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Original Article

Composition of phenolic compounds and antioxidant attributes of *Cyclea gracillima* Diels extracts

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ABSTRACT

Cyclea gracillima Diels is a Taiwanese native medicinal herb. However, there are currently few relevant reports on its biochemical activity. In this study, the antioxidant attributes of the ethanol and hot water extracts of this herb were assayed using *in vitro* models, including the following: 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl radical scavenging, Trolox equivalent antioxidant capacity, reducing power, and chelating ferrous ions. The following biochemical models were also assayed: inhibition of human low density lipoprotein oxidation, inhibition of human erythrocyte hemolysis, and scavenging oxygen radicals in human blood. The composition and content of flavonoids and phenolic acids in these extracts were also analyzed. The results showed that these extracts with high polyphenol levels presented remarkable antioxidant effects in all assays, especially when extracted with ethanol. Six phenolic acids (mainly ferulic acid, sinapic acid, and syringic acid) and 12 flavonoids (mainly narigenin, myricetin, naringin, and apigenin) were found in these extracts.

1. Introduction

Cyclea gracillima Diels, which belongs to the family Menispermaceae, is a Taiwanese native medicinal herb that usually inhabits the margins of thickets at the low altitudes of central and southern Taiwan [1]. Many menispermaceous plants are used in folk medicine for the treatment of cough, fever, lumbago, headache, edema, wind-dampness, diabetes, asthma, and snakebite [2,3].

Recent reports have indicated that *Cyclea peltata* Hook. F. & Thomson (Menispermaceae) grown in India contains flavonoids, tannins, alkaloids, diterpenes, and saponins that have multiple properties, including antioxidant, antidiabetic, gastric antisecretory, antiulcer, anticancer, diuretic, and hepatoprotective activities [4–10]. Although *C. gracillima* Diels

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is used to treat inflammation, edema, and throes in Taiwan [1], there have been almost no related investigations regarding its biochemical properties.

In this work, we first determined the total phenol, flavonoid, and condensed tannin content, as well as the composition and content of flavonoids and phenolic acids in the hot water and ethanol (EtOH) extracts of *C. gracillima* Diels by high performance liquid chromatography (HPLC). The antioxidant attributes of these extracts were also evaluated by *in vitro* models including 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl (DPPH) radical scavenging capacity, Trolox equivalent antioxidant capacity (TEAC), ferric ion (Fe^{3+}) reducing power, and ferrous ion (Fe^{2+}) chelating capacity, as well as biochemical models, including inhibition of cupric ion (Cu^{2+})-induced human low density lipoprotein (LDL) oxidation, inhibition of peroxyl radical-induced human erythrocyte hemolysis, and scavenging of oxygen radicals in human blood.

2. Materials and methods

2.1. Samples

C. gracillima Diels were gathered from the low altitude forest zone of central Taiwan, and then lyophilized using a freeze-drying system (Vastech Scientific Co., Ltd., Taipei, Taiwan) before use.

2.2. Chemicals

Acetonitrile (ACN), EtOH (95%), methanol (MeOH), acetic acid (CH_3COOH), and Folin-Ciocalteu's phenol reagent were obtained from Merck (Darmstadt, Germany). An Ultrapure water purification system (Lotun Co., Ltd., Taipei, Taiwan) was used to prepare distilled deionized water (dd H_2O). Phenolic acid standards: *p*-anisic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid, gallic acid, gentisic acid, *p*-hydroxybezoic acid, rosmarinic acid, sinapic acid, syringic acid, and vanillic acid; flavonoid standards: apigenin, catechin, daidzein, diosmin, epicatechin, eriodictyol, genistein, glycitein, hesperidin, hesperetin, isorhamnetin, kaempferol, luteolin, naringin, narigenin, myricetin, quercitrin, rutin, neohesperidin, morin, and quercetin; aluminum chloride; ascorbic acid; 2-2′-azinobis-(3-ethylbenz-thiazoline-6-sulphonic acid) (ABTS); ferrozine; EDTA; luminol; heparin; 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox); vanillin; DPPH; horseradish peroxidase; trichloroacetic acid (TCA); aluminum chloride ($AlCl_3$); ferrous chloride ($FeCl_3$); potassium ferricyanide ($K_3Fe(CN)_6$); ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$); copper sulfate ($CuSO_4$) and sodium dihydrogen phosphate were purchased from Sigma (St. Louis, MO, USA). Hydrochloric acid (HCl), sodium carbonate, sodium hydroxide (NaOH), sodium nitrite ($NaNO_2$), disodium hydrogen phosphate, and hydrogen peroxide (H_2O_2) were obtained from Wako Co. (Osaka, Japan).

