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Total Phenolics Content and Antioxidant Activity of Extracts from Dried Water Caltrop (*Trapa Taiwanensis* Nakai) Hulls

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ABSTRACT

This study was conducted to compare the total phenolics content and antioxidant activity of extracts from fresh (FS), freeze-dried (FD) and hot air-dried (HD) water caltrop hulls. The antioxidant properties, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity, reducing power, and cupric ion chelating ability were evaluated along with alpha-tocopherol and butylated hydroxytoluene (BHT) in this study. The results showed that total phenolics content of methanolic extracts ranged from 5.21-8.59 g GAE/100 g, and the extracts of FS exhibited a substantial total phenolics content (8.59 g GAE/100 g) compared with other dried samples. It was found that the antioxidative effect of water caltrop hulls was strongly concentration dependent. The dose response study indicated that before reaching a threshold level, there was a positive correlation between DPPH free-radical scavenging ability and concentration of methanolic extracts. At the point where the scavenging ability of DPPH free-radical was 79.3%, the dose of the methanolic extracts was 0.2 mg/mL for FS, 1.5 mg/mL for HD, 1.8 mg/mL for FD, and 1.3 mg/mL for BHT. The chelating ability of three different water caltrop hull extracts reached a threshold level of only 28% at a dose of 2 mg/mL. The total phenolics contents were higher, but IC_{50} was lower in FS extracts than in other antioxidants. Compared with other treatments with antioxidants, the reducing power of FS extracts was found to be significantly more pronounced than that of HD and FD extracts, BHT and α-tocopherol, at dose ranges of 0–1.0 mg/mL. This finding demonstrated that FS extracts is comparable to BHT in terms of its antioxidant properties in food application.

Key words: water caltrop hulls, total phenolics content, antioxidant activity

INTRODUCTION

Water caltrop (*Trapa Taiwanensis* Nakai) belongs to the family *Trapaceae*, one of free-floating plants grown in shallow water fields, ponds or swampy lands in tropical and sub-tropical countries[(1)]. The interesting features of water caltrop include color and shape of the outer cover in which the kernel is encased. Mature *Trapa* is 3-5 cm wide and 5-6 cm long, with one pair of spines in the shoulder and one pair of short spines in the abdomen. The water caltrop meat is covered with thick jet-black outer hulls, shaped like horns protruding from the head of a buffalo. The outer hull is hard, making it difficult to peel off to obtain the internal white fruit[(2)]. Water caltrop is one of the most popular vegetables in Asia, due to its special taste and medical function, and it is found in Taiwan, China and parts of Southeast Asia. Due to high activity of enzymes and phenolics content, the color of water caltrop hulls easily changes from the original pink color to dark brown during transportation and processing[(3)].

Vegetables contain a great quantity of non-nutritional antioxidants, such as flavoniods, flavone and total phenolic contents[(4-6)]. Flavonoids, a large group of plant total phenolics, are present in plant tissues, such as fruits, vegetables, nuts, seeds and leaves, in relatively high concentration. Flavonoids act as natural antioxidants[(7-10)]. Phenolic compounds, including phenolic acid and flavonoids, have been recognized as having health-related properties, including anticancer, antiviral, and anti-inflammatory activities[(11,12)]. Many researches have shown that vegetables can be used to prevent cancer or cardiovascular diseases due to the high amount of antioxidants including anthocyanin and total phenolic contents[(10,13)]. The nutrients and antioxidants present in vegetables are influenced during processing, particularly microwave heating, cooking, and stir-frying vegetables[(14,15)]. After different cooking treatments and precooking treatments was exhibited no profound effect on the antioxidant properties of brocco-

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li[15]. However, heating temperature and time significantly influenced the scavenging ability of DPPH free-radical of ethanolic extracts of purple yam[16]. Crozier[17] found that microwave heating and steam-cooking further reduced the phenolics content, particularly quercetin content, more so than stir-frying vegetables and fruits. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ) are commonly used as synthetic antioxidants in lipid-containing foods[18]. In the last two decades, there has been an increasing interest in replacing these synthetic antioxidants with natural alternatives[19], because of their possible role as promoters of carcinogenesis[20]. Fruits, vegetables, spices, nuts, seeds, leaves, roots, and barks have thus been exploited as potential sources of natural antioxidants[7].

