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Antioxidant Activity of Phenolic Compounds Extracted from Fresh and Dried Water Caltrop Pulp (*Trapa taiwanensis* Nakai)

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ABSTRACT

Water caltrop (*Trapa taiwanensis* Nakai) was used as the raw material to investigate the effects of drying treatments on the antioxidant activities and phenolic compounds of water caltrop. For methanolic extracts from fresh, freeze dried, and hot air dried samples, at 10 mg/mL, the scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals was 87.2%, 61.4% and 52.1%, respectively. At 10 mg/mL, the reducing power was 1.63, 0.81 and 0.52, respectively. At 40 mg/mL, the chelating ability on copper ions was 29.6%, 14.8% and 34.3%, respectively. The IC_{50}, half inhibition concentration is the concentration, at which the radicals or ions was scavenged or chelated by 50%. The total phenolics and flavonoids contents were higher in the fresh samples, while the IC_{50} values were lower. The HPLC analysis showed that four phenolic compounds (gallic acid, quercetin, ferulic acid, and hydroxycinnamic acid were found in water caltrop), and that only had three phenolic compounds (gallic acid, quercetin, and ferulic acid were found after drying). While the freeze-drying treatment could preserve more antioxidant activity of the water caltrop pulps than hot-air drying treatment, different drying water caltrop pulps exhibited different antioxidant mechanisms.

Key words: water caltrop, antioxidant activity, phenolic compounds, drying

INTRODUCTION

Vegetables are important to the human diet as they contain abundant dietary fibre, minerals and vitamins, and are often the important sources of natural nutritional antioxidants[1-5]. Vegetables contain a large quantity of non-nutritional antioxidants, such as flavoniods, flavones and phenolic compounds[1,6,7]. Flavonoids, a large group of plant phenolics, are present in plant tissues in relatively high concentrations and they act as antioxidants[8-10]. Previous research has shown that due to the high amount of antioxidants present in vegetables, including anthocyanin and phenolic compounds, they can be used to prevent cancer or cardiovascular diseases[8,11].

Water caltrop (*Trapa taiwanensis* Nakai) is the corm of sedge, which grows in water. Due to its special taste and medical function, it is one of the most popular vegetables in Asia, and is found in Taiwan, China, and parts of Southeast Asia. It is good for treating inflammation, swelling, sore mouth, and sore throats[12]. Phenolic compounds present in the water caltrop pulp cause browning easily[13]. It is difficult to store water caltrop because of the high content of moisture and carbohydrates. Therefore, farmers often carry out freezing and drying processes to achieve the objective of long-term storage. A few changes were reported in the contents and nutrients of many vegetables after freeze drying, and so did better color and appearance after the process[14]. The nutrients and antioxidants present in vegetables were influenced during processing. Masrizal, Giraus, and Driskell (1997)[15] used microwave heating, cooking, and stir-frying to cook vegetables, and found that the components such as vitamin C, irons and β-carotene, were affected. After cutting and freezing, changes occurred in the phenolic compounds present in lettuces. Crozier, Lean, McDonald, and Black (1997)[16] found that cooking reduces the quercetin content present in vegetables and fruits. Microwave heating and steaming caused more loss of quercetin than stir-frying. Ciou and Wang (2003)[17] found that the scavenging ability of ethanolic extracts of purple yam on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals decreased with increase in heating temperature and time. Lin and Chang (2005)[18] found that the broccoli samples, after different cooking treatments, still showed high reducing power, ferrous ion chelating ability, and DPPH radical scavenging ability. Nonetheless and Siddhuraju (2007)[19] found that the stability of antioxidant of dry heated *Tamarindus indica* seeds, through the concentration of extractable phenolics, relatively high, which might be due to the formation of Maillard reaction products.

To our knowledge, there are few reports on the

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effects of different drying procedures on the phenolic compounds of water caltrops. This work was thus aimed to study the effects of different drying procedures on the antioxidant properties and phenolic contents of water caltrops.