2.3. Preparation of hot water and ethanolic extracts of C. gracillima Diels

Twenty grams of *C. gracillima* Diels was extracted with 500 mL of EtOH for 24 hours. After filtrating using Whatman No. 1 filter paper, the extract was concentrated to dryness by a rotary evaporator (Panchun Scientific Co., Kaohsiung, Taiwan). For hot water extraction, 20 g *C. gracillima* Diels was refluxed with distilled water (500 mL) at 98 ± 2°C for 1 hour followed by filtrating and cooling. The extract was then lyophilized in the freeze-dryer system. Each extractive procedure was carried out in triplicate. These extracts were stored under nitrogen at −80°C until use.

2.4. Determination of phytochemicals in the extracts of C. gracillima Diels

Folin–Ciocalteu's phenol reagent was used to measure the content of total phenols as described by Julkunen-Titto [11]; gallic acid was used as a standard and the results were expressed as mg gallic acid equivalent (GAE)/g of dried extract. The total flavonoid content was detected using 5% $NaNO_2$, 10% $AlCl_3$, and 1M NaOH solutions according to the method of Zhishen et al [12]; (+)-catechin was used as a standard and the results were expressed as mg catechin equivalent (CE)/g of dried extract. The content of condensed tannin was measured with 4% vanillin (prepared in MeOH) and HCl as described by Liu et al [13]; (+)-catechin was used as a standard and the results were expressed as mg CE/g of dried extract. The conditions in the report of Chen et al [14] were used to analyze the compositions of phenolic acid and flavonoids in the *C. gracillima* Diels extracts as follows: stationary phase, Inspire C18 column (250 × 4.6 mm, 5 μm; Dikma Technologies Inc., Lake Forest, CA, USA); mobile phase, ACN (Solvent A) and H_2O with 2% CH_3COOH (Solvent B) (2–4% A from 0 to 25 minutes and kept at 4% A from 25 to 40 minutes; 4–10% A from 40 to 50 minutes; 10–15% A from 50 to 60 minutes; 15–18% A from 60 to 110 minutes; 18–20% A from 110 to 115 minutes; 20–22% A from 115 to 135 minutes; and 22–25% A from 135 to 150 minutes); flow rate, 0.8 mL/min. An HPLC system with a Shimadzu LC-10AT HPLC pump system, a Shimadzu SCL-10A system controller module (Kyoto, Japan), and an S-3210 photodiode-array detector (Schambeck SFD GmbH, Bad Honnef, Germany) were used in this work. Electrospray ionization mass spectrometers (ESI-MS) data were recorded on a Thermo Finnigan LCQ classic quadrupole ion trap HPLC-Mass (MS) system (Gentech, Arcade, NY, USA) under the following conditions: nebulizer pressure, 70 psi; capillary temperature, 200°C; dry gas flow, 11 L/min; electrospray voltage of the ion source, 3000 V; capillary exit, −159 V; and skimmer, 20 V. Identification of compounds was carried out by comparing their retention times, UV-Vis, and mass spectral data to those of the authentic reference standards.

2.5. Antioxidant assays in vitro

Each extract was dissolved in MeOH, and then different concentrations of extract solutions were prepared by serial dilution.

DPPH is a powerful reagent to evaluate the scavenging capacity of extracts for free radicals [11]. The scavenging effect on DPPH radicals was evaluated as described by Epsin et al [15]. Each solution (200 μL) was mixed with 50 μL of 1mM DPPH (prepared in MeOH). After a 30-minute reaction, the absorbance (Abs) was measured at 517 nm (Multiskan Spectrum;

Thermo Co., Vantaa, Finland). The percentage of DPPH radical scavenged was calculated by the following equation: scavenging effect (%) = [1 − (A_{de} − A_e)/A_d] × 100 (A_d: Abs of sample without extract; A_{de}: Abs of sample with extract; A_e: Abs of extract only). A (+)-catechin standard was used as a positive control for comparison.

The Fe^{3+} ion is a reducing agent used to estimate a molecule's reducing power [16]. The ability to reduce Fe^{3+} was measured using the method of Oyaizu [17]. Each solution (250 μL) was mixed with 250 μL of sodium phosphate buffer (0.2M, pH 6.6) and 250 μL of 1% $K_3Fe(CN)_6$ followed by incubating for 20 minutes at 50°C. After adding 10% TCA (250 μL), the mixture was centrifuged at 3750*g* for 10 minutes (Hermle Z300K centrifuge; Hermle Labortechnik GmbH, Wehingen Württ, Germany). Then, the supernatant (100 μL) was mixed with 100 μL of dd H_2O and 20 μL of a 1% ferric chloride solution. The Abs was measured at 700 nm after 10 minutes. L-Ascorbic acid and (+)-catechin were used as positive controls for comparison.