Water caltrop hulls are now commonly used as fertilizer or trash in Taiwan and other Asian countries. Finding a possible way to increase the utilization of water caltrop hulls as a source of antioxidants is the primary target of this study. The objectives of this study were to investigate the total phenolics and flavonoids content in the water caltrop hulls, and to compare the effect of freeze-drying and hot air-drying on the antioxidant properties of water caltrop hulls.

MATERIALS AND METHODS

I. *Materials*

Raw water caltrops were obtained from a local supplier in Tainan, Taiwan. Fresh caltrop hulls were manually removed and separated into three lots. The first lot of fresh caltrop hulls was blended by a domestic blender and used as the FS sample. The remaining sample lots were freeze-dried (FD) or hot air-dried (at 60°C) (HD) and powdered to obtain a 100 mesh size. The water caltrop hull samples (FS, FD, and HD) were stored at 4°C until further analysis was carried out.

II. *Methanolic Extraction*

Fifty grams of each treatment of water caltrop hulls, including FD, HD, and FS, were extracted by stirring with a magnetic stirrer and 250 mL of methanol at 25°C for 24 hr. The extract was filtered through Whatman No. 1 filter papers for removal of hull particles. The residue was re-extracted with 250 mL of methanol and filtrated. The extracts were pooled together and vacuum dried at 30°C. The dried extracts were powdered and stored at -20°C for the analysis of antioxidant activity.

III. *Total Phenolics Content*

Total phenolics content in the methanolic extracts of three water caltrops hulls were determined according to the method of Sato *et al.*[21] with minor modifications. One milligram of methanolic extracts was mixed with 400 μL of 10-fold diluted Folin–Ciocalteu reagent (Sigma Chem. Co., USA). After 3 min of incubation, 40 μL of 10% sodium carbonate was added and allowed to stand for 1 hr at 30°C. The absorbance at 735 nm was measured and converted to total phenolic content, according to the calibration curve of gallic acid (g GAE /100 g dry matter) (Sigma Chem. Co., USA). Estimation of the total phenolics content was carried out in triplicate and averaged.

IV. *Total Flavonoid Content*

Total flavonoid content was determined by the colorimetric method of Christel *et al.*[22]. Methanolic extract (0.5 mg) was mixed with 0.5 mL of 2% aluminum chloride (Sigma Chem. Co., USA) ethanol solution, and then incubated at room temperature for 1 hr. The absorbance was measured at 430 nm using a Hitachi U2000 UV-visible spectrophotometer (Hitachi Co., Japan). Total flavonoid contents were calculated using quercetin equilibrant (g QE /100 g dry matter) (Sigma Chem. Co., USA) from a calibration curve.

V. *Free-radical Scavenging Ability*

The scavenging ability of methanolic extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma Chem. Co., USA) free-radicals was estimated according to the method of Yamaguchi *et al.*[23]. An aliquot of methanolic extracts (50 mg/mL), α-tocopherol (vitamin E, Sigma Chem. Co., USA) (50 mg/mL), or butylated hydroxytoluene (BHT, Sigma Chem. Co., USA) (50 mg/mL) was mixed with a 100 mM Tris-HCl buffer (400 μL, pH 7.4) and then added to 500 μL of 250 mM DPPH in ethanol, respectively. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured at 517 nm against an aliquot blank. The scavenging ability was calculated as follows:

Scavenging ability (%) = [(ΔA_{517} of control − ΔA_{517} of sample) / ΔA_{517} of control] × 100.

The half-inhibition concentration (IC_{50}) was calculated as the antioxidant concentration required for providing 50% of antioxidative activity[24]. In other word, the lower IC_{50} values indicated the higher antioxidative activity in the methanolic extracts.

VI. *Chelating Effects on Cupric Ions*

The chelating effect was determined according to the method of Yang *et al.*[25]. Two milliliter of the test solution, including methanolic extract, and ethylene diamine tetraacetic acid (EDTA, Sigma Chem. Co., USA) were mixed with 30 mM of hexamine (Wako Chem. Co., Germany), 30 mM of potassium chloride (Sigma Chem. Co., USA) and 9 mM of copper sulfate, and 0.2 mL of 1 mM tetramethyl murexide (Sigma Chem. Co., USA). After reacting for 3

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min at room temperature, absorbance of the mixture was determined at 485 nm. Lower absorbance indicated higher cupric ion chelating ability of the test sample.

