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Characterization of Antioxidant Activities from Chestnut Inner Skin Extracts

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Abstract Total phenolics and antioxidant activities of water and 80% methanol extract of chestnut inner skin were investigated. The antioxidant properties of both extracts of chestnut inner skin were evaluated using different antioxidant tests, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activities, reducing power, ferric reducing ability of plasma (FRAP), and inhibitory effects on β-carotene bleaching. The 80% methanol extract of inner skin exhibited the higher DPPH, ABTS radical scavenging activities, reducing power, and FRAP than water extract of inner skin and did also in a concentration-dependent manner. However, inhibitory effects on β-carotene bleaching of 80% methanol extract was similar to those of water extract, 35.09 and 39.07% at 1 mg/mL, respectively. The total phenolic contents of water and 80% methanol extract from chestnut inner skin were 5,801.42 and 9,735.56 mg/100 g, respectively. High performance liquid chromatography (HPLC) analysis showed that gallic acid was the predominant phenolic compound in water and 80% methanol extract from inner skin. These water and 80% methanol extracts of chestnut inner skin can be utilized as an effective and safe source of antioxidants.

Keyworlds: chestnut inner skin, total phenolic, antioxidative activity, gallic acid

Introduction

Reactive oxygen species (ROS) generated from both living organisms and exogenous sources (1), initiate reactions which damage biological molecules and also play an important causative role in disease initiation (2). Lipid oxidation, caused by free radicals, is one of the major factors for the deterioration of food products during processing and storage. Effective synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been used for industrial processing but these are suspected of being responsible for liver damage and carcinogenesis (3). The emergence of natural extracts possessing antioxidation properties will help in reducing the current dependency on synthetic antioxidants in food applications. Recently, there is an increasing interest in finding natural antioxidants from plant materials to replace synthetic ones. Plants contain a large variety of substances possessing antioxidant activity, such as vitamin C, vitamin E, carotenes, xanthophylls, tannins, and phenolics (4). Sources of natural antioxidants are primarily plant phenolics that can be found in all parts of the plant (5). The plant phenolic compounds including flavonoids exhibit antioxidant properties due to their high redox potential (6).

They also exhibit a wide range of biological activity, antimicrobial activity, anticarcinogenicity, and antiproliferation, and many biological activities that can be attributed to their antioxidant properties (7,8). An increasing amount of evidence shows that the consumption of fruits, particularly nuts, has become more important in human nutrition, due to the protection provided by the antioxidant compounds (9). According to these authors, the tree nuts, chestnuts, walnuts, and pecans have the highest content of antioxidants. In recent years, the consumers have been showing an increased interest in chestnut fruits because of their nutritional qualities and potentially beneficial health effects. In fact, chestnut is also rich in carbohydrates and is a good source of essential fatty acids and minerals (10-13). In addition, they present several vitamins and appreciable levels of fiber (14). Although it has already been demonstrated that chestnut fruits (15) and leaves (16) contain phenolic compounds, nutrition components (17), antimicrobial (18), and antioxidant activities of different parts of chestnut (19), little is known about their antioxidant potential or about other chestnut extract, such as chestnut out skin, inner skin, leaf, and fruit. In line with the efforts to balance the conservation of plant resources for economic gains, especially in biopharming, wastage of valuable resources should be minimized. Therefore, this study investigated the antioxidative potential and isolate of active compounds of water and 80% methanol extract from chestnut inner skin which are normally wasted during frequent pruning.

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Materials and Methods

Materials and chemicals The chestnut inner skin was collected from Jinju, Korea in September, 2008. Folin-Ciocalteu's reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), potassium persulfate, potassium ferricyanide, trichloroacetic acid, ferric chloride, 2,4,6-tripyridyl-*S*-triazine (TPTZ), nitro-blue tetrazolium salt, β-carotene, ascorbic acid, α-tocopherol, butylated hydroxyanisole (BHA), and all solvents used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction from the inner skin of chestnut Each extract of freeze-dried chestnut inner skin (60 mesh particle size) was obtained as follows. Powdered chestnut inner skin (100 g) was suspended and extracted with 500 mL of water and 80% methanol at 100 and 80°C for 2 hr, respectively. The extracts were filtered through Whatman No. 2 filter paper (Whatman International Limited, Kent, England) and evaporated. Each extract was concentrated in a vacuum evaporator (Eyela NE, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 40°C. The extracts were place in a glass bottle and stored at –20°C until used. The lyophilized extracts were redissolved in water and 80% methanol to a concentration of 1 mg/mL.

