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In vitro Antioxidative Activities and Phenolic Composition of Hot Water Extract from Different Parts of *Cudrania tricuspidata*

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Abstract

We evaluated total phenolics and antioxidative activities of water extracts from different parts of *Cudrania tricuspidata* (specifically, the leaves, stems, roots, and fruits). The antioxidative activities of these samples were determined using five methods, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging, reducing power, ferric reducing ability of plasma (FRAP), and β-carotene/linoleic acid system. The water extract of leaves exhibited the higher DPPH, ABTS radical scavenging activities, reducing power, and FRAP than water extract of stem, roots, and fruits. Inhibition values on linoleic oxidation of water extracts from leaves, stems, roots, and fruits were calculated as 45.98%, 33.03%, 39.73%, and 25.48% at 10 mg/mL, respectively. The water extract of *C. tricuspidata* leaves had the highest amount of toal phenolics (73.60±0.28 mg/g). High-performance liquid chromatography (HPLC) analysis showed that quercetin is the predominant phenolic compound in water extract of leaves. Thus, our study verified that the water extract of leaves has strong antioxidant activities which are correlated with its high level of phenolic compounds, particularly quercetin. This water extract of *C. tricuspidata* leaves can be used as an effective and safe source of antioxidants.

Key words: *Cudrania tricuspidata*, total phenolics, antioxidant activities, quercetin

INTRODUCTION

Polyphenols are found at different concentrations in a variety of food types, including vegetables, fruits, chocolate, tea, coffee, wine, grape juice, and vinegar (1). Polyphenols are bioactive compounds that display antioxidant properties. Because of these properties, they are believed to be involved in the defense process against deleterious oxidative damage (2). Phenolic acids have been widely investigated as potential models for the development of new primary antioxidants, which can prevent and delay *in vitro* or *in vivo* oxidation processes (3). These phenolic compounds are powerful antioxidants that act in a structure-dependent manner; they can scavenge reactive oxygen species (ROS), and chelate transition metals which play vital roles in the initiation of deleterious free radical reactions (2). Because purified phenolic compounds are difficult to obtain and because extracts sometimes display more effective antioxidant activities than those of pure molecules, there is a growing interest for the use of plant extracts (4). Efforts have been made to search for selective and efficient antineoplasic agents to control tumor cell growth. Recent studies have shown that increased consumption of vegetables and fruits is associated with a decreased risk of cancer (5,6). Natural antioxidant phenolic acids and their derivatives, either present in the diet or synthetically prepared, were shown to have promising chemopreventive properties that are now being identified as promising agents for future development (7). To find new natural sources of active compounds, we studied the antioxidant potential of water extract from different parts of *Cudrania tricuspidata*.

C. tricuspidata is one of the most ubiquitous traditional herbal remedies in East Asia. This plant's beneficial effects have been traditionally associated with anti-inflammatory (8), anti-tumor (9), and α-glucosidase inhibition activity (10). Recently, it was reported that *C. tricuspidata* have antifungal (11), anti-lipid peroxidative (12), antioxidant (13) and cytotoxic activities (14).

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Although it has already been demonstrated that *C. tricuspidata* root (15) and root bark (16) contain phenolic compounds, little is known about their antioxidant potential or the other parts of *C. tricuspidata*, such as leaves, stem, and fruits. Since the antioxidant activities of water extracts from different parts of *C. tricuspidata* have not previously been reported, the objectives of this study were to measure the antioxidant activities of water extracts from different parts of *C. tricuspidata*, and identify the antioxidant active compounds of water extracts by HPLC.

MATERIALS AND METHODS

Chemicals

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), β-carotene, linoleic acid, polyoxyethyllene sorbitan monoplamitate (Tween-40), α-tocopherol, ascorbic acid, and all solvents used were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material

The different parts of *C. tricuspidata* were collected from Sancheong, Korea in September, 2007, which was authenticated by Instituted of Agriculture & Life Sciences, Gyeongsang National University where voucher specimens are maintained.