VII. *Reducing Power*

The reducing power of the methanolic extracts, α-tocopherol, and BHT were determined according to the method of Yen and Chen[26]. The aliquot of methanolic extract (20 mg/mL), α-tocopherol (20 mg/mL), or BHT (20 mg/mL) was mixed with an equal volume of 200 mM sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide (Sigma Chem. Co., USA), respectively. The mixture was incubated at 50°C for 20 min. An equal volume of 1% trichloroacetic acid was added to each mixture, which was then centrifuged at 4020 ×g for 10 min at 4°C. The upper layer of the solution was separated from the mixture and then mixed with distilled water and 0.12 mL of 0.1% ferric chloride ($FeCl_3$, Sigma Chem. Co., USA). The mixture was incubated at room temperature (25°C) for 14 min. The absorbance was measured at 700 nm against an aliquot blank in a Hitachi U-2000 UV-visible spectrophotometer. Increased absorbance of the reaction mixture indicated an increase of reducing power.

VIII. *Statistical Analysis*

All determinations were carried out for three replicates of each sample. For all analyses conducted, the data were subjected to an analysis of variance (ANOVA), where *F*-values were deemed to be significant and the mean value for each nutrient was compared using Duncan's new multiple-range test procedure, as specified by use of the Statistical Analysis System (SAS, 1990)[27].

RESULTS AND DISCUSSION

I. *Total Phenolics and Flavonoids Contents*

The total phenolics and total flavonoids content of methanolic extracts of water caltrop hulls, expressed as gallic acid equivalents (GAE) and quercetin equivalents (QE), was dependent upon the solvents and the methods used in the extraction (Table 1). The results indicated that FS extracts feature a substantial content of total phenolics as compared with FD and HD extracts, with total phenolic content being on the order of 5.21 – 8.59 g GAE / 100 g (dry basis, d.b.). In contrast to this, the HD extracts appeared to be the highest content of total flavonoids, as total flavonoids were on the order of 8.07 – 12.75 g QE / 100g (d.b.) (Table 1). Such an outcome suggested that hot air processing might produce some compounds by Maillard browning or enzymatic browning, as reflected by the determination of total flavonoids content. As compared to the other fruits and nuts, water caltrop hulls contained substantially higher amounts of

Table 1. Total phenolics and flavonoids contents of water caltrop hulls[1]

Water caltrop hulls[2]	Total phenolics (g GAE / 100 g)[3]	Total flavonoids (g QE / 100 g)[3]
FS	8.59 ± 0.03[a]	8.99 ± 0.08[b]
FD	5.21 ± 0.04[c]	8.07 ± 0.11[c]
HD	6.15 ± 0.04[b]	12.75 ± 0.11[a]

[a-c]Means with different letters within the same column are significantly different ($P < 0.05$).
[1]Each value is expressed as Mean ± S.D. ($n = 3$).
[2]FS, FD and HD are water caltrop hull extracts of fresh, freeze-dried and hot air dried, respectively.
[3]GAE: gallic acid equivalent; QE: quercetin equivalent.

Table 2. Total phenolics content of water caltrop hulls, nuts and by-product of fruits

Water caltrop hulls[1]	Total phenolics content	Fruit and nut source	Total phenolics content (g GAE /100 g dry matter)
FS	8.59	Red grape peels[2,3]	2.00
FD	5.21	Red grape seeds[4]	2.23
HD	6.15	Pomace (Manto Negro)[5]	2.63
		Stem (Manto Negro)[5]	11.60
		Almonds[6]	0.42
		Hazelnuts[6]	0.83
		Pine nuts[6]	0.06
		Brazil nuts[6]	0.31
		Macadamias[6]	0.16
		Peanuts[6]	0.40
		Pistachios[6]	1.66
		Walnuts[6]	1.56
		Peanuts with skin[7,8]	0.42
		Almonds with skin[7,8]	0.24