MATERIALS AND METHODS

I. *Materials*

Fresh dehulled water caltrops were obtained from a local supplier in Tainan, Taiwan. The fresh samples were stored at 4°C (moisture, 45-52%) from the farm to the lab. Freeze dried and hot air dried water caltrop pulps were used to prepare raw water caltrop powder. For freeze-drying, water caltrop pulps were dried in a freeze-dryer (FD-20L-6S, Kingmech Co., LTD, Taipei, Taiwan) (-40°C, 6 hr and rising to 20°C, 48 hr in vacuum), ground with a blender (DM-6, Rong Tsong Machinery Co., Taichung, Taiwan), and sieved through a 60-mesh (moisture, 5-7%) screen to obtain water caltrop pulps powder and stored at 4°C. For hot air-drying, water caltrop pulps were dried in an electric convection oven (60°C, 48 hr), then ground and sieved as above.

II. *Sample Extraction*

Fifty grams of fresh sample was homogenized in 250 mL of methanol, using the Osterizer (Sunbeam-Oster, IL, USA) at the "Hi" speed for 1 min. Fifty grams of each treatment of water caltrop hulls, including freeze and hot air dried samples were also added to 250 mL of methanol. Three kinds of samples were extracted by stirring, using a magnetic stirrer at 25°C for 24 hr. The extract was filtered through Whatman No. 1 filter papers to remove of hull particles. The residue was re-extracted with 250 mL of methanol and filtrated. The concentration of extract thus obtained was adjusted to 50 mg/mL by diluting with methanol and stored at -20°C for the further usage.

III. *Measurement of Radical-scavenging Ability*

The scavenging ability of water caltrop on the DPPH (Sigma Chemical Co., St. Louis, MO, USA) radicals was estimated according to the method of Yamaguchi *et al*. (1998)(20). An aliquot of water caltrop extract (50 mg/mL), α-tocopherol (vitamin E, Sigma, 50 mg/mL), or butylated hydroxytoluene (BHT, Sigma, 50 mg/mL) was mixed with the 100 mM Tris-HCl buffer (400 μL, pH 7.4) and then added to 500 μL of 250 mM DPPH in ethanol. The mixture was shaken vigorously and left to stand for 20 min at room temperature of 25°C in the dark. The absorbance of the resulting solution was measured at 517 nm in a Hitachi U-2000 spectrophotometer. The scavenging ability was calculated as follows:

scavenging ability (%) = [(A_{517} of control – A_{517} of sample) / A_{517} of control] × 100.

IV. *Measurement of Reducing Power*

The reducing power of the water caltrop extract, α-tocopherol, and BHT were determined according to the method of Yen and Chen (1995)[21]. The water caltrop 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 (pH 6.6) and 1% potassium ferricyanide (Sigma) and incubated at 50°C for 20 min. An equal volume of 1% trichloroacetic acid was the added to the mixture, which was then centrifuged at 4,000 ×g for 10 min at 4°C. The supernatant was mixed with 0.6 mL of distilled water and 0.12 mL of 0.1% ferric chloride (FeCl3, Sigma). The mixture was incubated at room temperature for 14 min and the absorbance was measured at 700 nm against a blank. Increased absorbance of the reaction mixture indicated the increased reducing power.

V. *Measurement of Cupric ions Chelating Ability*

Chelating ability was determined according to the method of Yang *et al*. (2000)[22]. Two milliliter of water caltrop extract was mixed with 30 mM hexamine (Wako Chem., Co., Germany), 30 mM potassium chloride (Sigma Chem., Co., USA), 9 mM copper sulfate and 0.2 mL of 1 mM tetramethyl murexide (Sigma Chem., Co., USA). After reaction for 3 min at room temperature, the absorbance of the mixture was determined at 485 nm. The lower absorbance indicated the higher cupric ions chelating ability of the test sample. The capability to chelate the cupric iron was calculated as follows:

chelating ability (%) = [(A_{485} of control – A_{485} of sample) / A_{485} of control] × 100.

VI. *Half inhibition Concentration, (IC_{50})*

Referring to the methods of Lai *et al*. (2001)[23], different concentrations of scavenging ability were entered in the equation:

$\ln [1 - (I_o/I_t)] = \pm kc$, where I_o represents the sample's scavenging ability; It represents the sample's highest scavenging ability; and $\ln [1 - (I_o/I_t)]$ was plotted into a graph against the concentration.

