Scavenging Capacity of Superoxide Radical and Screening of Antimicrobial Activity of *Castanea sativa* Mill. Extracts

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Abstract

Živković J., Zeković Z., Mujić I., Vidović S., Cvetković D., Lepojević Ž., Nikolić G., Trutić N. (2010): **Scavenging capacity of superoxide radical and screening of antimicrobial activity of** *Castanea sativa* Mill**. extracts**. Czech J. Food Sci., **28**: 61–68.

The superoxide radical scavenging and antimicrobial activity were examined of sweet chestnuts: seeds, peeled chestnut, brown seed coat, leaves, catkin, spiny burs, and chestnut bark as well as of Lovran's marrone leaves and grafted Italian marrone cultivar. Parts of chestnut were extracted under the same conditions with 50% ethanol as extractant and afterwards the dry extracts of the examined samples were obtained. The total phenolics and flavonoids contents were determined using standard spectrophotometrical methods. The capacity of the investigated extracts to remove radicals was evaluated by EPR method. The most efficient in scavenging $^{\bullet}O_2^-$ radicals proved to be the extracts of leaves of grafted Italian marrone (RI = 86%) and of Lovran's marrone cultivar (RI = 80%). Catkin, leaves, chestnut bark, and spiny burs extracts demonstrated the highest antimicrobial activity. Very significant and significant correlations were established between the antimicrobial activity of extracts and $^{\bullet}O_2^-$ radicals scavenging in all samples examined. The extracts of *Castanea Sativa* Mill. are important sources of components active in reducing the level of oxidative stress.

Keywords: antimicrobial activity; antioxidant; superoxide radical; sweet chestnut; total flavonoids; total phenolics

Reactive oxygen species (ROS), including superoxide radicals ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), and singlet oxygen ($^1O_2$), are generated as byproducts of normal metabolism (Wang & Jiao 2000). Both positive and negative biological functions are ascribed to superoxide ($^{\bullet}O_2^-$), while the hydroxyl radical ($^{\bullet}OH$) is exclusively negative due to its high reactivity (Halliwell & Gutteridge 1999). The food-derived antioxidants such as vitamins and phenolic phytochemicals have received growing attention because they are known to function as chemopreventive agents against oxidative damage. To counteract the deleterious effects of ROS, phenolic compounds, naturally distributed in plants, are effective (Chung 1998). They are also powerful reducing agents (Schanderl 1970).

Polyphenols have many favourable effects on human health, such as the inhibition of the low density proteins oxidisation (Frankel *et al.* 1993), thereby decreasing the heart disease risks (Williams & Elliot 1997). They also have anti-inflammatory activity and anti-carcinogenic properties (Miyake *et al.* 1999). On the other hand, the activity of these compounds as food lipid antioxidants is well known (Rice-Evans *et al.* 1997), and this fact

promoted studies of extracts from different plants containing them. Polyphenols are considered to be plant chemical defenses against pathogens and herbivores, and these compounds can exert detrimental effects in a multitude of ways (Bernays *et al.* 1989).

The resistance to antimicrobial agents has become an increasingly important and pressing global problem. For example, methicillin-resistant *Staphylococcus aureus,* which was present at low levels a decade ago, now accounts for 50% of all *S. aureus* isolates (Cushnie & Lamb 2005). Microorganisms have unfavourable effects on the quality, safety, and shelf life of foods. Synthetic antioxidants are widely used in the food industry, however, they also possess toxic and carcinogenic effects. Thereby, the interest in finding natural antioxidants, without undesirable side effects, has greatly increased (Baydar *et al.* 2004). Generally, it is accepted that phytochemicals are less potent anti-infective than the agents of microbial origin, i.e. antibiotics (Yamada 1991).

Chestnuts can be: used as a vegetable, steamed, boiled, puréed, used in stuffing for meat, used as a main component of soups, ground into flour for confectionery use. The wood is used for timber, paper, or fuel. Although it has already been demonstrated that chestnut fruits (Ribeiro *et al.* 2007) and leaves (Romussi *et al.* 1981) contain phenolic compounds, little is known about their antioxidant potential. It was recently demonstrated that *C. sativa* leaves extract possesses a pronounced *in vitro* antibacterial effect (Basile *et al.* 2000).