The Fe^{2+} ion can induce many radical reactions; hence, Fe^{2+} chelation is considered a useful therapeutic approach [18]. The ability to chelate Fe^{2+} was surveyed with ferrozine reagent per the method of Dastmalchia et al [19]. Each solution (200 μL) was mixed with 100 μL of $FeCl_2 \cdot 4H_2O$ (2.0mM) and 900 μL of MeOH. After 5 minutes, the reaction was carried out by the addition of 400 μL of ferrozine (5.0mM). After 10 minutes, the Abs at 562 nm was recorded. The chelating activity (%) was calculated by the equation: chelating activity (%) = [1 − (A_{ef} − A_e) / A_f] × 100 (A_{ef}: Abs of sample with extract; A_f: Abs of sample without extract; A_e: Abs of extract only). EDTA was used as a positive control for comparison.

TEAC is an effective method to assay antioxidant capacity [20]. The assay was performed as described by Scalzo et al [21]. The $ABTS^+$ solution (OD_{734} = 0.70 ± 0.03) was prepared by mixing ABTS, H_2O_2, and peroxidase, to final concentrations of 100μM, 50μM, and 4.4 units/mL, respectively. Each solution (30 μL) was mixed with 270 μL of the $ABTS^+$ solution and incubated for 1 minute. The Abs was read at 734 nm. The $ABTS^+$ scavenging percentage was counted relative to Trolox, and the TEAC value was expressed as mmole Trolox equivalent (TE)/g extract. L-Ascorbic acid and (+)-catechin were used as positive controls for comparison.

2.6. Antioxidant assays with human LDL, erythrocytes, and blood models

The inhibitory effect on Cu^{2+}-induced human LDL oxidation was estimated as described by Hsieh et al [22]. LDL was separated from the plasma of a fasting healthy volunteer. The cholesterol level of the isolated LDL was diluted to 50 μg/mL with 5mM phosphate buffered saline (PBS). The LDL (100 μL) was added to 130 μL of 5mM PBS, and 10 μL of each extract solution was incubated with 10 μL of 125μM $CuSO_4$ (in 5mM PBS) at 37°C. The level of conjugated dienes was measured at 232 nm at 5-minute intervals to obtain the kinetics of LDL oxidation during initiation, propagation, and termination. The lag time of LDL oxidation was recorded. The increase in lag time was calculated by the equation: LDL oxidation (%) = [(T_e − T) / T] × 100 (T_e: lag time of sample with extract; T: lag time of sample without extract). (+)-Catechin was used as a positive control for comparison.

The inhibitory effect on peroxyl radical-induced human erythrocyte hemolysis was evaluated as by Barreira et al [23]. Erythrocytes were separated from the blood of a fasting healthy volunteer by centrifugation at 1500*g* and 4°C for 10 minutes. The pellet was washed three times with 10 mL of 10mM PBS. The 20% erythrocyte suspension (in PBS, 0.1 mL) was mixed with 0.2 mL of 200mM 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) solution (in PBS) and 0.1 mL of each extract solution, followed by incubation at 37°C for 3 hours. After dilution with 0.5 mL of PBS, the mixture was centrifuged at 3000*g* and 25°C for 10 minutes. The Abs of the supernatant of the mixture was recorded at 540 nm. Hemolysis inhibition (%) was calculation by the equation: hemolysis inhibition (%) = [1 − (A_{ae}−A_e)/A_a] × 100 (A_a: Abs of sample without extract; A_{ae}: Abs of sample with extract; A_e: Abs of extract only). (+)-Catechin was used as a positive control for comparison.

The scavenging of oxygen radicals in human blood was performed as by Lu et al [24]. A chemiluminescence analyzing system (Tohoku electronic industrial Co., Ltd., Miyagi, Japan) was used to measure oxygen radicals. The system is composed of a photon detector, a chemiluminescence counter, and a water circulator. Each extract (0.1 mL, dissolved in glycerol/saline = 1/9, v/v) was added to 0.2 mL of heparinized human blood from a fasting man with rheumatoid arthritis and placed in a special chamber unit (Model No. TLU-21) including a stainless steel cell with magnetic stirrer bar in an absolutely dark chamber at 37°C. After 190 seconds, 1.0 mL of 0.01mM luminol (in normal saline) was added to the cell, and photon emission from whole blood was counted at 10-second intervals from 0 to 600 seconds. The scavenging of oxygen radicals (%) was calculated through the equation: scavenging effect (%) = [1 − CL_e / CL] × 100 (CL: chemiluminescence value of sample without extract; CL_e: chemiluminescence value of sample with extract). (+)-Catechin was used as a positive control for comparison.