DPPH free radical scavenging activity This was carried out according to Blois method with a slight modification (20). Briefly, a 1 mM solution of DPPH radical solution in ethanol was prepared, and then 4 mL of this solution was mixed with 1 mL of extracts; finally, after 30 min, the absorbance was measured at 517 nm (UV-1201; Shimadzu, Tokyo, Japan). The ascorbic acid and α-tocopherol were used as the positive control. This activity is given as percent DPPH scavenging that is calculated as;

$$\% \text{ DPPH scavenging} = [(\text{control absorbance} - \text{sample absorbance})/(\text{control absorbance})] \times 100$$

ABTS radical scavenging activity ABTS was dissolved in water to make a concentration of 7 mmol/L. ABTS was produced by reacting the ABTS stock solution with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 hr before use. For the study of samples, the ABTS stock solution was diluted with phosphate buffered saline (PBS, 5 mmol/L, pH 7.4) to an absorbance of 0.70 at 734 nm. After the addition of 0.98 mL of diluted ABTS to 20 μL of sample, the absorbance reading was taken 5 min after the initial mixing (21). This activity is given as %ABTS scavenging that is calculated as;

$$\% \text{ ABTS scavenging activity} = [(\text{control absorbance} - \text{sample absorbance})/(\text{control absorbance})] \times 100$$

Reducing power Similar to the methods performed by Oyaizu (22), we measured reducing power. Briefly, extracts in 1 mL of appropriate solvents were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide [$K_3Fe(CN)_6$] (1%), and then the mixture was incubated at 50°C for 30 min. Afterward, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, then centrifuged at 2,090×g for 10 min. Finally, 2.5 mL of the upper layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm (UV-1201; Shimadzu).

FRAP The FRAP assay was developed by Benzie and Strain (23). In short, 1.5 mL of working, prewarmed 37°C FRAP reagent (10 volumes 300 mmol/L acetate buffer, pH 3.6+1 vol of 10 mmol/L 2,4,6-tripyridyl-*S*-triazine in 40 mmol/L HCl+1 vol of 20 mmol/L $FeCl_3$) was mixed with 50 μL of test samples and standards. These were vortex-mixed and absorbance was measured at 593 nm against a reagent blank at a predetermined time after sample-reagent mixing. The test was performed at 37°C and the 0-4 min reaction time window was used.

β-Carotene linoleic acid bleaching inhibition The ability of the extract to inhibit the bleaching of the β-carotene linoleic acid emulsion was determined using a modification of the method described by Koleva *et al.* (24). In brief, 0.2 mg β-carotene dissolved in 1 mL chloroform, 20 mg of linoleic acid, and 200 mg of Tween 20 were transferred into a round-bottom flask. Once the chloroform had been removed under nitrogen, 50 mL distilled water was added and the mixture was vigorously stirred for 30 min. Thereafter, 5 mL aliquots of this emulsion were transferred into tubes containing either 200 μL of dissolved extract (31, 62, 125, 250, 500, and 1,000 μg/mL) or 200 μL of positive controls (1 mg/mL). After mixing, the absorbance (Abs_0) at 470 nm was recorded. The remaining samples were placed in a water bath at 50°C for a period of 2 hr. Thereafter, the absorbance of each sample was remeasured at 470 nm (Abs_{120}). The BHA and α-tocopherol were used as the positive control. The data (n=3) are presented as antioxidant activity % (AA%) values, calculated using;

$$AA\% = [1 - (Abs_0\text{sample} - Abs_{120}\text{sample})/(Abs_0\text{control} - Abs_{120}\text{control})] \times 100$$