So far, little is known about the possible application of chestnut in diet and therapy. With the intention to find new natural sources of active compounds, we studied the ability of different extracts of *Castanea sativa* Miller to scavenge superoxide ($^{\bullet}O_2^-$) radicals, and also their antimicrobial activities.

MATERIAL AND METHODS

Chemicals. Ethanol and methanol were obtained from Zorka Šabac (Serbia). Folin-Ciocalteu reagent was purchased from Sigma Chemical Co. (St. Louis, USA). Gallic acid and (+) catechin hydrate were obtained from Fluka AG, Chemische Fabric (St. Gallen, Switzerland). DEPMPO was purchased from Alexis Biochemical (Lausen, Switzerland). Hypoxantine and xantine oxidase were obtained from Sigma Chemicals Co. (St Louis, USA). Other chemicals and solvents used were of the highest analytical grade. Spectrophotometrical measuring was done using spectrophotometer Hewlett Packard 8452. For the extraction, the ultrasonic bathroom Branson model b-220 SmithKline Co., Shelton, USA (50/60 Hz, 125 W) was used. The EPR spectra were recorded on Varian E104-A EPR (Pal Alto, USA) spectrometer.

Sample preparation. Chestnut samples were collected in the area of the Una-Sana canton (B&H), in 2006. The investigation included three most predominant cultivars: sweet chestnut, Lovran's marrone, and grafted Italian marrone cultivar. Scan investigations were done with sweet chestnut, while with the other two cultivars only leaves were collected. There are many different types of chestnut, varying in shape and colour of fruit. One of them is "Lovran's marun" which is appreciated for the high quality of its fruit. The word "marron" usually signifies a large sort of domestic chestnut obtained by inoculation. In Italy, marron means a particular *Castanea sativa* cultivar of excellent quality.

The fruits were harvested in the chestnut ripening season, between the middle of September and the end of October. The results given for the seed refer to milled seed without burs. Separate parts of seed were investigated, like peeled chestnut (hand-peeled) and brown seed coat. In addition, leaves, catkin, spiny burs, and bark of tree were analysed.

The samples were milled for the analysis in a laboratory homogenisator. The mean particle diameter of the investigated samples was determined. To 50 g of the sample, 250 ml of 50% ethanol was added (sample solvent ratio was 1:5; w/v). The extraction was carried out using ultrasound (30 min). After the mass checking and possible solvent addition, the liquid extract was obtained by filtration through Whatman Grade #4 filter paper. A defined volume of the liquid extract was taken and the solvent was completely removed by evaporation under vacuum at the temperature of 40°C. In this way, dry extracts of the investigated chestnut samples were obtained, and the yields of the dry extracts were calculated. All dry extract samples were kept in the fridge.

Total phenolics. Total phenolics were determined in the dry extracts by Folin-Ciocalteu procedure (Singleton & Rossi 1965; Kähkönen *et al.* 1999). For the preparation of the calibration curve, 0.1 ml aliquots of 0.037, 0.072, 0.108, 0.144, and

0.180 mg/ml of ethanolic gallic acid solutions were mixed with 7.9 ml of H_2O, 0.5 ml of Folin-Ciocaleu reagent, and 1.5 ml of 20% sodium carbonate. The blank was distilled water instead of 0.1 ml of the investigated sample. The absorption was read after 2 h at the temperature of 20°C at 765 nm and the calibration curve was constructed. 0.1 ml of the methanolic 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 phenolics compounds in the investigated plant methanolic extracts was expressed as g of gallic acid equivalents (GAE) per 100 g of the dry extract sample (%; w/w), i.e. % GAE.

Total flavonoids. Total flavonoids content was measured by means of the aluminium chloride colorimetric assay (Markham 1989). An aliquot (1 ml) of 0.037, 0.074, 0.112, 0.149, 0.186 mg/ml methanolic catechin solutions or methanolic plant extracts (1 mg/ml) was added into 10 ml volumetric flask containing 1 ml of H_2O. Then 0.3 ml of 5% $NaNO_2$ was added and after 5 min, 0.3 ml of 10% $AlCl_3$, was added. At 6^{th} min, 2 ml of 1M NaOH was added and the total volume was made up to 10 ml with H_2O. The solution was well mixed 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 the dry extract sample (%; w/w), i.e. % CE.