[1]FS, FD and HD are water caltrop hull extracts of fresh, freeze-dried and hot air dried, respectively.
[2]Saura-Calixto (1998)[28].
[3]Bartolomé *et al*. (2004)[29].
[4]Guendez *et al*. (2005)[30].
[5]Antonia Llobera and Jaime Cañellas (2007)[31].
[6]Wu *et al*. (2004)[32].
[7]Kornsteiner *et al*. (2006)[33].
[8]All data are expressed as g of GAE / 100 g dry matter except peanuts and almonds with skin are expressed in fresh weight.

total phenolics at ranging from 2.00 to 11.60 g GAE /100 g (Table 2). However, nuts without hard hulls featured a high content of total phenolics, ranging between 0.06 – 1.66 g GAE/100 g. As increase of total phenolics in the hard hulls of nuts was also observed as expected because hard hulls of nuts were expected to contain a substantial amount of total phenolics, based on previous studies[28-30]. Based on this comparison, water caltrop hulls are a potential source of antioxidants in food application.

II. *Scavenging Ability of DPPH Free-radical and Chelating Ability*

The proton free-radical scavenging ability is a known mechanism for antioxidation. When DPPH, a proton free radical, encounters proton radical scavengers, its purple color fades rapidly as a measurement of absorption at 517 nm[23]. Figure 1 presents the dose-response curve for the free-radical scavenging ability of methanolic extracts of water caltrop hulls, BHT and α-tocopherol by the DPPH coloring method. Dose response studies showed a positive correlation between antioxidant activity and the concentration of methanolic extracts before reaching the threshold level. In general, the scavenging ability of the DPPH free-radical increased sharply with the increase in concentration of methanolic extracts to a certain extent, and then levels off with further increases of extract concentration. At a dosage of 2 mg/mL extracts, the highest DPPH scavenging ability is BHT, which is found to be 97.6% and followed by α-tocopherol, HD and FS extracts. The lowest DPPH scavenging ability of 78.83%was found in FD extracts. As dosage of methanolic extracts increased to 20 mg/mL, the same tendency of DPPH scavenging ability of each sample was observed. The scavenging ability of the DPPH free-radical was found to be 79.3% for methanolic extracts of FS at a dose of 0.2 mg/mL, for HD at dose level of 1.5 mg/mL, for FD at dose level of 1.8 mg/mL, and the same dose for BHT and α-tocopherol at level of 0.2 mg/mL. The results indicated that the methanolic extracts of FS exhibited the highest scavenging ability of the DPPH free-radical at low dose levels (≦ 1 mg/mL). In other words, FS extracts required lower doses than HD and FD to reach 80% DPPH scavenging ability. However, HD showed the highest DPPH free radical scavenging ability when the concentration of methanolic extracts increased to a high dose level (≧ 5mg/mL) (Figure 1). Moreover, the concentrations required for methanolic extracts of fresh water caltrop hulls were similar to those required for BHT.

The chelating ability of methanolic extracts of water caltrop hulls on cupric ions is presented in Figure 2. Similar to other antioxidative effects, the chelating ability of cupric ions also increased with the increase of concentration of extracts. Dose response studies indicated that the chelating ability of extracts of three different water caltrop hulls reached a threshold level of only 28% at a dose of 2 mg/mL. Compared with other antioxidants in this study, EDTA exhibited more pronounced effects than that of extracts of three water caltrop hulls at dose range of 0-20 mg/mL. The chelating ability of EDTA increased sharply to reach a threshold level up to 95% at a dose of 2 mg/mL.

As shown in Figures 1 and 2, the antioxidant activity provided by various antioxidants was strongly concentration dependent. In general, the antioxidant activity increased with increasing antioxidant concentration, and then leveled off, even with further increases in antioxidant concentration. As shown in Table 2, it was evident that the coefficients of determination (R^2) were high in all experiments (R^2 = 0.81–0.96). The half-inhibition concentration (IC_{50}) can be calculated as the antioxidant concentration required for providing 50% of antioxidant activity, such as the DPPH scavenging ability and cupric