Determination of total phenolics Total phenolics were determined by the spectrophotometric analysis (25). In brief, a 1 mL portion of appropriately diluted extracts was added to a 25-mL volumetric flask containing 9 mL of deionized distilled water. Reagent blank using deionized distilled water was prepared. One mL of Folin-Ciocalteu's phenol reagent was added to the mixture followed by shaking. After 5 min, 10 mL of a 7% Na_2CO_3 solution was added with mixing. The mixed solution was then immediately diluted up to 25 mL with deionized distilled water and mixed thoroughly. After 90 min at 23°C, the absorbance was read at 750 nm. The standard curve for total phenolics was generated using gallic acid standard solution (0-100 mg/L) under the same procedure as above. Total phenolics in chestnut inner skin were expressed as mg of gallic acid equivalents (GAE)/1 g of sample.

Quantification by high performance liquid chromatography (HPLC) Phenolic compounds were measured at 280 nm using Agilent HPLC (1100 Series; Agillent Co., Santa Clara, CA, USA). Separation was achieved with a LiChrospher RP-18 column (250×4 mm i.d., 5 μm, E. Merck Co., Darmstadt, Germany). The mobile phase consisted of acetonitrile:acetic acid:methanol:water (113:5:20:862, v/v/v/v). The flow rate was 1.0 mL/min and the injection volume was 20 μL. Compounds were detected by monitoring the elution at 280 nm. Identification of the phenolic compounds was carried out by comparing their retention times and absorption spectra to those of standards. Content of phenolic compounds was expressed in mg/100 g extract.

Statistical analysis All data were expressed as mean±

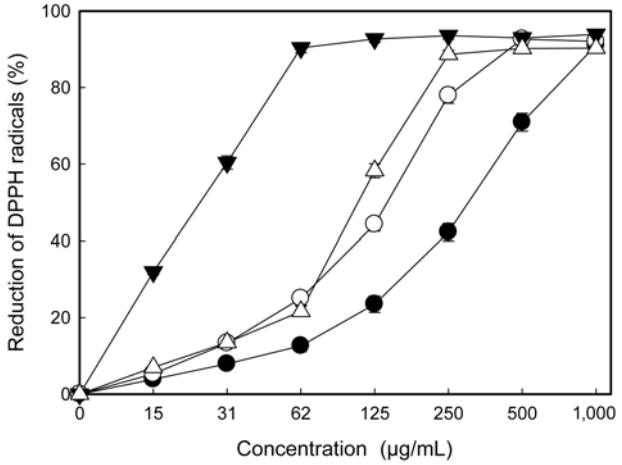

Fig. 1. DPPH radical scavenging activities of water and 80% methanol extract from chestnut inner skin. ●, water extract of the chestnut inner skin; ○, 80% methanol extract of the chestnut inner skin; ▼, ascorbic acid; △, α-tocopherol. Values are mean±SD (n=3).

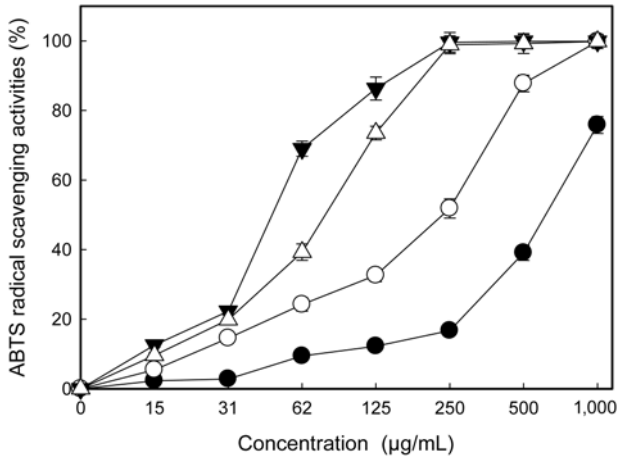

Fig. 2. ABTS radical scavenging activities of water and 80% methanol extract from chestnut inner skin. ●, water extract of the chestnut inner skin; ○, 80% methanol extract of the chestnut inner skin; ▼, ascorbic acid; △, α-tocopherol. Values are mean±SD (n=3).

standard deviation (SD). Analysis of variance was performed by the analysis of variance (ANOVA) procedures. Duncan's new multiple range test was used to determine the difference of means, and a value of $p<0.05$ was considered significant.