Scavenging of $^{\bullet}O_2^-$ radical. The ability of *Castanea sativa* Mill. extracts to scavenge superoxide radicals was tested using Hypoxantine/Xantine oxidase (HX/XO) reaction as an "$^{\bullet}O_2^-$ producing system".

The capacity of the investigated extracts to remove radicals is evaluated by the difference between the amplitudes of the EPR signals of the trapped radicals in radical-generating systems, with or without addition of the investigated extract. The results are presented as the relative inhibition (RI), which represents the relative decrease of the radical production:

RI = 100 × [peak amplitude (radical-generating system) – peak amplitude (radical-generating system + extract)]/[peak intensity (radical-generating system)]

Superoxide radicals were generated in a hypoxanthine/xanthine-oxidase system (HX/XO) consisting of hypoxanthine – 1.6mM, and xanthine oxidase – 1.6 IU/ml (Sigma-Aldrich, St. Louis, USA) dissolved in bidistilled 18MΩ deionised water. The final concentration of DEPMPO was 28mM. DEPMPO reacts with $^{\bullet}O_2^-$ to form DEPMPO/OOH adducts (Figure 1). The final concentration of the extracts was 0.2 mg/ml. The incubation time was 2 minutes. The samples with no extract served as controls. The final concentration of the extracts (previously dissolved in water) was 0.2 mg/ml.

EPR spectroscopy. 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 0.032 s; field centre 3410 G; scan range 200 G. The spectra were recorded using EW software (Scientific Software, Bloomington, USA). The samples were drawn into 10 cm long gas-permeable Teflon tubes (wall thickness 0.025 mm and internal diameter 0.6 mm; Zeus Industries, Raritan, USA). The measurements were performed using quartz capillaries in which Teflon tubes were placed. The recordings proceded 2 min after the beginning of the reaction, with the recording time of 4 minutes.

Antimicrobial activity. The disc-diffusion method was used as a screening test for antibacterial activity. Filter paper discs (6 mm in diameter) impregnated with sample solutions were placed on Mueller Hinton agar plates (Difco, Detroit, USA), which had been inoculated with the test organisms. From the primary isolation medium, 2–3 colonies of the investigated microorganisms were taken with a flamed loop, suspended in Mueller Hinton broth (Merck, Darmstadt, Germany), and subsequently incubated at 37°C. The suspension for both inoculations was prepared from the broth cultures. The number of cells in 1 ml of suspension for inoculation measured with a McFarland nephelometer was 1×10^7 CFU/ml. 1 ml of this suspension was homogenised with 9 ml of melted (45°C) Mueller Hinton poured into Petri dishes. For screening, sterile 6 mm discs (HiMedia®, Mumbai, India) were impregnated with 10 µl of 10 mg/ml of *C. sativa* Mill. extracts diluted in 30% ethanol. After incubation for 48 h in a thermostat at 37°C, the inhibition zone diameters (ZI, including disc) were measured and expressed in mm with 0.1 mm accuracy and the effect was calculated as a mean of triplicate tests. The presence of the inhibition zone indicated the activity of the tested extracts against bacteria: *Sarcina lutea* (ATCC 9341), *Staphylococcus aureus* (ATCC

25923), *Bacillus cereus* (ATCC 10876), *Lactococcus lactis* ssp. *lactis* (B-4449), *Micrococcus pyrogenes* var. *albus* (isolated from natural environment) (all Gram-positive), *Proteus mirabilis* (ATCC 35659 Gram-negative), and *Salmonella typhimuri* (ATCC 14028 Gram-negative). Penicillin (10 units/disc) and amoxicillin (25 µg/disc) obtained from Bioanalyse Co., Ltd. (Ankara, Turkey) were used as reference standards. In parallel with the antimicrobial investigation of *C. sativa* extracts, pure solvent was also tested, however, it did not exhibit any antimicrobial activity (data not shown). The bacteria were obtained from the stock cultures of Microbiology Laboratory, Faculty of Technology, University of Novi Sad.

Statistical analysis. All experiments were performed at least in triplicates. The results are presented as the mean values ± SD. Statistical analysis was carried out using Statistica 6.0 (StatSoft Inc, Tulsa, USA). Pearson correlation test was conducted to determine the correlations between the variables. Significant levels were defined at $P \leq 0.05$.

RESULTS AND DISCUSSION

The yields of dry extracts of sweet chestnut (Table 1), expressed in % (w/w), i.e. g/100 g of a sample, ranged from 1.82% for spiny burs to 12.79% for peeled sweet chestnut.