2.7. Statistical analysis

Phytochemical contents and antioxidant attributes were assayed in triplicate and the mean values were calculated. The data were subjected to analysis of variance and Duncan's multiple range tests were used to calculate differences between means. A significant difference was concluded at a level of $p < 0.05$.

3. Results

3.1. Antioxidant components in C. gracillima Diels extracts

Table 1 shows the yields from the two extraction processes. The yields of the EtOH and hot water extracts from dried *C. gracillima* Diels were 24.81% and 15.17%, respectively. The quantities of the antioxidant components, including total polyphenol (118.67 mg GAE/g) content, total flavonoid (60.83 mg CE/g), and condensed tannins (14.55 mg CE/g), in the EtOH extract were all higher than those in the hot water extract (105.07 mg GAE/g total polyphenols, 51.53 mg CE/g total flavonoids, and 10.50 mg CE/g condensed tannins; Table 1).

Table 1 – Extraction yields and contents of total phenols, total flavonoids, and condensed tannins in *Cyclea gracillima* Diels extracts.

Sample	Extraction yield (%)[a]	Content		
		Total polyphenols (mg GAE/g extract)	Total flavonoids (mg CE/g extract)	Condensed tannins (mg CE/g extract)
Ethanol extract	24.81 ± 1.23 a	118.67 ± 5.15 a	60.83 ± 3.29 a	14.55 ± 1.01 a
Water extract	15.17 ± 1.04 b	105.07 ± 4.88 b	51.53 ± 2.11 b	10.50 ± 0.89 b

Values (mean ± SD, $n = 3$) in the same column followed by a different letter are significantly different ($p < 0.05$).
CE = catechin equivalent; GAE = gallic acid equivalent; SD = standard deviation.
[a] Extraction yield (%) = (sample extract weight / sample weight) × 100%.

Through HPLC analysis, we found 6 phenolic acids (gallic acid, *p*-coumaric acid, ferulic acid, sinapic acid, syringic acid, and *p*-anisic acid), and 12 flavonoids (catechin, epicatechin, rutin, naringin, myricetin, hesperidin, diosmin, narigenin, luteolin, hesperetin, kamempferol, and apigenin) in these extracts (Table 2). Except for gallic acid, the EtOH extract had higher levels for each phenolic acid and flavonoid than the hot water extract. Therefore, the EtOH extract had higher total quantities of phenolic acids and flavonoids (118.67 mg/g) than the hot water extract (88.40 mg/g). The content of total phenolic acid (50.78 mg/g) in the EtOH extract was lower than the content of total flavonoid (54.36 mg/g); however, the reverse was true of the hot water extract (46.08 mg/g phenolic acid and 42.32 mg/g flavonoid). Ferulic acid (11.12 mg/g), sinapic acid (18.62 mg/g), and syringic acid (11.84 mg/g) were the major phenolic acids in the EtOH extract; similar results were found in the hot water extract (10.23 mg/g ferulic acid, 16.24 mg/g sinapic acid, and 8.24 mg/g syringic acid). Moreover, the major flavonoids in the EtOH and hot water extracts were narigenin (8.61 mg/g and 5.45 mg/g, respectively), myricetin (7.51 mg/g and 5.85 mg/g, respectively), naringin (6.89 mg/g and 6.18 mg/g, respectively), and apigenin (6.87 mg/g and 6.27 mg/g, respectively).

3.2. *Antioxidant capacities of* C. gracillima *Diels extracts*

C. gracillima Diels extracts displayed high capacity for quenching DPPH radicals (Figure 1A). These extracts were also capable of reducing Fe^{3+} (Figure 1B) and chelating Fe^{2+} (Figure 1C). For the three assays, the EtOH extract had significantly better antioxidant effects than the hot water extract ($p < 0.05$); the higher concentrations of the extracts, the greater the antioxidant effect (Figures 1A, 1B, and 1C). The EC_{50} values of DPPH radical scavenging activity, Fe^{3+} reducing power, and Fe^{2+} chelating power were, respectively, 0.14 mg/mL, 0.21 mg/mL, and 1.18 mg/mL for the EtOH extract, and 0.17 mg/mL, 0.29 mg/mL, and 1.67 mg/mL for the hot water extract (Table 3). For the TEAC assay, the antioxidant level of the EtOH extract (0.63 mmol TE/g) was also significantly

Table 2 – Composition of flavonoids and phenolic acids of *Cyclea gracillima* Diels extracts.