Results and Discussion

Scavenging effect on DPPH radical Methanol and 80% methanol were the most suitable solvent in the extraction in polyphenolic compounds from plant tissue, due to its ability to inhibit the action of polyphenol oxidase that causes the oxidation of polyphenol, antioxidant activity, and its ease of evaporation compared to water (26,27).

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Hence, DPPH is often used as a substrate to evaluate antioxidative activity of antioxidants. Figure 1 illustrates a significant decrease in the concentration of DPPH radical due to the scavenging ability of the extracts of chestnut inner skin and standards. We used ascorbic acid and α-tocopherol as standards. The scavenging effect of water and 80% methanol extract of chestnut inner skin and standards on the DPPH radical decreased in the order: ascorbic acid>α-tocopherol>80% methanol extract>water extract, which were 93.57, 88.67, 77.92, and 42.23%, respectively, at the concentration of 250 μg/mL. These results indicate that both chestnut inner skin extracts have a significant effect on scavenging free radical. Free radical scavenging activity is also shown in a concentration-dependent manner. Yen *et al.* (28) reported that peanut hulls with total phenolic compounds greater than 0.1671 mg/g of hulls displayed strong antioxidant activity.

Scavenging effect on ABTS radical The scavenging activities of water and 80% methanol extract of chestnut inner skin on ABTS are shown in Fig. 2 and compared with ascorbic acid and α-tocopherol as standards. Chestnut inner skin extracts were capable of scavenging ABTS in a concentration-dependent manner. Water and 80% methanol extract at 500 μg/mL of chestnut inner skin exhibited 87.74 and 39.07% scavenging activity on ABTS radical, respectively. As a comparison, ascorbic acid and α-tocopherol exhibited 99.86 and 99.23% ABTS radical scavenging activities at the same dose. These results showed that both chestnut inner skin extracts show stronger ABTS radical scavenging activities, though the activities of the tested samples were lower than that of ascorbic acid and α-tocopherol.

Reducing power The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity, and radical scavenging. Like the antioxidant activity, the reducing power of chestnut extracts increased with increasing amount of samples. All of the amounts of both chestnut inner skin extracts showed higher activities than control with a statistically significant difference ($p<0.01$). Reducing power of water and 80% methanol extract of chestnut inner skin and standard compounds exhibited the following order: ascorbic acid>α-tocopherol>80% methanol extract of chestnut inner skin>water extract of chestnut inner skin. The ascorbic acid, α-tocopherol, 80% methanol extract of chestnut inner skin, and water extract of chestnut inner skin, exhibited reducing power (unit: optical density) of 7.80, 4.28, 2.20, and 1.59 at the concentration of 1 mg/mL, respectively (Fig. 3). Barreira *et al.* (19) reported that the reducing power also increased with higher concentration and the values obtained for all the extracts from chestnut were excellent. The extracts obtained with flowers and skins showed similar values, while leaf showed marginally good results. Based on these observations, it is suggested

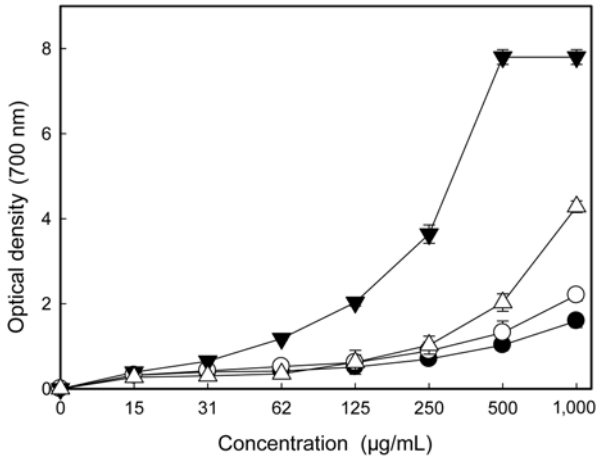

Fig. 3. Reducing power of water and 80% methanol extract from chestnut inner skin. ●, water extract of the chestnut inner skin; ○, 80% methanol extract of the chestnut inner skin; ▼, ascorbic acid; △, α-tocopherol. Values are mean±SD (n=3).