The highest content of total phenolic compounds (3.28% GAE) was found in the extracts of sweet chestnut catkin, while the lowest one (0.42% GAE) was obtained with sweet chestnut seeds. Total flavonoid content ranged from 0.09% CE in peeled sweet chestnut to 0.69% CE in chestnut bark of sweet chestnut.

The parts of chestnut with the highest content of polyphenols included catkin, chestnut bark, leaves, and brown seed coat. The lowest content of polyphenols was detected in seeds and peeled chestnut, because chestnut is fruit rich in carbohydrates (especially starch) and low in the fat content (Ertürk *et al.* 2006). Chestnuts have an average starch content of 22.3 g/100 g of raw edible portion. This fact places chestnut fruit among the main sources of starch (Pizzoferrato *et al.* 1999).

The ability of the chestnut extracts to scavenge radicals was tested using EPR spin-trapping technique. This technique enables the detection of different ROS species by EPR spectroscopy qualitatively (different species can be distinguished) and with greater sensitivity (Bačić & Mojović 2005).

One of the most commonly used spin traps, 5,5-dimethylpyrroline-N-oxide (DMPO), is the most versatile and commonly used spin trap for measuring and identifying oxygen radicals. However, it has several fundamental limitations.

Table 1. The yield of dry extract, total phenolics and total flavonoids contents

Extracts	Yield of dry extract (% w/w)	Total phenolics content (% expressed as GAE)	Total flavonoids content (% expressed as CE)
Sweet chestnut			
Seeds	7.09 ± 0.091	0.42 ± 0.067	0.17 ± 0.008
Peeled chestnut	12.79 ± 0.092	0.59 ± 0.029	0.09 ± 0.003
Brown seed coat	3.30 ± 0.083	1.19 ± 0.126	0.65 ± 0,021
Catkin	10.04 ± 0.046	3.28 ± 0.154	0.60 ± 0.031
Leaf	4.94 ± 0.038	1.40 ± 0.011	0.26 ± 0.008
Chestnut bark	3.40 ± 0.089	1.70 ± 0.097	0.69 ± 0.056
Spiny burs	1.82 ± 0.066	0.49 ± 0.023	0.13 ± 0.017
Lovran's marrone			
Leaf	7.03 ± 0.063	2.43 ± 0.056	0.61 ± 0.024
Grafted Italian marrone			
Leaf	6.18 ± 0.045	1.71 ± 0.065	0.42 ± 0.039

Table 2. RI of $^{\bullet}O_2^-$ radical production in the X/XO system induced by *C. sativa* extracts

Extracts	RI (%)
Sweet chestnut	
Seeds	67 ± 0.05
Peeled chestnut	54 ± 0.01
Brown seed coat	70 ± 0.03
Catkin	70 ± 0.04
Leaf	73 ± 0.06
Chestnut bark	77 ± 0.07
Spiny burs	79 ± 0.03
Lovran's marrone	
Leaf	80 ± 0.03
Grafted Italian marrone	
Leaf	86 ± 0.04

One major limitation is the short half-life of the DMPO-superoxide adduct, DMPO-OOH, in aqueous biological media. The half life of DMPO-OOH in buffer solutions at pH 7 is approximately 50 s (Roubaud *et al.* 1997). On the other hand, the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) is very efficient for the detection of $^{\bullet}O_2^-$. It forms a superoxide adduct with a half-life of almost 15 min while DEPMPO/$^{\bullet}$OOH is about 10 times higher compared to the respective DMPO adduct (Buettner 1978). In comparison to DMPO, DEPMPO is suitable for a more accurate detection of $^{\bullet}O_2^-$. The amount of oxygen within the investigated system must also be taken into account when dealing with oxygen consuming systems (Bačić *et al.* 2008). When, experiments with the systems consuming oxygen are performed in quartz capillaries or flat cells, both the production of oxygen radical species and the rate of conversion of DEPMPO adducts vary with the changes of pO_2, thus making the observed system very difficult to analyse (Bačić & Mojović 2005). Since the adduct conversion appears to be rather slow compared to the radical generation, DEPMPO spin-trap can be efficiently used for the detection of oxygen-centred radicals produced by the systems *in vivo* (Mojović *et al.* 2005).