After computing the curve equation linear regression, an inhibition constant was obtained, and the value of $\ln [1 - (I_o/I_t)]$ was divided by the inhibition constant. A half inhibition concentration (IC_{50}) meant the concentration at which the radicals or ions was scavenged or chelated by 50%. The rate of antioxidation increases as IC50 value decreases.

VII. *Determination of Total Phenolics Contents*

The total phenolics contents of the fractions were determined according to the method of Sato *et al*. (1996)(24) with minor modification. One milliliter of each fraction was mixed with 400 μL of diluted Folin-Ciocalteu reagent (Sigma). After incubating for 3 min at room temperature, 40 μL of 10% sodium carbonate was added and then the mixture was incubated for 1 hr at room temperature. The absorbance at 735 nm was measured and converted to phenolics contents according to the calibration curve of gallic acid (mg GAE /g sample dry base, Sigma).

VIII. *Determination of Total Flavonoids Contents*

Total flavonoids content was determined by the colorimetric method described previously[25]. The mixture included 0.5 mL of water caltrop extracts and 0.5 mL of 2% aluminum chloride ($AlCl_3$, Sigma) ethanol solution. After reaction for 1 hr at room temperature, the absorbance was measured at 430 nm. Total flavonoids contents were calculated as quercetin (mg QE /g sample dry base, Sigma) from the calibration curve.

IX. *High Performance Liquid Chromatography (HPLC) Analysis*

Referring to the method of Lin *et al*. (2000)[26], 2 g of sample was added into 35.5 mL of 80% of methanol (containing 0.2% BHA, Sigma), and then added to 10 mL of 6 M HCl, shaken and mixed for 2 hr in a 90°C water bath, followed by placing in cold water for 30 min and methanol was added to 100 mL. The mixture was kept under ultrasonic waves for 5 min, and then centrifuged at 4,020 ×g (4°C) for 15 min. The sample was stored at 4°C and waiting to be tested. Two milliliter of sample suspension was used from the above extract. From 0.45 μm of filtrate, 20 μL of filtrate was used taken for HPLC analysis (Model L-7100 pump, L-2450 Diode Array Detector, Hitachi Co., Japan). Mightysil RP-18 GP 250×4.6 (5 μm) (Kanto Inc., Japan) was used as the separating column. Methanol (containing 300 μL/L trifluoroacetic acid, Sigma) was used as the mobile phase. With a flow rate of 0.5 mL/min, the absorbance at 280 nm was measured.

Phenolic standards, caffeic acid, chlorogenic acid, cinnamic acid, ferulic acid, gallic acid, *p*-hydroxybenzoic acid, hydroxycinnamic acid, kaempferol, narigenin, protocatechuic acid, quercetin, rutin, syringic acid and vanillin (all from Sigma) were used at a concentration of 10 ppm in methanol for the tentative identification of unknown peaks.

X. *Statistical Analysis*

Three samples extracted from three dried water caltrops were prepared for assays of every antioxidant attribute. All data were expressed as mean ± standard deviation. Analysis of variance was performed by SAS ANOVA procedures (SAS Institute Inc., Cary, NC, USA, 1988). Duncan's new multiple range tests were used to determine the differences of means, and $p < 0.05$ was considered to be statistically significant. For the correlations, the CORR procedure (SAS) was used to determine the correlation coefficient (r) between total flavonoids or total phenolics and each antioxidant attribute.

RESULTS AND DISCUSSION

I. *Scavenging Ability of DPPH Radical of Water Caltrop*

The scavenging abilities of methanolic extracts of water caltrop pulps from different drying treatment on DPPH radicals were shown in Figure 1. At concentration of 10 mg/mL, the DPPH scavenging ability of methanol extracted from the fresh water caltrop was 84.15%, which was greater than freeze dried extracts (61.01%) and hot air dried extracts (52.95%). With the increase of concentrations, there was an increasing trend in the scavenging ability. At a concentration of 20 mg/mL, there was a gradual phenomenon of stable trend. The scavenging ability of α-tocopherol and BHT methanolic solutions at a concentration of 10 mg/mL was 97.61% and 95.24%, respectively, which is 1.13-1.15 folds higher than that of fresh water caltrop, 1.56-1.60 folds higher than that of freeze dried extracts at 50°C, and 1.80-1.86 folds higher than that of hot air dried extract. Ciou and Wang (2003)[17] pointed out that α-tocopherol, BHT, BHA could provide hydrogen atoms, eliminate DPPH within a short period of time to obtain stability, and resist free radical materials, and their antioxidation power is also stronger than the fresh yam sample. The proton radical-scavenging ability is known to be one of the various mechanisms for antioxidation. DPPH