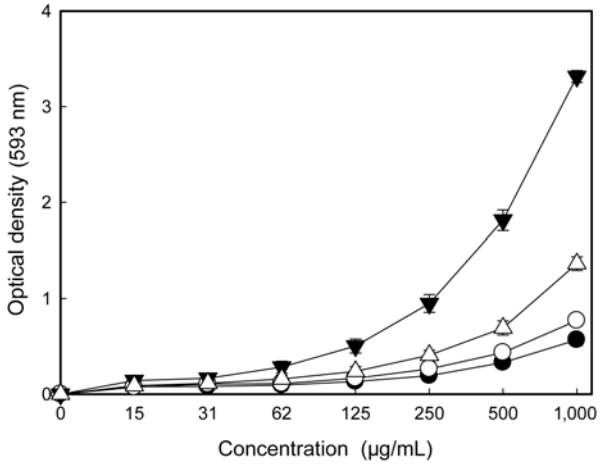

Fig. 4. Ferric reducing ability of plasma of water and 80% methanol extract from chestnut inner skin. ●, water extract of the chestnut inner skin; ○, 80% methanol extract of the chestnut inner skin; ▼, ascorbic acid; △, α-tocopherol. Values are mean±SD (n=3).

that chestnut inner skin has a remarkable potency to react with free radicals to convert them into more stable non-reactive species and to terminate radical chain reaction.

FRAP In this assay, samples are used in a redox-linked reaction whereby the antioxidants present in the sample act as the oxidants. Reduction of the ferric-tripyridyltriazine to the ferrous complex forms an intense blue color which can be measured at a wavelength of 593 nm. The intensity of the color is related to the amount of antioxidant reductants in the samples. In the present study, the trend for ferric ion reducing activities of water and 80% methanol extract from chestnut inner skin was shown in Fig. 4. For extracts of chestnut inner skin, the absorbance clearly increased, due to the formation of the Fe^{2+}-TPTZ complex with increasing concentration. FRAP of water and 80% methanol extract of chestnut inner skin and standards decreased in the order: ascorbic acid>α-tocopherol>80% methanol extract of chestnut inner skin>water extract of chestnut inner skin, which were 3.31, 1.36, 0.76, and 0.56, respectively, at the concentration of 1 mg/mL. Similar to the results obtained from the DPPH and ABTS assay, extracts of chestnut inner skin showed relatively strong ferric ion reducing activity. A strong correlation between the mean values of the total polyphenol content and FRAP deserves detailed attention, as polyphenols in chestnut inner skin were likely capable of reducing ferric ions. Consistently, others have reported similar correlations between polyphenols and antioxidant activity measured by various methods (29,30). Additionally, many of the phenolics have been shown to contain high levels of antioxidant activities.

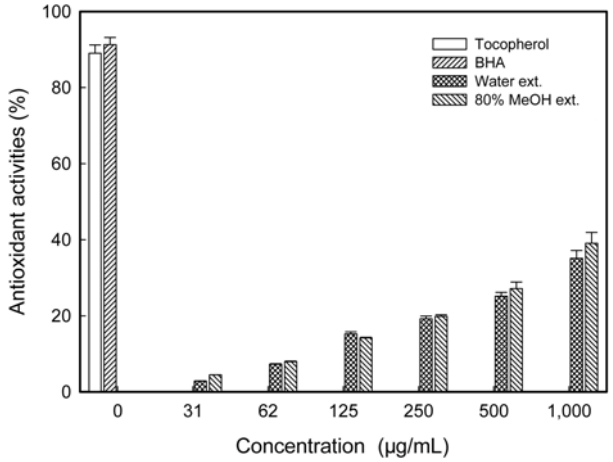

Fig. 5. Antioxidant activity of water and 80% methanol extract from chestnut inner skin measured by β-carotene bleaching method. Values are mean±SD (n=3).