Extraction from the different parts of *C. tricuspidata*

Water extracts of freeze-dried components of *C. tricuspidata* (60 mesh particle size) were obtained as follows. The different parts of *C. tricuspidata* were in powdered form (100 g) and were suspended and extracted with 1,000 mL of water at 100°C for 2 hr. The extracts were filtered through Whatman No. 2 filter paper (Whatman International Limited, Kent, England) and evaporated to dryness. Each water extract was concentrated in a vacuum evaporator at 40°C. Water filtrate was frozen and lyophilized. The extracts were place in a glass bottle and stored at -20°C until used. The lyophilized extracts were re-dissolved in water to a concentration of 10 mg/mL.

DPPH free radical-scavenging activity

The free radical-scavenging activity was carried out according to Blois method with a slight modification (17). 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 extract solution in water containing 0.3～10 mg/mL of dried extract; finally, after 30 min, the absorbance was measured at 517 nm (UV-1201, Shimadzu, Tokyo, Japan). This activity is given as percent DPPH scavenging that is calculated as % DPPH scavenging = [(control absorbance − extract absorbance)/ (control absorbance)] × 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 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 (18). This activity is given as percent ABTS scavenging that is calculated as % ABTS scavenging activity = [(control absorbance − extract absorbance)/ (control absorbance)] × 100.

Reducing power

This assay was carried out by Oyaizu (19). 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, which was then centrifuged at 3,000 rpm 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, Tokyo, Japan).

Ferric reducing ability of plasma (FRAP)

The FRAP assay developed by Benzie and Strain was used (20). Briefly, 1.5 mL of working solution, 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 sample and standards. This was vortex mixed and absorbance at 593 nm was read 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. (21). In brief, 0.2 mg β-carotene dissolved in 1 mL chloroform, 20 mg of linoleic acid and 200 mg of 20 were transferred into around-bottom flask. Once the chloroform had been removed under nitrogen, 50 mL distilled H_2O was added and the resulting mixture was vigorous stirred for 30 min. Thereafter, 5 mL aliquots of this emulsion were transferred tubes containing either 200 μL of dissolved extract (1.25, 2.5, 5, and 10 mg/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 data (n=3) are presented as antioxidant activity % (AA%) values, calculated using $AA\% = [1 - (Abs_0 sample - Abs_{120} sample)/ (Abs_0 control - Abs_{120} control)] \times 100$.

Determination of total phenolics

Total phenolics were determined by the spectrophotometric analysis (22). In brief, a 1 mL portion of appropriately diluted extracts was added to a 25 mL volumetric flask containing 9 mL of ddH_2O. A reagent blank using ddH_2O was prepared. One mL of Folin-Ciocalteu's phenol reagent was added to the mixture and then shaken. After 5 min, 10 mL of a 7% Na_2CO_3 solution was added with mixing. The mixed solution was then immediately diluted to volume (25 mL) ddH_2O and mixed thoroughly. After 90 min at 23°C, the absorbance was read at 750 nm. The standard curve for total phenolics was made using gallic acid standard solution (0～100 mg/L) under the same procedure as above. Total phenolics in water extract from different parts of *C. tricuspidata* were expressed as milligrams of gallic acid equivalents (mg/g GAE) of dried sample.

Determination of flavonols

One gram of sample was mixed with 40 mL of 60% aqueous ethanol and 5 mL of 6 M HCl. After refluxing at 95°C for 2 hr, the hydrolysed solution was filtered into a 50 mL volumetric flask and subsequently made up to the volume with 60% aqueous ethanol. Approximately 1 mL of the final solution was allowed to cool under running water and filtered through a 0.45 μm filter (Nylon Acrodisc 13 Gelman, Ann Arbor, MI, USA) prior to injection for Agilent HPLC (1100 series, Agilent Co., Santa Clara, CA, USA) analysis. The analysis of flavonols (myricetin, quercetin, and kaempferol) in sample was carried out by the following HPLC method. Agilent 1100 series liquid chromatograph system, comprising vacuum degasser, quaternary pump, auto-sampler, thermostated column compartment, and diode array detector, was used. The column was a LiChrospher RP-18 column (250 mm×4.6 mm i.d., 5 μm, E. Merck Co., Darmstadt, Germany). Mobile phase consisted of 30% acetonitrile in 0.025 M KH_2PO_4 buffer solution (v/v); the pH of the mobile phase was adjusted with 6 M HCl to 2.5. The flow rate was 1.0 mL/min. The column was operated at 30°C. The sample injection volume was 20 μL. UV spectra were recorded from 200 and 400 nm, and peak areas were measured at 370 nm (23).

