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RESEARCH ARTICLE

Studies on the antioxidant activity and phenolic compounds of enzyme-assisted water extracts from Du-zhong (*Eucommia ulmoides* Oliv.) leaves

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Abstract

Enzyme-assisted water extracts (EWEDL) and ethanol extracts of Du-zhong leaves (EEDL) were evaluated for their antioxidant activities using the DPPH radical-scavenging assay, Fe^{2+}-chelating assay, and inhibition ability of the linoleic acid peroxidation assay. In general, the antioxidant activity of Du-zhong leaf extracts increased with increasing concentration. Based on the two extracting methods with different antioxidative reactions, it was shown that the enzyme-assisted water extracting method was more effective for antioxidant extraction from Du-zhong leaves. By HPLC-MS analysis, the main phenolic compounds (geniposidic acid, epicatechin, and chlorogenic acid) identified in EWEDL and EEDL were similar. EWEDL and EEDL had total phenolic contents of 13.84 ± 0.11 and 14.72 ± 0.14 mg chlorogenic acid equivalents (CAE) in each gram of extract, respectively. However, there was no positive correlation between total phenolic content and antioxidant activities of EWEDL and EEDL measured by the three different assays.

Keywords: *Antioxidant; extraction; enzyme; Du-zhong leaves; phenolic compound;* Eucommia ulmoides

Introduction

Du-zhong (*Eucommia ulmoides* Oliv.) is an important tonic herb widely used in China, Japan, and Korea. According to ancient records, Du-zhong possesses many pharmacological effects, including reinforcement of the muscles and lungs, prevention of abortion, lowering of blood pressure, and antioxidant activity[1,2]. Previously, only the bark of the Du-zhong was thought to contain the medicinally effective components, but lately, interest has focused on the leaves, as the bark of *Eucommia ulmoides* Oliv. tree can only be peeled off after 20 years, and after that the tree will probably die[3]. Moreover, it has been reported that Du-zhong leaves contain similar medical components and nutrient components to bark[4]. In Japan, Du-zhong tea, an aqueous extract of Du-zhong leaves and a popular beverage, is used in the treatment of hypertension, and is thought to be a functional healthy food[2,5]. Thus, there has been increasing interest in research study of Du-zhong leaves.

Previous literature reveals that several studies have been performed on the extraction of bioactive components from Du-zhong leaves, including aqueous extraction[6,7], organic solvent extraction (ethanol, methanol, and acetone)[3,8], and physical extraction (ultrasonic technique, microwave technique, and supercritical fluid extraction)[9,10]. Application of these procedures presents some disadvantages, such as the loss of bioactive components due to ionization, hydrolysis, and oxidation during extraction, and the consumption of a large amount of solvent and energy[10]. Enzyme extraction, as a fairly new procedure, has been studied by some researchers. Treatment with several enzymes (mainly cellulase, pectinase, and hemicellulase) can disintegrate and hydrolyze cell-wall materials (mainly cellulose and pectin) for better separation and solvent extraction of the intracellular components[11,12]. Enzyme-assisted solvent extraction has been widely used for bioactive component extraction in plant materials such as sweet potato, orange peel, carrot, and soybean[11,13,14]. In particular, enzyme-assisted water

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extraction enhances the extraction yield, and improves product quality compared with the original aqueous process without enzymes in oil extraction. Moreover, it eliminates solvent consumption and the energy requirement[15,16]. However, few reports are related to the application of enzyme extraction in Chinese medicinal herbs.

Natural antioxidants from plant extracts have attracted increasing interest due to consumer concern about the safety of synthetic antioxidants in food[17]. The extracts from Du-zhong leaves, being a potential resource of natural antioxidant, are capable of scavenging reactive oxygen species, inhibiting Fenton reaction-induced oxidative damage in biomolecules[2,18]. Recently, it was indicated that the inhibitory activity of water extracts of Du-zhong (leaves, roasted cortex, and raw cortex) on the peroxidation of linoleic acid measured by the thiocyanate method followed the order leaves > roasted cortex > raw cortex at 60 h of incubation, and all water extracts of Du-zhong were found to possess inhibitory effects on the oxidative modification of low-density lipoprotein (LDL) induced by Cu^{2+}[19,20]. However, investigations into the effects of different extraction methods on the antioxidant activity of Du-zhong leaves are still relatively rare.