Compound	MS signal	Content (mg/g extract)	
		Ethanol extract	Water extract
Gallic acid	$[M-H]^-$ *m/z* 169; fragment *m/z* 125	0.34 ± 0.01 b	4.25 ± 0.17 a
Catechin	$[M+H]^+$ *m/z* 291; fragments *m/z* 123,139	2.16 ± 0.08 a	1.37 ± 0.04 b
Epicatechin	$[M+H]^+$ *m/z* 291; fragments *m/z* 139, 165	0.99 ± 0.03 a	0.91 ± 0.02 b
p-Coumaric acid	$[M-H]^-$ *m/z* 163; fragments *m/z* 128, 119	2.42 ± 0.10 a	2.28 ± 0.09 b
Ferulic acid	$[M-H]^-$ *m/z* 193; fragment *m/z* 134	11.62 ± 0.45 a	10.23 ± 0.87 b
Sinapic acid	$[M-H]^-$ *m/z* 223; fragments *m/z* 208, 164, 149	18.62 ± 0.92 a	16.24 ± 0.87 b
Syringic acid	$[M-H]^-$ *m/z* 197; fragments *m/z* 153, 138	11.84 ± 0.65 a	8.24 ± 0.14 b
Rutin	$[M+H]^+$ *m/z* 611; fragments *m/z* 465, 303	4.84 ± 0.09 a	4.13 ± 0.13 b
p-Anisic acid	$[M-H]^-$ *m/z* 151; fragments *m/z* 135, 107	5.94 ± 0.10 a	4.84 ± 0.06 b
Naringin	$[M+H]^+$ *m/z* 581; fragments *m/z* 419, 273, 153	8.61 ± 0.45 a	5.45 ± 0.14 b
Myricetin	$[M+H]^+$ *m/z* 319; fragments *m/z* 153	7.51 ± 0.41 a	5.85 ± 0.28 b
Hesperidin	$[M+H]^+$ *m/z* 611; fragments *m/z* 449, 303, 153	0.53 ± 0.02 a	0.40 ± 0.02 b
Diosmin	$[M+H]^+$ *m/z* 609; fragments *m/z* 563, 299, 284	4.99 ± 0.13 a	4.21 ± 0.15 b
Narigenin	$[M+H]^+$ *m/z* 273; fragments *m/z* 147, 153	6.89 ± 0.38 a	6.18 ± 0.16 b
Luteolin	$[M+H]^+$ *m/z* 287; fragments *m/z* 241, 153, 135	4.56 ± 0.13 a	3.88 ± 0.16 b
Hesperetin	$[M+H]^+$ *m/z* 303; fragments *m/z* 177, 153	5.62 ± 0.21 a	3.02 ± 0.10 b
Kamempferol	$[M+H]^+$ *m/z* 287; fragments *m/z* 153, 121	0.79 ± 0.03 a	0.65 ± 0.02 b
Apigenin	$[M+H]^+$ *m/z* 271; fragments *m/z* 153, 119	6.87 ± 0.24 a	6.27 ± 0.25 b
Flavonoids		54.36 ± 2.20 a	42.32 ± 1.47 b
Phenolic acids		50.78 ± 2.23 a	46.08 ± 2.20 b
Total		105.14 ± 4.43 a	88.40 ± 3.67 b

Values (mean ± SD, $n = 3$) in the same row followed by a different letter are significantly different ($p < 0.05$).
MS = Mass; SD = standard deviation.

