to the initial conditions, 95%A, 10 min. Flow rate was set at 0.300 ml/min. The column was operated at room temperature (22 °C). The sample (solution of extracts and standard, rutin) injection volume was 10 µl and the injection was performed manually. Crude extracts of C. sativa were dissolved in the following mixture: methanol : water = 2 : 3 (v/v). All solutions were filtered through 0.45 µm pore size nylon filters (Rotilabo–Spritzenfilter 13 mm, Roth, Karlsruhe, Germany) before injecting them into the HPLC system.

RESULTS AND DISCUSION

The yield of dry extract of sweet chestnut (Table 1), expressed in % (w/w), i.e. g/100 g of a sample, was from 4.940 ± 0.038% for sweet chestnut leaves to 10.580 ± 0.078% for catkins of the grafted Italian ″marrone″ cultivar

Total phenolics content and total flavonoids

The highest content of total phenolic compounds (3.959 ± 0.012%GAE) occurred in leaf extracts of the grafted Italian ″marrone″ cultivar, while lowest content (1.396 ± 0.011 %GAE) was in leaf extracts of sweet chestnut, (Table 1). Total flavonoid content ranged from 0.325 ± 0.021%CE for sweet chestnut leaves, to 2.190 ± 0.032%CE for catkins from grafted Lovran Marrone.

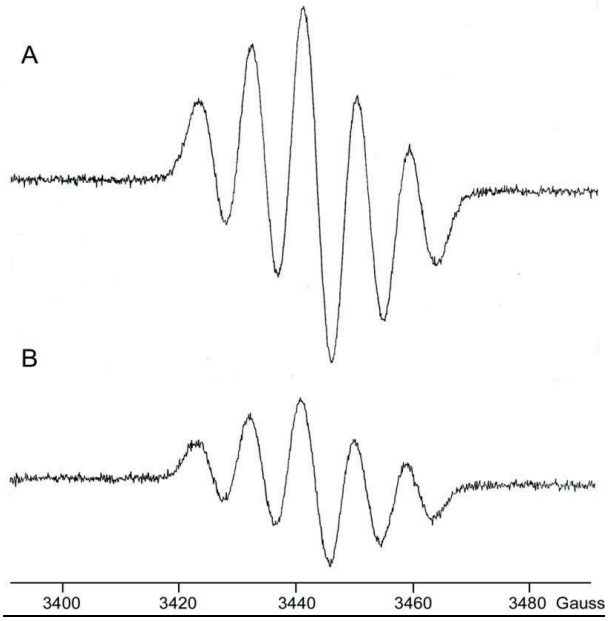

Fig 1a. EPR spectra of DPPH radicals with no addition of extracts (blank). The final concentration of DPPH radical was 11.8 x 10-4 mM

Fig 1b. EPR spectra of DPPH radicals the same as blank but with 0.2 mg/ml of catkin of sweet chestnut water extract.

Slika 1a:EPR spektar DPPH radikala bez dodavanja ekstrakta (slepa proba). Konačna koncentracija of DPPH radikala je 11,8 x 10-4 mM

Slika 1b:EPR spectra of DPPH radikala koji ima isti sastav kao i slepa proba ali mu je dodato i 0,2 mg/ml rese pitomog kestena rastvorenog u vodi.

DPPH radicals scavenging activity

Stable DPPH radical was used to investigate antiradical activity in C. sativa. The EPR spectrum of stable DPPH radical is easily recognized in a blank probe by its five lines of relative intesities 1:2:3:2:1 and hyperfine splitting constant a_N = 9.03 G (Fig.1a).

In all cases examined, there was no change in shape of the EPR spectra with added C. sativa .extracts, rather the relative intesity of EPR signals was reduced (Fig.1b). The antiradical activity (AA) of 0.2 mg/ml aqueous extracts of various cultivar and parts of C. sativa on DPPH radical indicated that highest AA values (37.50 ± 0.96%) occurred in catkins of sweet chestnut as did total phenolic and flavonoid content (Table 2).

There appeared to be more antioxidant components present in these extracts, which could promptly react with DPPH radicals, than in extracts from other tissues. Catkins of grafted Italian ″marrone″ cultivar showed the lowest value, AA = 9.56 ± 0.09 %. Phenolic compounds in plants are powerful in vitro antioxidants because of their ability to donate hydrogen or electrons and to form stable radical intermediates [20].

Hydroxyl radicals scavenging activity

The capacity of various extracts of C. sativa extracts to inhibit hydroxyl radical generated by the Fenton reaction (Fe^{2+}/H_2O_2) varied among tissues with catkin (59.09 ±1.78%) and leaf (48.18 ± 1.32%) tissue of grafted Italian marrone cultivar showed the highest AA values. Catkins of grafted Italian Marrone had significant total phenolic and flavonoid content (Table 1).