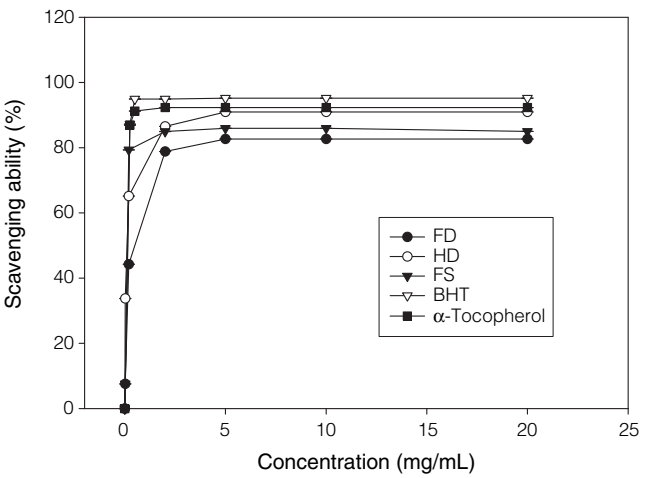

Figure 1. DPPH radical-scavenging activity of methanolic extracts of various water caltrop hulls, BHT, and α-tocopherol. *Each value represents Mean ± S.D. (n = 3). ** FS, FD and HD are water caltrop hull extracts of fresh, freeze-dried and hot air dried, respectively.

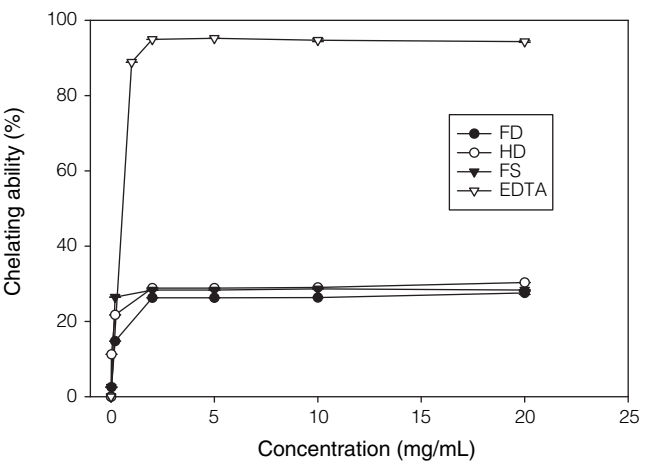

Figure 2. Cupric ion-chelating ability of methanolic extracts of various water caltrop hulls and EDTA. *Each value represents Mean ± S.D. (n = 3). ** FS, FD and HD are water caltrop hull extracts of fresh, freeze-dried and hot air dried, respectively.

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ion chelating ability, by interpolation from linear regression analysis (Figures 1 and 2). In other words, the lower IC_{50} values indicated higher antioxidative activity in the methanolic extracts. The IC_{50} of the methanol extracts of water caltrop hulls for the DPPH free-radical scavenging ability ranged from 0.07 to 0.25 mg/mL, depending on the processing methods (Table 3). According to the measurement results of the half-inhibition concentrations (IC_{50}), FS extracts and α-tocopherol exhibited the most effective DPPH scavenging ability, followed by BHT and then the remaining water caltrop hulls extracts (Table 3). The IC_{50} values for BHT and α–tocopherol were 1.0 ~ 3.5 fold higher than that of three water caltrop hull extracts. In other words, similar concentration of FS extracts was required for BHT and α-tocopherol to reach a similar extent of DPPH scavenging ability. The FS extracts having the lowest IC_{50} exhibited the highest DPPH free-radical scavenging ability compared with FD and HD. Our results demonstrated that treatment of water caltrop hulls significantly influence the DPPH free-radical scavenging ability compared with BHT and α-tocopherol. Therefore, to reach similarly extensive reducing power, the concentration required for the FS extract was significantly lower than that required for BHT and α-tocopherol.

Table 3. The concentration of fifty percent inhibition (IC_{50}) of water caltrop hulls, BHT, and α-tocopherol[1]

Antioxidants[2]	IC_{50} (mg / mL)
FS	0.07 ± 0.00^{d}
FD	0.25 ± 0.02^{a}
HD	0.11 ± 0.01^{b}
BHT	0.09 ± 0.01^{c}
α-Tocopherol	0.07 ± 0.00^{d}

[a-e]Means with different letters are significantly different at a specific antioxidant attribute ($P < 0.05$).
[1]Each value is expressed as mean ± S.D. ($n = 3$).
[2]FS, FD and HD are water caltrop hull extracts of fresh, freeze-dried and hot air dried, respectively.