The main objectives of this work were to prepare extracts from Du-zhong leaves using enzyme-assisted water extraction and ethanol extraction, and evaluate the antioxidant activity with different methods including the radical-scavenging effect, Fe^{2+}-chelating ability, and inhibition ability of linoleic acid peroxidation. The results for antioxidant activity have been compared with those of a synthetic antioxidant (tertiary butylhydroxyquinone (TBHQ)). Furthermore, the total phenolic content (TPC) has also been evaluated.

Materials and methods

Materials and reagents

Dried Du-zhong leaves were purchased from a medicinal herbs base (Hanzhong, China), homogenized to a fine powder, and stored at 5°C until use.

Cellulase (15,000 U/g), linoleic acid, trichloroacetic acid (TCA), and thiobarbituric acid (TBA) were purchased from Shanghai Chemical Co. (Shanghai, China). Epicatechin, chlorogenic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH), and {4,4′-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bisbenzenesulfonic acid} (Ferrozine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tertiary butylhydroxyquinone (TBHQ) was purchased from Liyuan Industries, Ltd. (Beijing, China). All other chemicals and solvents were of HPLC (high performance liquid chromatography) grade or analytical reagent grade.

Preparation of enzyme-assisted water extracts of Du-zhong leaves (EWEDL)

One hundred grams of Du-zhong leaf powder were finely mixed with 1000 mL distilled water (pH 4.5, adjusted with citric acid) in a large beaker. One gram of cellulase was added and the mixture was stirred on a magnetic stirrer for 5 min. This mixture was placed in a water bath (Model HH-S; Jiangsu Zhenjiang Instrument Co. Ltd., Jiangsu, China) at 50°C. After 2.5 h of enzyme treatment, the mixture was vacuum-filtered through Whatman No. 2 filter paper using a Buchner funnel. The residue was washed with distilled water twice. Combined filtrates were evaporated under vacuum below 40°C using a rotary evaporator (Model R52; Shanghai Yarong Biochemistry Instrument Co., Shanghai, China) to a final volume of approximately 50 mL. Then the extract was freeze-dried *in vacuo* to powder form by a freeze dryer (Model LGJ102; Sihuan Instrument Co., Beijing, China), and stored at –36°C until further use.

Preparation of ethanol extracts of Du-zhong leaves (EEDL)

Du-zhong leaf powder (100 g) was extracted using a Soxhlet extractor for 2 h with 500 mL of ethanol under reflux conditions. The extract was vacuum-filtered through Whatman No. 2 filter paper using a Buchner funnel, the filtrates were evaporated under vacuum below 40°C to a final volume of approximately 50 mL, and then the extract was freeze-dried *in vacuo* to powder form, and stored at –36°C until further use.

Determination of total phenolic content

The total phenolic content of the extract was determined according to the method of Sokmen *et al.*[21]. One milligram of extract was taken in a volumetric flask, 46 mL distilled water and l mL Folin–Ciocalteau reagent were added, and the flask was shaken thoroughly. After 3 min, 3 mL solution of Na_2CO_3 (2%, w/v) was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance was measure at 760 nm using a spectrophotometer (Hitachi UV-Vis model U-3110 spectrophotometer; Tokyo, Japan). The total phenolic amount was calculated as chlorogenic acid equivalents (CAE) from a calibration curve.

Determination of DPPH radical-scavenging activity

The radical-scavenging activity assay was performed as described, with some modifications[22]. An ethanol solution (1 mL) of Du-zhong leaf extract (0.16–10 mg/mL), or TBHQ (standard, 0.02–1.25 mg/mL), was mixed with 100 mM Tris-HCl buffer (2 mL, pH 7.4) and then added to 2 mL of 1.5 mM DPPH in ethanol. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance was read using a spectrophotometer at 517 nm. The DPPH radical-scavenging effect was calculated using the following equation:

$$\text{Scagenging effect (\%)} = \left(1 - \frac{A_{\text{sample}} \text{ at } 517 \text{ nm}}{A_{\text{blank}} \text{ at } 517 \text{ nm}}\right) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The scavenging effect of the sample was expressed as 50% effective concentration (EC_{50}), which represented the concentration of sample having 50% DPPH radical-scavenging effect. All tests were carried out in triplicate.