Figure 1 shows a characteristic EPR signal obtained in the X/XO system using DEPMPO. Table 2 shows that all the extracts investigated exhibited a significant capacity for $^{\bullet}O_2^-$ radicals scavenging.

The most efficient in $^{\bullet}O_2^-$ radicals scavenging proved to be the extracts of leaves of grafted Italian marrone (RI = 86%) and Lovran's marrone cultivar (RI = 80%). A high antioxidant activity was also expressed by the extract of spiny burs (RI = 78%). Those extracts possessed high contents of total phenolics and total flavonoids (Table 1). For the investigated extracts, the following order of the strength to scavenge $^{\bullet}O_2^-$ radical was obtained: leaves > catkin > spiny burs > chestnut bark > brown seed coat > seeds > peeled chestnut.

Unlike the synthetic antioxidants, which are phenolic compounds with varying degrees of alkyl substitution, the natural antioxidants can be phenolic compounds (flavonoids, phenolic acids, and tannins), nitrogen-containing compounds, carotenoids, tocopherols, or ascorbic acid and its derivatives (Velioglu *et al.* 1998).

In out previous research (Živković *et al.* 2008), we investigated the capacity of sweet chestnut ex-

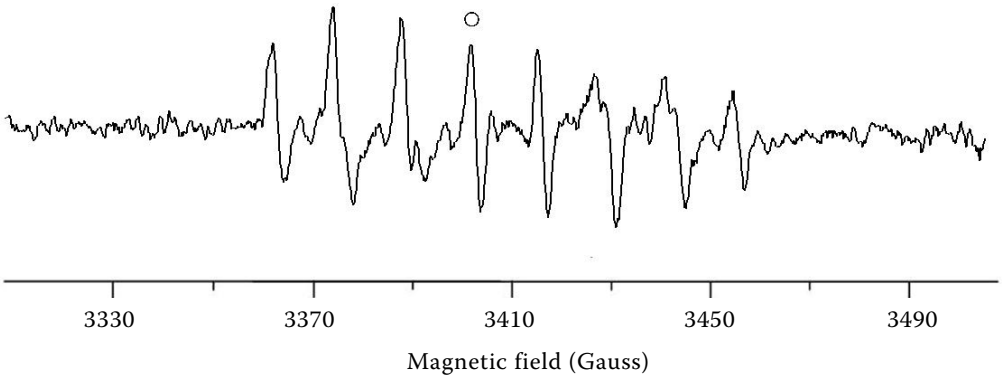

Figure 1. EPR signal of DEPMPO adducts obtained in the X/XO system. Open circle marks the line of DEPMPO/OOH signal whose amplitude was measured. A weak signal of DEPMPO/OH adduct can also be observed, however, it does not affect the amplitude or shape of two central lines of DEPMPO/OOH signal (Bačić *et al.* 2007)

Table 3. Antimicrobial activity of *C. sativa* extracts and reference standards – inhibition zone in diameter (mm) around the discs (6 mm)

Extracts	Test microorganisms						
	Staphylococcus aureus	*Sarcina lutea*	*Bacillus cereus*	*Proteus mirabilis*	*Lactococcus lactis* ssp. *lactis*	*Micrococcus pyrogenes* var. *albus*	*Salmonella typhimurium*
Sweet chestnut							
Seeds	–	–	–	–	10.67 ± 1.53	–	–
Peeled chestnut	–	–	–	–	–	–	–
Brown seed coat	9.67 ± 0.58	7.33 ± 0.58	9.33 ± 0.58	10 ± 0	15 ± 2.5	12.33 ± 1.15	–
Catkin	15.33 ± 0.58	13.66 ± 0.58	10 ± 0	11.33 ± 0.47	10.33 ± 0.72	15.5 ± 2.59	17.82 ± 0.78
Leaf	14 ± 4.89	11 ± 1	10 ± 0	12 ± 0	14.5 ± 2.5	14.33 ± 1.15	13.67 ± 0.88
Chestnut bark	14.67 ± 0.58	9.33 ± 0.58	10 ± 0	11 ± 0	12.33 ± 1.53	13.67 ± 0.58	11.65 ± 0.55
Spiny burs	9.33 ± 0.58	10 ± 0	10 ± 1	11.33 ± 0.47	10 ± 1	14.16 ± 1.25	15.83 ± 1.82
Lovran's marrone							
Leaf	16 ± 3.46	13 ± 1	10.33 ± 0.58	12.66 ± 0.47	11.5 ± 0.84	16.33 ± 0.58	17.83 ± 1.19
Grafted Italian marrone							
Leaf	14 ± 1.73	9 ± 1	9.67 ± 0.58	11.33 ± 0.47	14 ± 1.73	14.33 ± 1.15	12.47 ± 0.61
Standards							
Amoxicillin	27.3 ± 1.15	55.0 ± 1.0	29.0 ± 0	26.33 ± 0.57	8 ± 0	36 ± 0.58	16 ± 0
Penicillin	30.3 ± 2.25	37.7 ± 0.49	34.0 ± 0	34.33 ± 0.57	12 ± 0.58	52 ± 0	15 ± 0