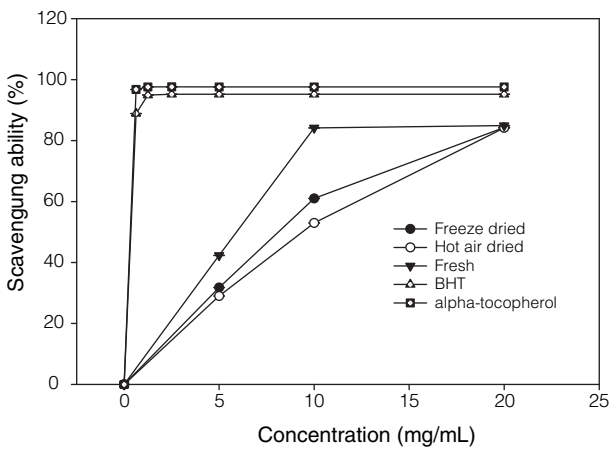

Figure 1. Scavenging ability of methanolic extracts from water caltrop pulps on DPPH radicals. Each value was expressed as mean ± standard deviation ($n = 3$).

is one of the compounds that possesse proton-free radicals and show a characteristic absorption at 517 nm (purple). When DPPH encounters proton radical scavengers, its purple color would fade rapidly[27]. The results implied that the antioxidant activity of water caltrop may be attributed to its proton-donating ability.

II. *Reducing Power of Water Caltrop*

The presence of reductants (antioxidants) in the sample would result in the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. The Fe^{2+} could, therefore, be monitored by measuring the formation of Perl's Prussian blue with absorbance at 700 nm. Increased absorbance at 700 nm indicated an increase in reducing power. The strengthening compounds of reducing power had a stronger peroxide reducing ability. As shown in Figure 2, although the greatest reducing power was observed in α-tocopherol or BHT relative to water caltrop, the reducing power of water caltrop was evident.

The reducing power of methanolic extracts from the water caltrops from different treatments is shown in Figure 2. At concentration of 5 mg/mL, the reducing power of methanol extracts from the fresh water caltrop was 0.78, which is higher than that of freeze dried extracts (0.35) and hot air dried extracts (0.26). With the increase in the concentrations, there was an increasing trend in the reducing power.

At concentration of 10 mg/mL, there was a gradual phenomenon of stable trend. Comparing the α-tocopherol and BHT methanol solutions at concentration of 5 mg/mL, BHT methanol solutions had a reducing power of 1.65 and α-tocopherol had a reducing power of 1.45, which is 2.0 folds higher than that of the fresh water caltrop, 3.1 folds higher than that of freeze dried extracts and 5.1 folds higher than that of hot air dried extracts. However, at concentration of 20 mg/mL, the reducing power of the fresh water caltrop and methanol extracts was 2.20, which is higher than α-tocopherol and BHT. These results revealed that water caltrop extract is an electron donor and could react with free radicals, convert them into more stable products and terminate the radical chain reaction[28].

III. *Cupric Ions Chelating Ability of Water Caltrop*

As shown in Figure 3, at concentration of 5 mg/mL, the Cu^{2+}-chelating ability of methanol extracted from the hot air dried extracts was 11.64%, which is greater than the freeze dried extracts (2.08%) and fresh extracts (1.34%). With the increase in the concentration, there was an increasing trend in the scavenging ability. With regard to methanol extracts at 20 mg/mL, *H. marmoreus* chelated 84.2% of cupric ions[29]. Compared with any antioxidants in this study, EDTA exhibited more pronounced effect than extracts of three treatments of water caltrop pulps at dose ranges of 0-40 mg/mL. The