Determination of Fe^{2+}-chelating ability

The Fe^{2+}-chelating ability was determined according to the method of Decker and Welch[23], described by Chou *et al.*[24]. Fe^{2+} was monitored by measuring formation of the ferrous iron–Ferrozine complex. Du-zhong leaf extract (1.25–20 mg/mL), or TBHQ (standard, 0.02–1.25 mg/mL), was mixed with 2 mM $FeCl_2$ and 5 mM Ferrozine at a ratio of 10:1:2. The mixture was shaken and left at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. A lower absorbance of the reaction mixture indicated a higher Fe^{2+}-chelating ability. The ability to chelate the ferrous iron was calculated by the following equation:

$$\text{Chelating effect (\%)} = \left(1 - \frac{A_{\text{sample}} \text{ at 562 nm}}{A_{\text{blank}} \text{ at 562 nm}}\right) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The Fe^{2+}-chelating ability of the sample was expressed as 50% effective concentration (EC_{50}), which represented the concentration of sample having 50% Fe^{2+}-chelating ability. All tests were carried out in triplicate.

Determination of lipid peroxidation in linoleic acid system

Thiobarbituric acid reactive substances (TBARS) were determined according to the modified method of McDonald and Hultin[25], described by Chun *et al.*[26]. Emulsions were prepared by homogenizing 10% linoleic acid and 5% Tween 40 in 100 mL of distilled water. One milliliter of emulsion was added to a glass tube containing 1 mL ethanol solution of Du-zhong leaf extract (0.62–20 mg/mL), or TBHQ (standard, 0.02–1.25 mg/mL), and 2 mL of phosphate buffer (0.2M, pH 7.4). Tubes were incubated at 50°C for 10 h. The reaction was terminated by adding 2 mL TCA (20%, w/v), followed by 0.5 mL TBA (2%, w/v). The mixture was vortexed and heated in a boiling water bath for 90 min. After cooling with tap water for 10 min, the solution was centrifuged for 15 min at 2000*g*. The absorbance of the upper layer was measured at 532 nm. The inhibition percentage of lipid peroxidation of the sample was calculated by the following equation:

$$\text{Inhibition effect (\%)} = \left(1 - \frac{A_{\text{sample}} \text{ at 532 nm}}{A_{\text{blank}} \text{ at 532 nm}}\right) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The inhibition of lipid peroxidation of the sample was expressed as 50% inhibition concentration (IC_{50}), which represented the concentration of sample having 50% inhibition effect on the lipid peroxidation of linoleic acid. All tests were carried out in triplicate.

HPLC-MS instrumentation and conditions

Polyphenol analyses from EWEDL and EEDL were carried out on an HP 1100 HPLC system equipped with a diode array detector (DAD) (Agilent Technologies, Palo Alto, CA, USA) and interfaced with an Adilent 1100 LC/MSD Ion Trap (Agilent Technologies) mass spectrometer (MS) with an electrospray interface (ESI). Separation was carried out using a Waters C_{18} reverse column (250 × 4.6 mm i.d., 5 µm; Waters, CA, USA). The samples were analyzed according to the method of Li *et al.*, with minor modification[9]. Samples were dissolved in methanol, filtered through a 0.45 µm nylon filter, and injected (10 µL) into a HPLC-MS system. CH_3OH-H_2O-CH_3COOH (20:80:1, v/v) was used as the mobile phase, the flow rate was 1 mL/min, the column was at room temperature, and the detecting wavelength was set at 240 nm.

The mass spectrometer was programmed to operate in full scan MS mode from m/z 50 to 2200. Mass spectra were acquired in negative mode with ion spray voltage at 3.5 kV, capillary temperature at 350°C, and capillary voltage at –85.5 V. Nitrogen was used as the drying gas at 5 L/min and 325°C.

In EWEDL and EEDL, quantification of epicatechin and chlorogenic acid were achieved by comparison with an external standard of known phenolic compounds and expressed as milligrams per gram of extract; standard curves were obtained for each standard. Because of the lack of geniposidic acid standard, the amount of geniposidic acid was calculated as chlorogenic acid equivalents (CAE) from a calibration curve.