Antimicrobial Activity

Extracts of C. sativa showed strong antibacterial activity against Staphylococcus aureus, one of the most common Gram-positive bacteria causing food poisoning. Rauha et al., (2000) showed that the growth of S. aureus was inhibited very effectively by flavone, flavonols and naringenin, which indicates that flavonoides in chestnut extracts may be the active antimicrobial substances. Extracts were also active against the Gram-positive bacteria Sarcina lutea and Bacillus cereus Catkin and leaf extracts of all cultivars had high antimicrobial activity. Tannins in general are thought to provide chemical defense for plants against pathogens and herbivores, and these compounds can exert detrimental effects in the multitude of ways [2].

Not all 30% ethanol extracts inhibited growth of Escherichia coli and the two yeasts tested - Saccharomyces cerevisiae and Candida albicans (Table 3).

The strain of E. coli used showed the lowest sensitivity to the antibiotics used as reference standards: amoxicillin (21.33±1.15mm) and penicillin (10±0mm). This lack of activity of extracts on the pathogenic bacteria E.coli may have resulted from an inherent high resistance of this strain towards antibiotics in general and therefore on tested extracts in particular.

HPLC of extracts

HPLC techniques are widely used for both separation and quantitation of phenolic compounds. Quantitative determination of individual flavonoid glycosides in food is difficult because most reference compounds are not commercially available. Furthermore, more than 50 different glycosides of the most common flavonoids have been described [4]. Hydrolysis of all glycosides to aglycons offers a practical method for the quantitative determination of flavonoids in food [12]. Using Method B (in sample preparation) acid hydrolysis was used to prepare extracts for HPLC analysis.

Table 3: Antimicrobial activity of *Castanea sativa* extracts obtained as described in method A and reference standards
Table 3: Antimikrobna aktivnost ekstrakata dobijenih po metodi A i referentnih standarda

Extracts	Microorganisms			
	Staphylococcus aureus	*Sarcina lutea*	*Bacillus cereus*	*Proteus mirabilis*
	ZI	ZI	ZI	ZI
Sweet chestnut				
Leaf	14 ± 4.89	11 ± 1	10 ± 0	12 ± 0
Catkin	15.33 ± 0.58	13.66 ± 0.58	10 ± 0	11.33 ± 0.47
Lovran's marrone cultivar				
Leaf	16 ± 3.46	13 ± 1	10.33 ± 0.58	12.66 ± 0.47
Grafted Italian ″marrone″ cultivar				
Leaf	14 ± 1.73	9 ± 1	9.67 ± 0.58	11.33 ± 0.47
Catkin	16.67 ± 0.58	15.66 ± 0.58	9.33 ± 0.58	12 ± 0
Standards	**Microorganisms**			
	S. aureus	*S. lutea*	*B. cereus*	*P. mirabilis*
	ZI	ZI	ZI	ZI
Amoxicillin	27.3 ± 1.15	55.0 ± 1.0	29.0 ± 0.0	26.33 ± 0.57
Penicillin	30.3 ± 2.25	37.7 ± 0.49	34.0 ± 0.0	34.33 ± 0.57

Table 4: Mean particle size, total phenolics contents and flavonoids contents of extract of *Castanea sativa* obtained as described in method B
Tablica 4: Srednji promjer čestica, prinos suhog ekstrakta, sadržaj ukupnih fenola i flavonoida ekstrakata dobijenih na način dat u metodi B

Extracts	Mean particle size (mm)	Total Phenolics (HPLC) (%; w/w, i.e. % rutin)	Total Phenolics (%; w/w, i.e. % GAE)	Total Flavonoides (%; w/w, i.e. % CE)
Sweet chestnut				
Leaf	0.27	0.065	0.105	0.024
Catkin	0.237	0.083	0.077	0.082
Lovran's marrone cultivar				
Leaf	0.42	0.057	0.116	0.038
Grafted Italian ″marrone″ cultivar				
Leaf	0.396	0.041	0.061	0.015
Catkin	0.34	0.021	0.074	0.014

In Table 4 was given results of amount of the total phenolics in the extracts. The highest content of total phenolic compounds determined by HPLC and spectrophotometrically according to the Folin-Ciocalteau method, occurred in dry extract of catkins from the grafted Italian ″marrone″ cultivar (0.021 %rutin), while the lowest content (0.083 %rutin) for catkins was obtained from sweet chestnut.

CONCLUSIONS

All extracts evaluated from the different chestnut sources could be used as protective reagents against oxidative stress provoked by DPPH and hydroxyl radicals. Extracts also contained compounds with high antimicrobiological activity. The extracts of C. sativa leaves and catkins may be a potential source of natural antioxidants for use as a food supplement or for use in pharmaceutical industry.

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