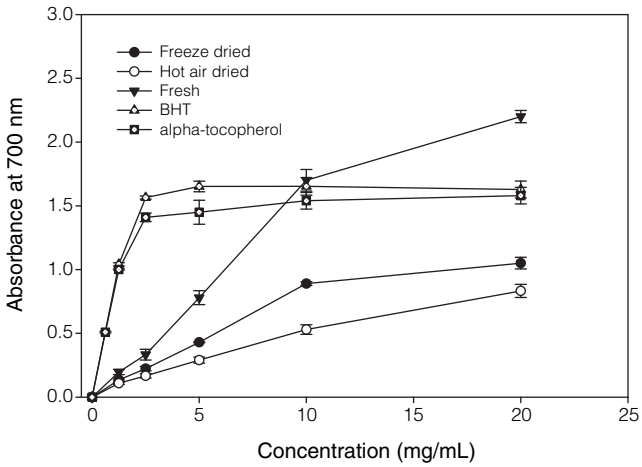

Figure 2. Reducing power of methanolic extracts from water caltrop pulps. Each value was expressed as mean ± standard deviation (n = 3).

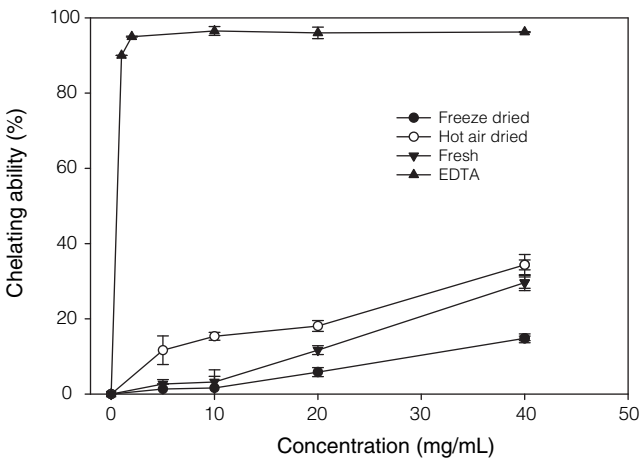

Figure 3. Chelating ability of methanolic extracts from water caltrop pulps on cupric ions. Each value was expressed as mean ± standard deviation (n = 3).

chelating ability of EDTA increased sharply to reach a threshold level up to 95%, at a dose of 2 mg/mL.

However, among the various samples, hot air dried extracts were found to register the highest Cu^{2+}-chelating ability. Most of the natural antioxidant compounds, such as ascorbic acid, are relatively unstable[30]. This loss of chelating ability due to prolong heating can be minimized by recovery or even enhancement of the antioxidant activity due to the formation of advanced Maillard reaction products. Since cupric ions were commonly found in food systems[31], and moderate cupric-ion chelating ability of hot-air dried of water caltrop pulp extracts would be beneficial.

IV. *Half-inhibition Concentrations (IC_{50}) for Antioxidant Activity of Water Caltrop*

As shown in Figures 1, 2 and 3, the antioxidant effect

exerted various antioxidants were strongly concentration dependent. In general, the antioxidant activity increased with increasing antioxidant concentration to a certain extent and then leveled off with further increase in antioxidant concentration. On the basis of the half-inhibition concentration (IC_{50}) results, BHT was found to show the most effective DPPH radical-scavenging ability, followed by α-tocopherol and water caltrop extract in descending order (Table 1). The difference was statistically significant ($P < 0.05$). The IC_{50} values for water caltrop extracts were about 64.7–106.7 folds higher than that of α-tocopherol. In other words, to reach a similar extent of DPPH-scavenging ability, the concentration required for water caltrop extract was significantly higher than that required for α-tocopherol. The increasing on reducing power and the chelating ability of cupric ions were still occured. In terms of reducing power and cupric ions chelating ability, it seemed that water caltrop extracts was no effective.