Statistical analysis

All results were obtained in triplicate and data are presented as mean ± standard deviation. The mean values of data were analyzed by one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($p < 0.05$) using SPSS software (SPSS, version 12.0).

Results and discussion

Proton radical-scavenging action is known as an important mechanism for measuring antioxidant activity. DPPH was used to determine the proton radical-scavenging action of EWEDL, EEDL, and TBHQ, and results are shown in Figure 1. Both extracts and TBHQ were capable of quenching DPPH radicals in a concentration-dependent manner. At a concentration of 0.16–10 mg/mL, the scavenging activity of EWEDL on DPPH radicals increased with increasing concentration of EWEDL. In particular, the DPPH radical-scavenging activity of EWEDL increased significantly with concentration from 0.16 to 0.31 mg/mL, and leveled off as the concentration further increased; a similar result was found for EEDL at a concentration of 0.16–2.5 mg/mL and TBHQ at a concentration of 0.02–0.16 mg/mL. These results implied that the antioxidant activity of extracts from Du-zhong leaves might be attributed to their proton-donating ability, because both EWEDL and EEDL might prevent reactive radical species from reaching biomolecules by means of hydrogen and/or electron donation[22].

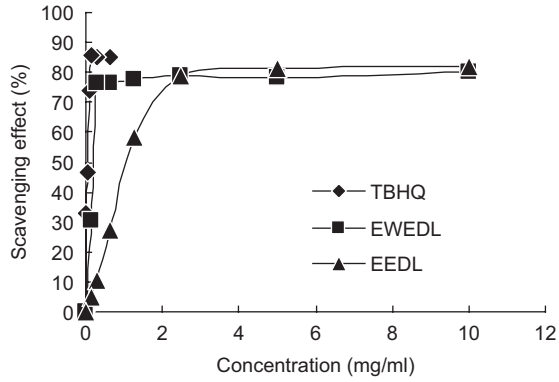

Figure 1. Scavenging effect of enzyme-assisted water extracts (EWEDL) and ethanol extracts (EEDL) of Du-zhong leaves on α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals. Values are expressed as mean (n=3). Tertiary butylhydroxyquinone (TBHQ) was used as the standard.

The EC_{50} value, i.e. the effective concentration of a sample for 50% reduction of free radicals, was determined from the plotted graph of scavenging activity against concentration of Du-zhong leaf extract. The quality of scavenging activity for EWEDL, EEDL, and TBHQ was evaluated by EC_{50} value as shown in Table 1. A low EC_{50} value indicates strong antioxidant activity in a sample. Based on Table 1, EWEDL provided a significantly lower ($p<0.05$) EC_{50} value of 0.10 ± 0.01 mg/mL than that of EEDL (1.35 ± 0.03 mg/mL). However, when compared to the standard, TBHQ, both EWEDL and EEDL showed lower radical-scavenging activity; TBHQ had a significantly lower ($p<0.05$) EC_{50} of 0.04 ± 0.01 mg/mL.

Food is often contaminated with transition metal ions, which may be introduced by manufacturing methods. Bivalent transition metal ions play an important role as catalysts of oxidative processes, leading to the formation of hydroxyl radicals and hydroperoxide decomposition reactions via Fenton chemistry; these processes can be delayed by iron chelation and deactivation[27,28]. Since Fe^{2+} has also been shown to cause the production of oxyradicals and lipid peroxidation, minimizing the Fe^{2+} concentration in the Fenton reaction affords protection against oxidative damage. Therefore, the ability of Du-zhong leaf extracts to chelate Fe^{2+} was evaluated, and the result is presented in Figure 2. EWEDL showed 25.06–75.76% ability to chelate Fe^{2+} at a concentration of 0.62–20 mg/mL, and its chelating ability increased with concentration of the extract; however, the highest chelating ability of EEDL was only 33.43% in the same concentration range. The chelating ability of EWEDL was about 2.5–50 times that of EEDL at the same concentration level. The result showed that EWEDL had a better Fe^{2+}-chelating ability, and EEDL possessed limited chelating effects under the experimental conditions. In the case of Du-zhong leaves, the result implied that enzyme-assisted water extraction might be a better extraction method than ethanol extraction for extracting Fe^{2+}-chelating agent. However, TBHQ in the range of 0.02–0.62 mg/mL showed no detectable Fe^{2+}-chelating ability. Chou *et al.* reported a similar result of Fe^{2+}-chelating ability for butylated hydroxytoluene (BHT) and α-tocopherol using the same method[24].