β-Carotene-linoleic acid bleaching inhibition In this oil-water emulsion-based system, linoleic acid undergoes thermally induced oxidation, thereby producing free radicals that attack the β-carotene's chromophore resulting in a bleaching effect. An extract that inhibits β-carotene bleaching can be described as a free radical scavenger and a primary antioxidant. As shown in Fig. 5, the water and 80% methanol extract from chestnut inner skin demonstrated an ability to inhibit the bleaching of β-carotene by scavenging linoleate-derived free radicals. The water and 80% methanol extract from chestnut inner skin were also capable of inhibiting the bleaching of β-carotene in a manner dependent on concentration. The inhibitory effect of water and 80% methanol extract from chestnut inner skin and standards decreased in the order BHA (91.28%)> α-tocopherol (89.03%)>80% methanol extract of chestnut inner skin (39.07%)>water extract of chestnut inner skin (35.09%) at 1 mg/mL, respectively. Since the extract seems to contain lower concentration of antioxidant substances than positive controls, the anti-bleaching activity of the extract is lower than BHA or α-tocopherol. This is the general trend observed in other studies using extracts. Collectively, these data strongly suggest that electrons donated from the plant extract can scavenge free radicals.

Total phenolics and analysis of extract by HPLC The total phenolics of water and 80% methanol extract from

Table 1. Total phenolics and phenolic compounds content of water and 80% methanol extract from chestnut inner skin

Compounds	Content (mg/100 g extract)	
	Water ext.	80% MeOH ext.
Total phenolics	5,801.42±184.51[1)]	9,735.56±226.48
Gallic acid	31.04±3.18	57.05±5.29
Catechin	7.54±1.27	8.58±0.95
Epicatechin	6.60±0.39	6.63±0.17
Chlorogenic acid	5.66±0.83	6.34±0.62

[1)]Values are mean±SD (n=3).

chestnut inner skin was determined (Table 1). The results showed that the total phenolics content of 80% methanol extract (9,735.56±226.48 mg of GAE/100 g) was relatively higher than that of water extract (5,801.42±184.51 mg/100 g) from chestnut inner skin. Since the water and 80% methanol extract of chestnut inner skin exhibited the strong antioxidant activity, it was subjected to further analysis by HPLC. Water and 80% methanol extract contained a variety of phenolic compound. By comparing the retention time of these compounds with those of standards, 4 phenolic compounds were identified (Table 1). Furthermore, the HPLC indicated that gallic acid (31.04±3.18 and 57.05 ±5.29 mg/100 g) was the predominant compounds in water and 80% methanol extract, followed by catechin (7.54±1.27 and 8.58±0.95 mg/100 g). Flavonoids and phenolic acids are important contributing factors to the antioxidant activity of the human diet. Based on the results for the phenolic composition of water and 80% methanol extract from chestnut inner skin, we can conclude that these compounds (particularly gallic acid and catechin) mediate the antioxidant activities of water and 80% methanol extract.

More potential activities were found in the 80% methanol extract than the water extract. This is probably because 80% methanol is a better solvent for extraction of antioxidant substances compared to the water. In line with this, Heo *et al*. (31) reported that individual phenolics showed their characteristic antioxidant capacities, while the mixtures of 2 or 3 phenolics revealed that the summation of antioxidant capacities of individual phenolics lead to total antioxidant capacity. Consequently, the activities determined in the extracts could be attributed to the phenolic compounds found in the extracts.

This study has demonstrated the antioxidant activity of water and 80% methanol extract from chestnut inner skin. The results obtained in this work are noteworthy, not only with respect to the antioxidant activities of water and 80% methanol extract from chestnut inner skin, but also the content of a variety of phenolic compounds. The activity of water and 80% methanol extract is attributed to these phenolic compounds and in particular to gallic acid. Finally, our results verified that water and 80% methanol extract of chestnut inner skin have very strong antioxidant activities and the results of this study show that the extract of chestnut can be used as easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

Acknowledgments

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