Determination of phenolic compounds

Phenolic compounds were measured at 280 nm using an Agilent HPLC (1100 series, Agilent Co., Santa Clara, CA, USA). Separation was achieved with a LiChrospher RP-18 column (250 mm×4.6 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 to those of standards. Content of phenolic compounds was expressed in mg/100 g extract (24).

Statistical analysis

All data were expressed as mean±SD. Each experimental set was compared with one-way analysis of variance (ANOVA) and Duncan's multiple-range test ($p<0.05$) using SAS program (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Scavenging effect on DPPH radical

The DPPH radical scavenging activities of the water extracts from different parts of *C. tricuspidata* were estimated by comparing the percentage inhibition of DPPH radicals by the extracts and those of α-tocopherol and ascorbic acid. It was found that the radical-scavenging activities of positive control and water extract from different parts of *C. tricuspidata* increased with increasing concentration (Fig. 1). The ability to scavenge DPPH radicals of water extracts from different parts of *C. tricuspidata* were in the order of leaves (90.63%)> root (58.68%)> stem (28.49%)> fruit (7.37%) at the concentration of 1.25 mg/mL. A 1.25 mg/mL of α-tocopherol and ascorbic acid exhibited 92.84% and 95.14% scavenging activities, respectively. All of the extract had a scavenging activities on the DPPH radicals in a dose dependent manner. The non-enzymatic antioxidants like vitamin E, vitamin C and glutathione (GSH) also play a critical role in scavenging the free radicals (25). Nonetheless, when compared to α-tocopherol and ascor-

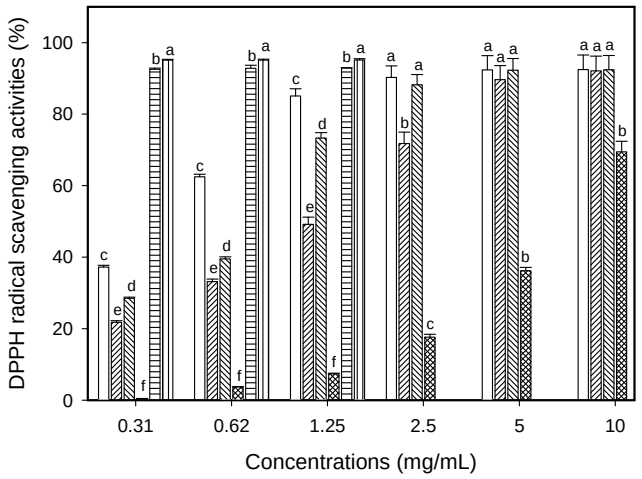

Fig. 1. DPPH radical scavenging activities of water extract from different parts of *C. tricuspidata*. ▭: water extract of leaves, ▨: water extract of stems, ▩: water extract of roots, ▩: water extract of fruits, ▭: α-tocopherol, ▥: ascorbic acid. [a-f]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

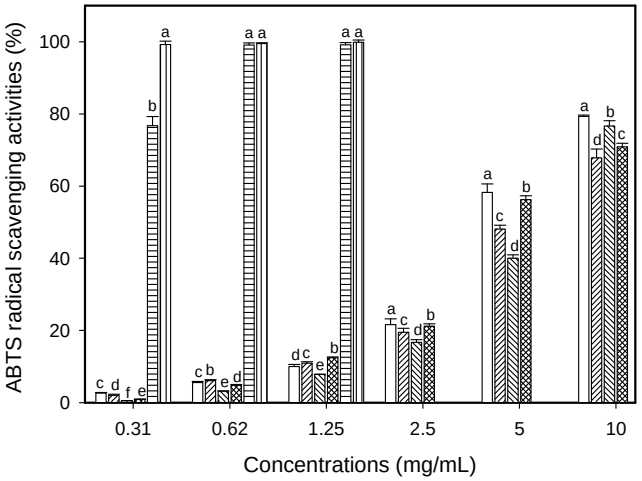

Fig. 2. ABTS radical scavenging activities of water extract from different parts of *C. tricuspidata*. ▭: water extract of leaves, ▨: water extract of stems, ▩: water extract of roots, ▩: water extract of fruits, ▭: α-tocopherol, ▥: ascorbic acid. [a-f]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