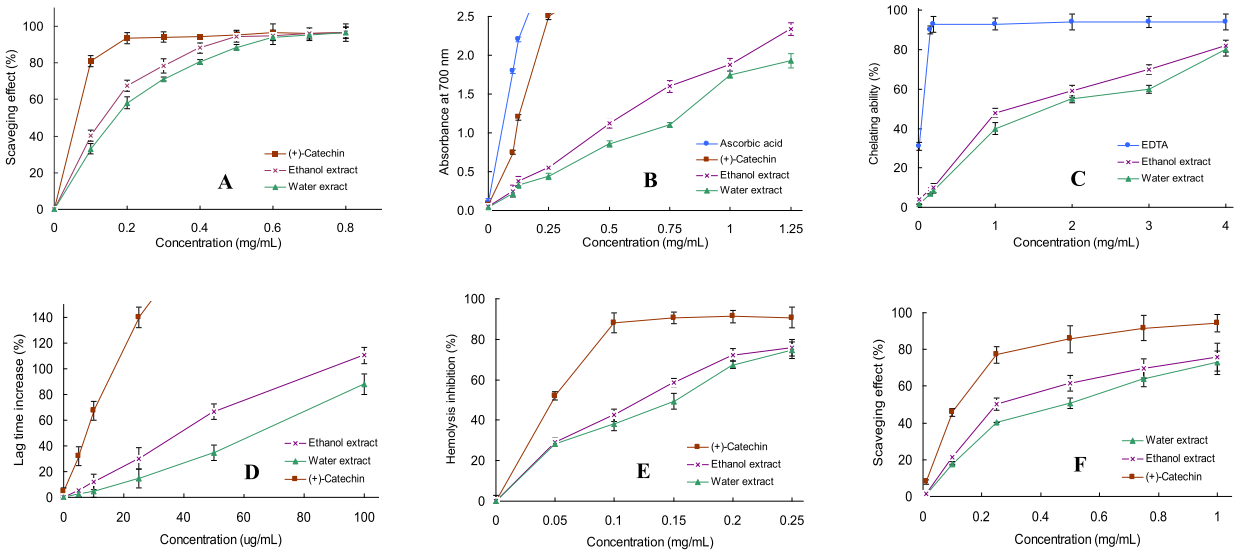

Figure 1 – Antioxidant effects of *Cyclea gracillima* Diels extracts: (A) DPPH radical scavenging activity, (B) Fe^{3+} reducing power, (C) Fe^{2+} chelating power, (D) inhibition of human LDL oxidation, (E) inhibition of human erythrocyte hemolysis, and (F) scavenging oxygen radicals in human blood. DPPH = 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl; LDL = low density lipoprotein.

higher than that of the hot water extract (0.51 mmol TE/g, $p < 0.05$; Table 3).

The EtOH extract showed greater inhibition of Cu^{2+}-induced human LDL oxidation than the hot water extract (Figures 1D and 2A); the EC_{50} values, indicating the increase in 50% lag time of LDL oxidation, were 38.60 μg/mL and 64.47 μg/mL for the EtOH and the hot water extracts, respectively (Table 3). The lag time of the LDL oxidation was prolonged with increasing extract concentration (Figure 1D). LDL oxidation induced by Cu^{2+} ion in the absence of extract took 87.2 minutes. With the addition of 50 μg/mL of the extracts, lag times were increased to 117.3 minutes (hot water extract) and 145.4 minutes (EtOH extract) (Figure 2A), an increase of 34.5% and 66.7%, respectively (Figure 1D).

C. gracillima Diels extract suppressed peroxyl radical-induced human erythrocyte hemolysis in a concentration-dependent manner. As we saw with other assays, the EtOH extract had a greater effect than the hot water extract (Figure 1E); the EC_{50} values were 0.12 mg/mL and 0.15 mg/mL for the EtOH and hot water extracts, respectively (Table 3).

With regard to the scavenging of oxygen radicals in human blood, the EtOH extract still exhibited a stronger effect than the hot water extract (Figures 1F and 2B); their EC_{50} values were 0.24 mg/mL (EtOH extract) and 0.47 mg/mL (hot water extract) (Table 3). As seen previously, higher concentrations of the extracts scavenged higher levels of the oxygen radical (Figure 1F). The chemiluminescence level for the original blood (without extracts) was 22626 counts/10 s. Blood treated with 0.25 mg/mL of the extracts showed decreased levels (hot water extract, 13485 counts/10 s; EtOH extract 11267 counts/10 s); the extracts increased scavenging by 40.4% and 50.2%, respectively (Figure 2B).

Table 3 – EC_{50} values of *Cyclea gracillima* Diels extracts in antioxidant assays.

Sample	EC_{50}						TEAC (mmol TE/g)
	LDL oxidation inhibition[a] (μg/mL)	Erythrocyte hemolysis inhibition[b] (mg/mL)	Scavenging oxygen radicals[b] (mg/mL)	Scavenging DPPH radicals[b] (mg/mL)	Reducing power[c] (mg/mL)	Chelating power[b] (mg/mL)	
Ethanol extract	38.60 ± 1.58 b	0.12 ± 0.01 b	0.24 ± 0.02 b	0.14 ± 0.01 b	0.21 ± 0.01 b	1.18 ± 0.06 b	0.63 ± 0.01 b
Water extract	64.47 ± 5.76 a	0.15 ± 0.01 a	0.47 ± 0.03 a	0.17 ± 0.01 a	0.29 ± 0.02 a	1.67 ± 0.02 c	0.51 ± 0.02 c
(+)-Catechin	0.77 ± 0.01 c	0.05 ± 0.01 c	0.12 ± 0.01 c	0.06 ± 0.01 c	0.06 ± 0.00 c	—	1.24 ± 0.01 a
EDTA	—	—	—	—	—	0.01 ± 0.01 a	—
Ascorbic acid	—	—	—	—	0.02 ± 0.00 d	—	—

Values (mean ± SD, $n = 3$) in the same column followed by a different letter are significantly different ($p < 0.05$).
DPPH = 2,2-diphenyl-1-(2,4,6-trinitrophenyl)-hydrazyl; LDL = low density lipoprotein; SD = standard deviation; TE = Trolox equivalent; TEAC = Trolox equivalent antioxidant capacity.
[a] The effective concentration increasing 50% lag time of oxidation.
[b] The effective concentration providing 50% antioxidant effect.
[c] The concentration when $A_{700} = 0.5$.