V. *Total Flavonoids Contents and Total Phenolics Contents of Water Caltrop*

The concentration of flavonoids in the extracts, expressed as quercetin equivalents (QE), was determined in the methanol extraction, as shown in Table 2. The amount of flavonoid compounds in the fresh water caltrop extracts was 7.01 mg/g, which is higher than that of freeze dried extracts (1.15 mg/g) and hot air dried extracts (1.27 mg/g). Phenolic compounds such as BHT and gallate were known to be effective antioxidants[32]. Yen *et al.* (1993)[33] found that the antioxidant activity of the methanolic extract from peanut hulls was correlated with its content of total phenols. Therefore, the high content of total phenols in all methanolic vegetable extracts might explain their high antioxidant properties. In general, extracts or fractions with a high antioxidant activity showed a high antioxidant content as well, but good correlations might not be found among them. The correlation coefficients between total flavonoids content

Table 1. Antioxidant activities of water caltrops, BHT, and α-tocopherol as showed by half-inhibition concentrations (IC_{50})

Antioxidant attribute	Samples	IC_{50} *(mg / mL)
Scavenging ability on DPPH radicals	Fresh	5.83 ± 0.35[c]
	Freeze dried	8.13 ± 0.29[b]
	Hot air dried	9.60 ± 0.21[a]
	BHT	0.09 ± 0.01[d]
	α-Tocopherol	0.07 ± 0.00[d]
Reducing power	Fresh	___**
	Freeze dried	___
	Hot air dried	___
	BHT	0.83 ± 0.01[a]
	α-Tocopherol	0.94 ± 0.01[a]
Chelating ability on cupric ions	Fresh	___
	Freeze dried	___
	Hot air dried	___
	EDTA	0.03 ± 0.00

* Each value was expressed as mean ± standard deviation ($n = 3$).
** No effect.
[a-d] Means with different letters within the same column at a specific antioxidant attribute are significantly different ($P < 0.05$).

Table 2. Total flavonoids and phenolics contents of methanolic extracts from various dried water caltrops

Samples	Total phenolics[1] (mg GAE /g)[2]	Total flavonoids[1] (mg QE / g)[2]
Fresh	64.51 ± 0.57[a]	7.01 ± 0.98[a]
Freeze dried	63.72 ± 1.88[a]	1.15 ± 0.07[b]
Hot air dried	63.80 ± 0.51[a]	1.27 ± 0.01[b]

[1] Each value was expressed as mean ± standard deviation ($n = 3$).
[2] GAE = gallic acid equivalent ; QE = quercetin equivalent
[a-b] Means with different letters within the same column at a specific antioxidant attribute are significantly different ($P < 0.05$).

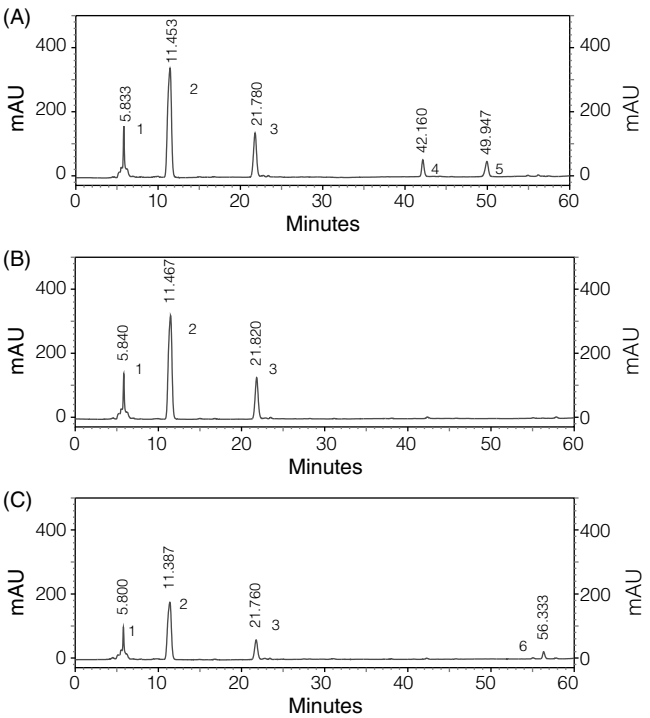

Figure 4. HPLC analysis of the different methanolic extracts from water caltrop pulps. (A) Fresh (B) Freezed dried (C) Hot air dried (Peak 1. gallic acid 2. quercetin 3. ferulic acid 4. hydroxycinnamic acid 5. unknown 6. unknown)

and antioxidant activities were 0.99, 0.97 and 0.99 for scavenging ability on DPPH radicals, reducing power and chelating ability on cupric ions, respectively. It seems that total flavonoids content was responsible for the antioxidant activities of dried water caltrops.