bic acid, the DPPH radical scavenging activities of the extract was found to be low.

An almost linear correlation between DPPH radical scavenging activity and concentrations of polyphenolic compounds in various vegetable and fruits have been reported (26,27). This indicated that DPPH radical scavenging activities of water extracts from different parts of *C. tricuspidata* were related to the amount of antioxidative substance extracted from different parts of *C. tricuspidata* by water. These results revealed that water extract of *C. tricuspidata* leaves have free radical scavengers, which possibly act as primary antioxidants.

Scavenging effect on ABTS radical

The water extracts from different parts of *C. tricuspidata* exhibited ABTS radical scavenging activities to different extents in a concentration-dependent manner, although the activities levels of all of the tested samples were lower than that of α-tocopherol and ascorbic acid.

The water extract from leaves of *C. tricuspidata* exhibited the highest radical scavenging activities when reacted with the ABTS radicals (Fig. 2). In contrast, the water extract of stems only showed low activities. Fig. 2 demonstrates the steady increase in the percentage inhibition of the ABTS radicals by the water extract from *C. tricuspidata* leaves. Maximum inhibition was achieved above 10 mg/mL.

Reducing power

In the reducing power assay, the presence of reductants (antioxidants) in the samples results in the reduction of Fe^{3+} to Fe^{2+} by the donation of an electron. The amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (28). Increasing absorbance at 700 nm indicates an increase in reductive ability. Fig. 3 shows the dose-response curves for the reducing powers of the water extracts from different parts of *C. tricuspidata*. It was found that the reducing powers of all the water extracts also increased with the increase of their concentrations. The water extracts from different parts of *C. tricuspida-*

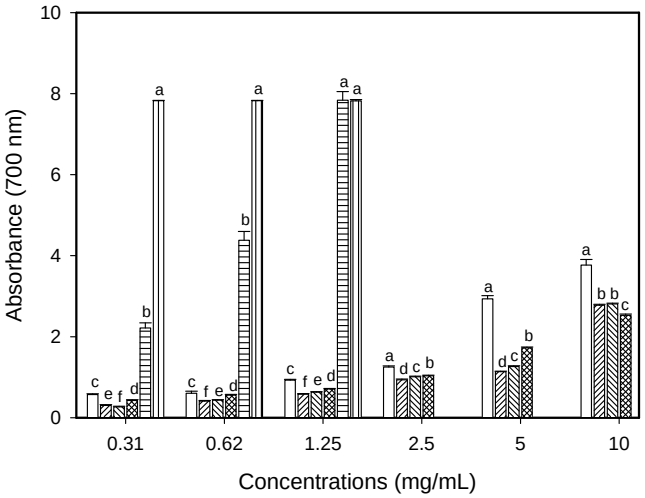

Fig. 3. Reducing power of water extract from different parts of *C. tricuspidata*. ▭: water extract of leaves, ▨: water extract of stems, ▩: water extract of roots, ▩: water extract of fruits, ▭: α-tocopherol, ▥: ascorbic acid. [a-f]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

ta, including leaves, stem, root, and fruit, exhibited a good reducing power of 3.77, 2.78, 2.81, and 2.53 at 10 mg/mL, respectively. From these observations, it is suggested that water extract of leaves has a remarkable potency to react with free radicals to convert them into more stable non-reactive species and to terminate radical chain reaction.