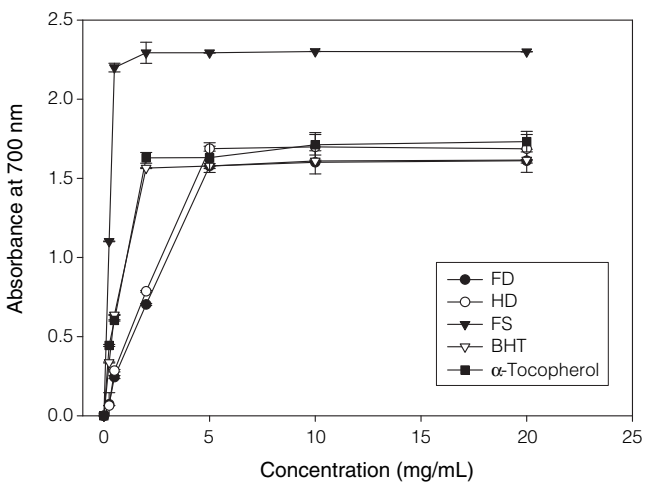

Figure 3. Reducing power of methanolic extracts of various water caltrop hulls, BHT, and α-tocopherol. *Each value represents Mean ± S.D. ($n = 3$). ** FS, FD and HD are water caltrop hull extracts of fresh, freeze-dried and hot air dried, respectively.

III. *Reducing Power*

The effect of methanolic extracts on the reducing power was compared to that of BHT and α-tocopherol in this study (Figure 3). It was found that the reducing power of water caltrop hull treatments was concentration dependent. Dose response studies indicated that before reaching a threshold level, there was a positively linear relationship between the reducing power and the concentration of extracts of different hulls. Compared with treatments of other antioxidants, the reducing power of FS extracts was found to be significantly more pronounced than that of HD and FD extracts, BHT, and α-tocopherol at ranges of 0 – 1.0 mg/mL. Reducing power of FS extracts reached its threshold level at dose level above 1.0 mg/mL. The absorbance at 700 nm was found to be 0.70 – 1.63 mg/mL for HD, FD, BHT and α-tocopherol, but up to 2.29 mg/mL for FS extracts at a dose level of 2.0 mg/mL. In other words, to reach a similar absorbance of reducing power at 700 nm with FS extracts, the concentration required for HD and FD extracts was about 3-fold that required for FS extracts.

IV. *The Relationship between Antioxidants Activity and Total Phenolics or Flavonoids Content*

Phenolic compounds are believed to account for a major portion of the antioxidant activity in many plants. Although some studies have demonstrated a linear correlation between content of total phenolic compounds and antioxidant[36], we found this may not be true across the three types of methanolic extracts of water caltrop hulls. The FS extracts with the highest total phenolics content exhibited the highest reducing power but no DPPH free-radical scavenging ability. The same phenomenon was existed in total flavonoids content. Among all the sample extracts, HD methanolic extracts was reported to possess the highest content of total flavonoids which exhibited the highest DPPH free-radical scavenging ability at a high dose level of methanolic extracts. Overall, there is no high correlation between antioxidant activity and total phenolics or flavonoids content. Thus, antioxidant activity is not fully contributed by total phenolics or flavonoids.

CONCLUSIONS

As compared to nuts, water caltrop hulls featured a reasonably substantial content of total phenolics. Regarding the antioxidant potential, water caltrop hulls

are an excellent source of polyphenols. The results of this study demonstrate that water caltrop hulls exhibit extremely high antioxidative capability, particularly in FS extracts, as measured by their reducing power. In addition, different dehydration processes might deteriorate the antioxidative effect. The results indicated that water caltrop hulls (more specifically, FS) are a potential source of antioxidants for application in food products, as compared with the commercial antioxidants of BHT and α-tocopherol. Nevertheless, on the basis of the results obtained, and based upon the consumption of water caltrop hulls, the alleged antioxidant properties might be somewhat beneficial to the antioxidant protection system of the human body, shielding against oxidative damage.

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