The amount of total flavonoids of fresh samples is shown in Table 3. It is assumed that low temperature processing might accelerate the oxidative and hydrolytic enzyme to destroy antioxidants in vegetables and fruits. Dewanto *et al*. (2002)[34] and Caro *et al*. (2004)[35] had reported that heating treatments lead to a slight decrease in the amount of total phenolics and total flavonoids in the tomato and several fruits, respectively. However, some researchers mentioned that heat treatment resulted in higher total flavonoids than fresh samples because many biochemical reaction occurred during heat processing[36,37].

Results of HPLC analysis are shown in Figure 4 and Table 3. The water caltrop extracts had only six compounds that were found from 14 authentic phenolic compounds standards. As shown in Table 3, the amount of gallic acid detected in the fresh water caltrop extracts, freeze dried extracts and hot air dried extracts was 21.05, 14.02 and 6.23 μg/mL dry weight, respectively. The amount of quercetin was 97.03, 90.14 and 84.01 μg/mL dry weight, respectively. The amount of hydroxycinnamic acid detected in the fresh water caltrop extracts was 7.90 μg/mL dry weight, but no hydroxycinnamic acid was detected in the freeze dried extracts and hot air dried extracts because heating or drying, caused the loss of hydroxycinnamic acid. No vanillin, rutin and cinnamic acid were detected. According to Yurttas *et al*. (2000)[38] method, the retention time was confirmed with standards to exact phenolic compounds. However, the concentration of phenolics in the extracts, expressed as mg/GAE g sample, was dependent on the solvent and the method used in the extraction, as shown in Table 2. The amount of total phenolic compounds in the water caltrop extracts was 63.80–64.51 mg/g. There was no significant difference ($P < 0.05$) among three extracts (Table 2). The correlation coefficient between total phenolics content and antioxidant activities was 0.99, 0.94 and 0.99 for scavenging ability on DPPH radicals, reducing power and chelating ability on cupric ions, respectively. Similarly, total phenolics content was responsible for the antioxidant activities of dried water caltrops.

CONCLUSIONS

Based on the results obtained, the alleged higher antioxidant properties that the methanolic extracts displayed might be somewhat beneficial to the antioxidant protection system of the human body against oxidative damage. Therefore, the methanolic extracts from three different pulps of water caltrop might be a potential antioxidant supplement for application in food products. Since six phenolic compounds were found in the extract, the study on the contribution of phenolic compounds to antioxidant properties of methanolic extracts is still in progress. Due to the fact that most of the nature antioxidant compounds are relatively unstable, different drying water caltrop pulps could be taken as different antioxidant alternatives.

ACKNOWLEDGEMENTS

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Table 3. Comparison of the HPLC retention time of the selected major peaks of the methanolic extracts form various treatment water caltrop pulps with phenolic standards

Peak Numbers	Retention Time	Standards	Content * (μg / 100 g, dry weight)		
			Fresh	Freeze dried	Hot air dried
Peak 1	5.83	gallic acid	21.05 ± 0.02[a]	14.02 ± 0.04[b]	6.23 ± 0.09[c]
Peak 2	11.45	quercetin	97.03 ± 0.35[a]	90.14 ± 0.15[b]	84.01 ± 0.23[c]
Peak 3	21.78	ferulic acid	32.10 ± 0.14[a]	26.72 ± 0.09[b]	12.03 ± 0.11[c]
Peak 4	42.16	hydroxycinnamic acid	7.90 ± 0.05	nd**	nd
Peak 5	49.95	unknown	-***	nd	nd
Peak 6	56.33	unknown	nd	nd	-***

* Each value was expressed as mean ± standard deviation ($n = 3$).

** nd means not detected

***- means no relative standard components

[a-c] Means with different letters within the same rows at a specific antioxidant attribute are significantly different ($P < 0.05$)

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