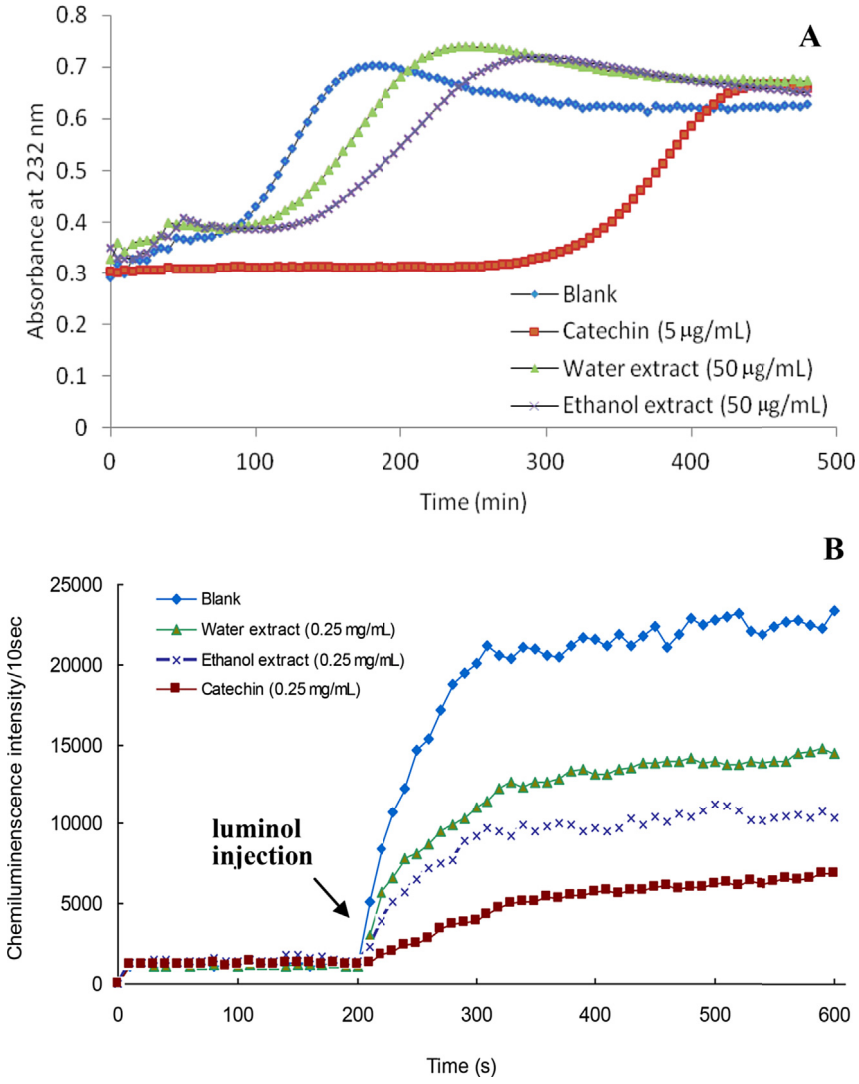

Figure 2 – Antioxidant activity assays for *Cyclea gracillima* Diels extracts: (A) inhibition of Cu^{2+}-induced oxidation of human LDL (50 μg/mL); (B) scavenging oxygen radicals in human blood (0.25 mg/mL). LDL = low density lipoprotein.

4. Discussion

Free radicals and reactive oxygen species (ROS) are generated in the human body through varied endogenous systems, exposure to various physiochemical conditions, and pathological situations. For perfect physiological function, an oxidant-antioxidant balance is regulated by endogenous antioxidant defense mechanisms [25]. If this protection does not adequately remove dangerous radicals, oxidative stress ensues [26]. Therefore, substantial oxidative damage to proteins, DNA, and lipids is induced, and many human diseases (e.g., atherosclerosis, cardiovascular disease, and cancer) can be triggered as well [27]. An external source of antioxidants can be applied to assist in resisting this oxidative stress. Many such natural antioxidants occur in plant sources, such as polyphenols, flavonoids, and condensed tannins [28].