Table 1. Antioxidant activities of extracts from Du-zhong leaves and tertiary butylhydroxyquinone (TBHQ) as expressed by IC_{50} or EC_{50}.

Antioxidant reaction	Extract	IC_{50} or EC_{50} (mg/mL)
DPPH radical-scavenging	EWEDL	0.10 ± 0.01^{b}
	EEDL	1.35 ± 0.03^{c}
	TBHQ	0.04 ± 0.01^{a}
Fe^{2+}-chelating ability	EWEDL	1.45 ± 0.03
	EEDL	ND
	TBHQ	ND
Lipid peroxidation	EWEDL	16.62 ± 0.10^{b}
	EEDL	16.74 ± 0.09^{b}
	TBHQ	0.76 ± 0.02^{a}

Note. EWEDL, enzyme-assisted water extracts of Du-zhong leaves; EEDL, ethanol extracts of Du-zhong leaves; ND, not detected. Values are expressed as mean ± standard deviation (n = 3).
[a–c]Means with different superscript letters in same antioxidant reaction were significantly different at the level of $p < 0.05$.

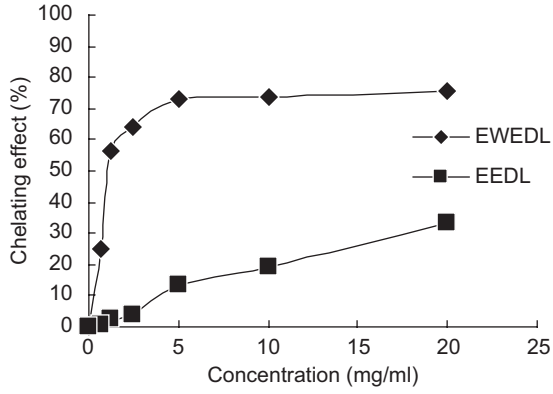

Figure 2. Chelating effect of EWEDL and EEDL on Fe^{2+}. Values are expressed as mean (n=3). TBHQ was used as the standard.

EWEDL in our research showed an EC_{50} of 1.45 ± 0.03 mg/mL (Table 1), but EC_{50} values for EEDL and TBHQ were not obtained in the Fe^{2+}-chelating ability assay.

In the present study, the effect of the Du-zhong leaf extracts on the inhibition of lipid peroxidation in linoleic acid was determined by the TBA method, in which the amount of thiobarbituric acid reactive substances (TBARS), namely malonaldehhyde (MDA), a secondary lipid peroxidation product, was determined by measuring the absorbance at 532 nm[29]. From Figure 3, it can be seen that, in the range 0.31–20 mg/mL, EWEDL and EEDL exhibited 5.31–62.12% and 5.16–62.35% inhibition effect of linoleic acid oxidation, respectively. This result indicated that EWEDL and EEDL showed moderate inhibition activity in the linoleic acid peroxidation system, compared to the DPPH radical-scavenging activity assay. The IC_{50} value (Table 1) for EWEDL was 16.62 ± 0.10 mg/mL, which was slightly lower than the IC_{50} value for EEDL (16.74 ± 0.09 mg/mL), but no significant difference existed between them ($p<0.05$). The highest inhibition activity in the linoleic acid peroxidation system was found to be exhibited by TBHQ, as positive control, which had a significantly lower ($p<0.05$) IC_{50} value at 0.76 ± 0.02 mg/mL than those of EWEDL and EEDL.

Due to the differences observed in the antioxidant activities of EWEDL and EEDL, their phenolic compounds were studied by HPLC in a reversed-phase column coupled with a diode array detector and mass spectrometer (HPLC-DAD-MS). Identification of the main phenolic compounds in EWEDL and EEDL was carried out by comparing HPLC retention time, ultraviolet (UV) absorption, and MS fragment pattern with those of the standards and literature data. The chromatograms of EWEDL and EEDL are shown in Figure 4, while Table 2 and Figure 5 show retention times, MS spectral data, and identification results for peaks numbered in the chromatogram.