tracts concerning the removal of lipid peroxidation. It was found that all the extracts protected liposomes from lipid peroxidation. Also, the extracts could be used as protective reagents due to the obstruction of oxidative stress provoked by $^{\bullet}OH$ and H_2O_2 and, at the same time, they were extremely resistant to the radical effects of this degradation process. For these reasons, the effect of lipid peroxidation on their scavenging capacity is minimal. Especially, these extracts are very efective scavengers of superoxide radicals in all respects.

The results of antimicrobial activity determination of 30% (v/v) ethanol extracts of *C. sativa* and standards are shown in Table 3.

All the investigated extracts showed antibacterial activity. The highest activity of the extracts was found against *M. pyrogenes* var. *albus*, *S. aureus*, and *S. typhimurium*. Catkin and leaves extracts showed the highest antimicrobial activity. A high activity was also exhibited by the extracts of chestnut bark, spiny burs, and brown seed coat. On the other hand, the seeds and peeled chestnut (with the exception of *L. lactis* ssp. *lactis*), revealed no antimicrobial activity.

The antimicrobial activity of the analysed extracts was exhibited in the following order: catkin > leaves > chestnut bark > spiny burs > brown seed coat >>> seeds and peeled chestnut.

The results obtained in the determination of total phenolics, total flavonoids, antioxidant and antimicrobial activities of chestnut extracts are shown in Tables 1–3, and were analysed by means of the correlation analysis. The values of the correlation coefficients (r), which express the strength of the relationship between two variables, are given in Table 4.

The total phenolic content correlated significantly with total flavonoids (r = 0.73). A very significant correlation ($P < 0.01$) was obtained between the antimicrobial activity of the extracts and $^{\bullet}O_2^-$ radical scavenging with all the seven bacterial strains investigated. The total phenolic content correlated significantly with the antimicrobial activity of the extracts toward *S. lutea* (r = 0.76) and *S. aureus* (r = 0.78). A significant linear correlation ($P < 0.05$) was determinated between the total flavonoids and antimicrobial activity (*S. aureus*) (r = 0.71).

Table 4. Correlations between antimicrobial activity and $^{\bullet}O_2^-$ radicals scavenging

Parameter	*Staphyloco-ccusaureus*	*Sarcina lutea*	*Bacillus cereus*	*Proteus mirabilis*	*Lactococcus lactis* ssp. *lactis*	*Micrococcus pyrogenes* var. *albus*	*Salmonella typhimurium*	Total phenolics	Total flavonoids
S. lutea	0.95**								
B. cereus	0.94**	0.94**							
P. mirabilis	0.95**	0.95**	0.99**						
L. lactis ssp. *lactis*	NS	NS	0.69*	0.68*					
M. pyrogenes var. *albus*	0.96**	0.97**	0.99**	0.99**	NS				
S. typhimurium	0.82**	0.89**	0.77*	0.80**	NS	0.82**			
Total phenolics	0.78*	0.76*	NS	NS	NS	NS	NS		
Total flavonoids	0.71*	NS	NS	NS	NS	NS	NS	0.73*	
Scavenging of $^{\bullet}O_2^-$ radical	0.74*	0.68*	0.77*	0.78*	0.73*	0.77*	0.67*	NS	NS

*correlation is significant at 0.05 level; **correlation is significant at 0.01 level; NS – no significant correlation ($P > 0.05$)

The extracts of leaf, catkin, brown seed coat, spiny burs, and chestnut bark of *C. sativa* can be used as natural antioxidants with the application in diet and therapy.

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Received for publication July 14, 2009
Accepted after corrections December 19, 2009

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