Ferric reducing ability of plasma (FRAP)

In the FRAP assay, samples are used in a redox-linked reaction where 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 reductant in the samples. In the present study, the trend for ferric ion reducing activities of water extract from different parts of *C. tricuspidata* is shown in Fig. 4. For water extract of leaves, stem, root, and fruit, the absorbance clearly increased, due to the formation of the Fe^{2+}-TPTZ complex, with increasing concentration. The highest reducing activity was found in water extract of leaves, compared to those of the other extracts of parts (Fig. 4). Similar to the results obtained from the DPPH and ABTS assay, water extract of leaves showed relatively strong ferric ion-reducing activity. Water extract of stem showed lower ferric ion-reducing activities.

The water extracts from leaves, stem, root, and fruit of *C. tricuspidata* exhibited good reducing activities of 1.31, 0.63, 0.91, and 0.89 at 10 mg/mL, respectively.

A strong correlation between the mean values of the total polyphenol content and FRAP (r^2=0.6477) deserves closer attention, as it implies that polyphenols in water extracts from different parts of *C. tricuspidata* are capable of reducing ferric ions (29). Some authors have reported similar correlations between polyphenols and antioxidant activity measured by various methods (30,31).

β-Carotene-linoleic acid bleaching inhibition

In this oil-water emulsion-based system, linoleic acid undergoes thermally induced oxidation, and thereby produces free radicals that attack the β-carotene's chromophore, resulting in a bleaching effect (21). An extract that inhibits β-carotene bleaching can be described as a free-radical scavenger and a primary antioxidant (32). As shown in Fig. 5, the water extract from different parts of *C. tricuspidata* demonstrated an ability to inhibit the bleaching of β-carotene by scavenging linoleate-derived free radicals. The water extract from different parts of *C. tricuspidata* were also capable of inhibiting the bleaching of β-carotene in a manner dependent on concentration. The inhibition effect of four water extracts from *C. tricuspidata* decreased in the following order: leaves (45.98%)> root (39.73%)> stem (33.03%)> fruit (25.48%) at 10 mg/mL, respectively. However, this activity was less than that seen with the positive controls, α-tocopherol (89.03%) and BHA (91.28%) at a concentration of 1 mg/mL. This difference is likely the result of the extracts containing a much lower concentration of antioxidant compounds than the positive controls.

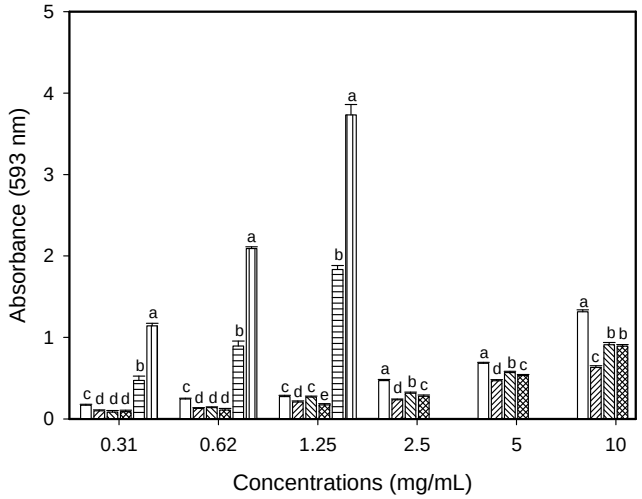

Fig. 4. Ferric reducing activities of water extract from different parts of *C. tricuspidata*. ▭: water extract of leaves, ▨: water extract of stems, ▩: water extract of roots, ▩: water extract of fruits, ▭: α-tocopherol, ▥: ascorbic acid. [a-f]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