Peaks 2 and 3 were identified as epicatechin and chlorogenic acid, respectively, by comparing to the HPLC retention times and mass spectra of authentic standards and literature data[30–32]. Du-zhong leaves have been reported to contain epicatechin and chlorogenic acid[10,18], and thus, peak 2 of the HPLC chromatograms for EWEDL and EEDL (Figure 4), having an $[M–H]^-$ ion at m/z 292, could be epicatechin, which has a molecular weight (MW) of 290. Also, peak 3 may be chlorogenic acid, which has an MW of 354. Because of the lack of corresponding reference compound, peak 1was tentatively identified as geniposidic acid (fragment m/z 373) by comparing to MS spectra of literature data[32,33]. It should be noted that peaks 1, 2, and 3 had signals with higher molecular weights at 747, 431, and 707 m/z (Figure 5), respectively, in their MS spectra. One possible explanation for this result is that the main phenolic compounds in EWEDL and EEDL were present as their glycosidic compounds. Glycosidic compounds are common in plant material. Tong *et al*. reported that geniposidic acid was present as linking to the aglycone form in Du-zhong leaves and bark[33]. As shown in Figure 4 and Table 2, the main phenolic compounds (geniposidic acid, epicatechin, and chlorogenic acid) identified in EWEDL and EEDL were similar. Several studies have reported the same phenolic compounds identified in Du-zhong leaf extracts[3,10,18]. This result showed that there was no difference in main phenolic compounds extracted by the enzyme-assisted water method, compared to ethanol extraction, in Du-zhong

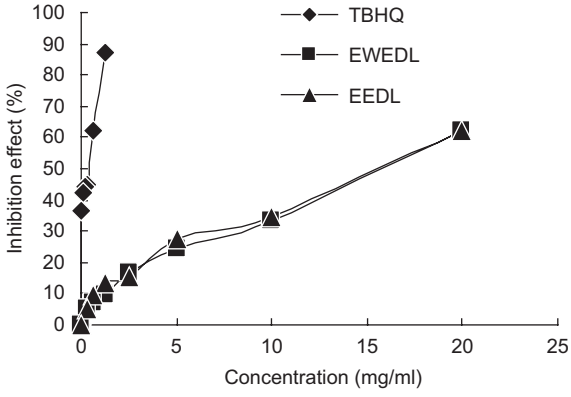

Figure 3. Inhibition effect of EWEDL and EEDL on linoleic acid peroxidation. Values are expressed as mean (n=3). TBHQ was used as the standard.

Table 2. Identification of phenolic compounds contained in enzyme-assisted water extracts of Du-zhong leaves (EWEDL) and ethanol extracts of Du-zhong leaves (EEDL).

Peak	Retention time (min)	m/z	Molecular weight	Identification
1	5.0	373, 747	374	Geniposidic acid
2	8.9	292, 431	294	Epicatechin
3	12.1	191, 353, 707	354	Chlorogenic acid