Many *Menispermaceae* plants have good antioxidant activities. Zulkefli et al [29] indicated that the MeOH extract of *Tinospora crispa* (Patawali) showed high antioxidant activity for DPPH radical scavenging, reducing power, and metal chelating because of the presence of high levels of polyphenol; apigenin and magnoflorine were the major antioxidant compounds. Praveen et al [30] reported that *Tinospora cordifolia* (Guduchi) had abundant phenolic contents and presented with prominent scavenging effects on DPPH radicals, reducing power, and metal chelating capacity. Meena and Santhy [7] demonstrated that administration of a MeOH extract of *Cyclea peltata* could increase the activities of catalase, superoxide dismutase, and glutathione peroxidase, and decrease the level of malondialdehyde in liver and kidney tissues of Dalton's Ascites Lymphoma (DAL) mice. Chellappan et al [5] also found that the hydroalcoholic extract of *C. peltata* had a good scavenging effect on ABTS free radicals.

In this work, we first examined the ability of hot water and EtOH extracts of *C. gracillima* Diels in DPPH radical scavenging, TEAC, reducing power, and Fe^{2+} ion chelation assays to

determine their antioxidant activities. Water and EtOH extractions are allowed to be used for functional food exploitation in Taiwan. Both of the extracts had high activity in these assays, which is likely caused by their rich amounts of phenolic compounds. The EtOH extract had a greater effect than the hot water extract owing to its higher level of polyphenols. Ferulic acid, sinapic acid, and syringic acid were the major phenolic acids, and narigenin, myricetin, naringin, and apigenin were the major flavonoids in these extracts.

ROS include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), among others. High concentrations of ROS are extremely harmful to organisms and can be detected in almost all cancer tissues. Effective reduction of overproduced ROS during various environmental stresses by antioxidants is necessary for health [31]. Free radicals and ROS can induce LDL oxidation, which is considered a significant biomarker for atherosclerosis. The oxidized LDL can unceasingly damage blood vessels and lead to formation of foam cells and plaques, the symptoms of atherosclerosis. Therefore, inhibition of LDL oxidation is regarded as the most important therapeutic approach to block atherosclerosis development [32]. Free radical induced lipid peroxidation in biological membranes is related to many pathological events, as well as aging. Erythrocyte membranes with rich polyunsaturated fatty acids are very susceptible to free radical-mediated lipid peroxidation, which ultimately results in hemolysis. Hence, the model for free radical-induced erythrocyte hemolysis is suitable for investigating oxidative damage of biomembranes [33].

Plant-derived antioxidants have remarkable protective effects against LDL oxidation and oxidative damage. Investigation into and evaluation of the antioxidant attributes of these natural products mostly focus on phenolic compounds [34]. Ness and Powles [35] illustrated that phenolic compounds can efficiently quench free radicals and ROS owing to their conjugated ring structures and hydroxyl groups. The consumption of polyphenol-rich foods such as fruits, vegetables, and herbs can diminish oxidative damage [36]. Saputri and Jantan [32] reported that the MeOH extract of *Tinospora crispa* L. (*Menispermaceae*) could inhibit human LDL oxidation, an activity ascribed to its phenolic compounds. Amresh et al [37] demonstrated that a 50% EtOH extract of *Cissampelos pareira* (L.) Hirsuta (*Menispermaceae*) containing a large amount of polyphenols exhibited significant ROS scavenging effects.

We first explored the hot water and EtOH extracts of *C. gracillima* Diels, which revealed notable effects on the inhibition of human LDL oxidation and human erythrocyte hemolysis, as well as scavenging oxygen radicals in human blood. In addition, the EtOH extract that contained a higher phenolic content presented greater effects than the hot water extract in all assays.

5. Conclusions

To the best of our knowledge, this is the first study to determine the composition and flavonoid and phenolic acid content in the hot water and EtOH extracts of *C. gracillima* Diels, as well as their antioxidant effects. These extracts of *C. gracillima* Diels, especially the EtOH extract, contained high levels of polyphenols and exhibited prominent antioxidant effects, tested by scavenging DPPH radicals, TEAC, reducing power, Fe^{2+} ion chelation, inhibition of human LDL oxidation, inhibition of human erythrocyte hemolysis, and scavenging of oxygen radicals in human blood. Ferulic acid, sinapic acid, and syringic acid were the major phenolic acids, and narigenin, myricetin, naringin, and apigenin were the major flavonoids in these extracts. These results illustrate that *C. gracillima* Diels has a potential benefit for health promotion.

Conflicts of interest

All authors declare no conflicts of interest.

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