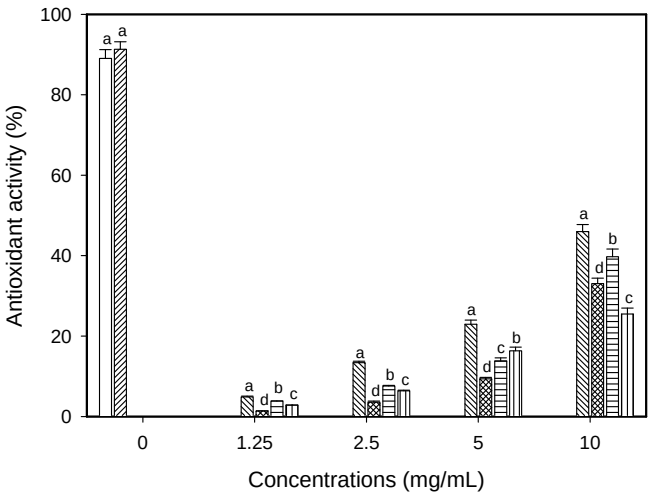

Fig. 5. Antioxidant activity of water extract from different parts of *C. tricuspidata* by β-carotene-linoleic acid bleaching method. ▭: α-tocopherol, ▨: BHA, ▩: water extract of leaves, ▩: water extract of stems, ▭: water extract of roots, ▥: water extract of fruits. [a-f]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

Table 1. Total phenolic content of water extract from different parts of *C. tricuspidata*

	Content (mg GAE/g[1)])
Leaves	73.60±0.28[2)a3)]
Stems	57.44±1.21[c]
Roots	70.56±0.36[b]
Fruits	56.42±1.98[c]

[1)]Gallic acid equivalent (mg/g).
[2)]The values are means±SD of three experimental data.
[3)]Data were significantly different with one-way ANOVA followed by Duncan's multiple range test at the 0.05 level of significance.

Table 2. Phenolic composition of water extract from leaves of *C. tricuspidata*

	Content (mg/100 g)
Quercetin	106.67±2.83[1)]
Kaempferol	49.24±2.35
Myricetin	44.28±1.18
Gallic acid	41.83±2.14
Epicatechin	7.66±0.17
Catechin	4.87±0.08
Chlorogenic acid	1.51±0.21
Epicatechin gallate	1.00±0.06
Epigallocatechin	ND
Epigallocatechin gallate	ND

[1)]The values are means±SD of three experimental data.
ND: Not detected.

Total phenolics of *C. tricuspidata* and phenolic composition of leaves

The Folin-Ciocalteau assay is a fast and simple method to determine total phenolics content in plant materials (33). Phenolics or polyphenols are secondary plant metabolites that are ubiquitously present in plants and plant products. Many of the phenolics have been shown to contain high levels of antioxidant activities. The total phenolic content of the water extracts from different parts of *C. tricuspidata* are presented in Table 1. The water extract from *C. tricuspidata* leaves exhibited the highest total phenolic content at 73.60 mg GAE/g , followed by the roots (70.56 mg GAE/g)> stems (57.44 mg GAE/g)> fruits (56.42 mg GAE/g). Since the water extract of *C. tricuspidata* leaves exhibited the strongest antioxidant activity, it was subjected to further analysis by HPLC. Water extract of *C. tricuspidata* leaves contained a variety of phenolic compounds. By comparing the retention time of these compounds with those of standards, three flavonols (quercetin, kaempferol, and myricetin) and five phenolic compounds (gallic acid, epicatechin, catechin, chlorogenic acid, and epicatechin gallate) were identified (Table 2). Furthermore, the HPLC results (Table 2) indicated that quercetin (106.67 mg/100 g) was the predominant flavonol in this extract, followed by kaempferol (49.24 mg/100 g) and myricetin (44.28 mg/100 g). Flavonoids and phenolic acids are important contributing factors to the antioxidant activity of the human diet. Based on the results of the phenolic composition of water extract from *C. tricuspidata* leaves, we can conclude that these compounds (particularly quercetin and gallic acid) contribute to the antioxidant activities of the extract. The results obtained in this work are noteworthy, not only with respect to the antioxidant activities of water extract of *C. tricuspidata* leaves, but also with respect to the variety of phenolic compounds found to be present in the extract. The activity of this extract is attributed to these phenolic compounds and, in particular, to quercetin, and gallic acid. Finally, our results verified that water extract of *C. tricuspidata* leaves has very strong antioxidant activities and can be used as an effective antioxidant source, although the antioxidant activities of water extract from leaves were lower than that of α-tocopherol and ascorbic acid.

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