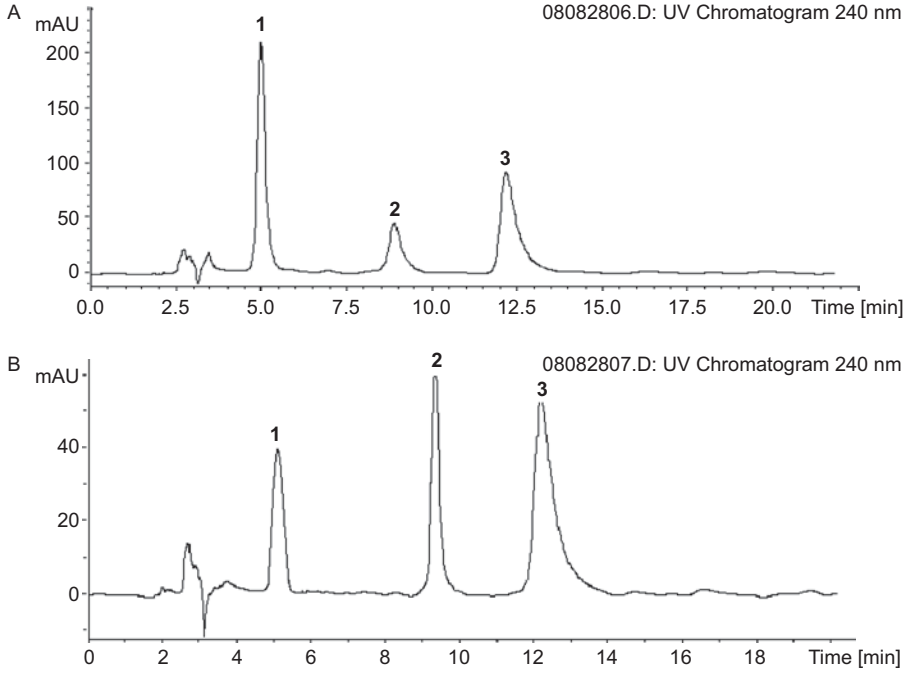

Figure 4. High performance liquid chromatography-diode array detection (HPLC-DAD) chromatograms of (A) ethanol extracts of Du-zhong leaves (EEDL) and (B) enzyme-assisted water extracts of Du-zhong leaves (EWEDL). Peaks: 1, geniposidic acid; 2, epicatechin; 3, chlorogenic acid.

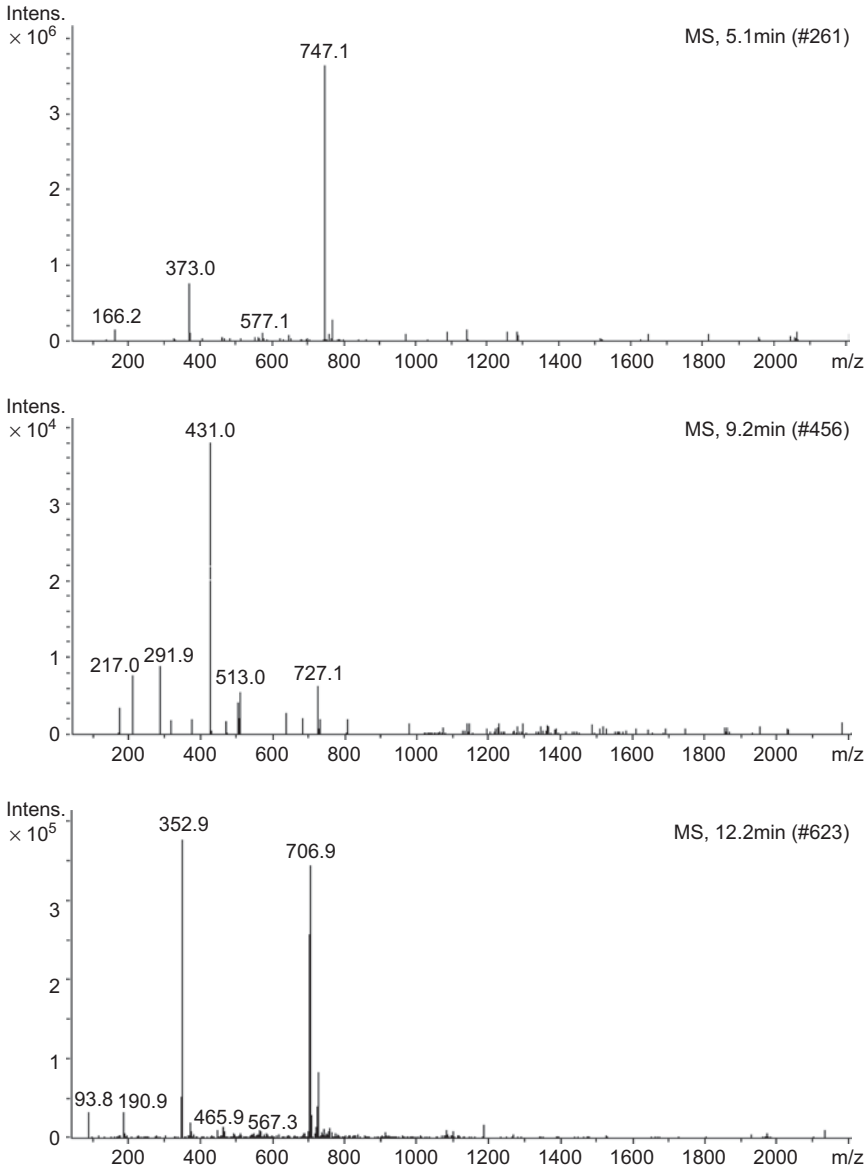

Figure 5. Mass spectra of peak 1 (A), peak 2 (B), and peak 3 (C) in HPLC chromatograms for EWEDL and EEDL.

Table 3. Concentrations of main phenolic compounds in EWEDL and EEDL (mg/g of extract).

Extract	Geniposidic acid[a]	Epicatechin	Chlorogenic acid
EWEDL	0.58 ± 0.03^{b}	2.26 ± 0.05^{b}	5.82 ± 0.06^{b}
EEDL	2.14 ± 0.04^{c}	1.93 ± 0.05^{c}	8.45 ± 0.9^{c}

Note. EWEDL, enzyme-assisted water extracts of Du-zhong leaves; EEDL, ethanol extracts of Du-zhong leaves. Values are expressed as mean ± standard deviation ($n = 3$).
[a]Quantified as chlorogenic acid.
[b,c]Means with different superscript letters in same column were significantly different at the level of $p < 0.05$.

Table 4. Total phenolic content (TPC) of extracts from Du-zhong leaves, mg/g of extract, as chlorogenic acid equivalents (CAE).

Extract	TPC (mg CAE/g of extract)
EWEDL	13.84 ± 0.11^{a}
EEDL	14.72 ± 0.14^{a}

Note. EWEDL, enzyme-assisted water extracts of Du-zhong leaves; EEDL, ethanol extracts of Du-zhong leaves. Values are expressed as mean ± standard deviation ($n = 3$).
[a]Means with same superscript letter in same column were not significantly different at the level of $p < 0.05$.

leaves; however, differences were found in contents of the three main phenolic compounds (Table 3). Hence, differences observed in antioxidant activity could be explained by differences of the main phenolic contents, not the main phenolic compounds in EWEDL and EEDL.

Phenolic compounds in plants are powerful antioxidants, which may significantly contribute to the overall antioxidant activity. Total phenolic contents were determined for both Du-zhong leaf extracts (Table 4). The total phenolic content for EWEDL was 13.84 ± 0.11 mg chlorogenic acid equivalents (CAE) in each gram of extract, which was slightly lower than that of EEDL (14.72 ± 0.14 mg CAE in each gram of extract); however, no significant difference existed between EWEDL and EEDL ($p < 0.05$). This result implied that enzyme-assisted water extraction

might also be an effective extracting method for phenolic compounds, compared to ethanol extraction, in Du-zhong leaves. Several studies have reported a significant positive correlation between total phenolic content and antioxidant activity of extracts from plant materials, such as mushroom and *Sorbus domestica* fruits[34,35]. Nevertheless, our study showed no positive correlation between total phenolic content and scavenging ability of EWEDL and EEDL on DPPH radicals. This finding was the same as results obtained from the Fe^{2+}-chelating assay and lipid peroxidation assay. The result could be due to the fact that the total phenolic content did not include all the antioxidants[17]; EWEDL and EEDL could possess different antioxidant compounds from phenolic compounds, such as β-carotene and tocopherol[4].

In conclusion, the extracting method and the evaluation method both significantly affected the antioxidant ability of Du-zhong leaves, based on enzyme-assisted water extraction and ethanol extraction results. Compared to EEDL, EWEDL showed higher antioxidant activity when evaluated by DPPH radical-scavenging assay and Fe^{2+}-chelating ability assay. EWEDL and EEDL showed moderate antioxidant activity when determined by lipid peroxidation assay. By the three antioxidant methods used in our research, the extracts from Du-zhong leaves could not be comprehensively evaluated for antioxidant activity; however, they may serve as potential dietary sources of natural antioxidants for human nutrition and health. In EWEDL and EEDL, there was no significant difference in total phenolic yield. Nevertheless, enzyme-assisted water extraction has some advantages, such as moderate extracting conditions, without organic solvent residues and so on, compared to ethanol extraction. These advantages are important in terms of natural antioxidants applied to the food industry; therefore, enzyme-assisted water extraction is a possible effective way of bioactive component extraction from plant materials.

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***Declaration of interest*:** The authors report no conflicts of interest.

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