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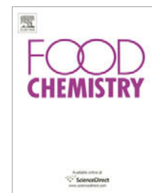

Screening, determination and quantification of major antioxidants from *Balanophora laxiflora* flowers

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ABSTRACT

In this study, antioxidant phytochemicals of *Balanophora laxiflora* flowers were detected by online HPLC–DPPH method. Accordingly, five phytochemicals including 1-*O*-(*E*)-caffeoyl-*β*-D-glucopyranose (**1**), 1-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranose (**2**), caffeic acid (**3**), 1,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (**4**), and 1-*O*-(*E*)-caffeoyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (**5**) were isolated using the developed screening method. Of these, compounds **1** and **5** were found to be major bioactive phytochemicals, and their contents were determined to be 10.8 mg and 9.5 mg per gramme of crude extract, respectively. In addition, compared with (+)-catechin, a well-known antioxidant, compounds **4** and **5** exhibited stronger DPPH radical and superoxide radical scavenging activities than (+)-catechin. These results demonstrated that the flower extracts of *B. laxiflora* have excellent antioxidant activities and thus have great potential as a source for natural health products.

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1. Introduction

Reactive oxygen species (ROS) including superoxide radical, hydroxyl radical, singlet oxygen, and hydrogen peroxide are often generated as byproducts of biological reactions or from exogenous factors (Cerutti, 1991). ROS have been found to play an important role in several diseases such as ageing, atherosclerosis, inflammatory injury, cancer, and cardiovascular disease (Halliwell, 1997). Thus, a potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases. In addition, many studies have demonstrated the potential of plant products as antioxidants against various diseases induced by free radicals (Hou et al., 2003), and it has been determined that the antioxidant effect of plants is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (Pietta, 2000). Therefore, recent studies have shown that phenolic compounds have some bioactivities such as antioxidant, antivirus, antitumor, antiinflammation, hepatoprotection and the prevention of cardiovascular diseases, as well as the bioactivities of these phenolic compounds which might be related to their antioxidant properties (Li, Liu, Zhang, & Yu, 2008).

Balanophora laxiflora Hemsl. (Balanophoraceae), a native species, is widely distributed in woodlands from middle or low altitude in Taiwan, and it is traditionally used as a medicinal plant. In our previous study, it was found that the extract from the female flower of *B. laxiflora* exhibited a good antioxidant activity (Cheng, Ho, Chen, & Wu, 2008), and therefore it might be a good candidate for further development with antioxidant potential. However, the potential bioactivities of male and female flowers of *B. laxiflora* have not been studied. Thus, the antioxidant activities of methanolic extracts from male and female flowers of *B. laxiflora* were evaluated by various *in vitro* antioxidant models, including DPPH radical scavenging assay, superoxide radical scavenging assay, and reducing power assay. On the other hand, in order to conduct rapid screening the active compounds from flowers of *B. laxiflora*, an online HPLC–DPPH technique was also applied in this study.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), potassium ferricyanide, gallic acid, ferric chloride, Folin–Ciocalteu reagent, and (+)-catechin were all purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents used in this experiment were of analytical grade.

2.2. Plant materials

B. laxiflora was collected in Taichung County, in the midlands of Taiwan. The plant species was identified by Dr. Chao-Lin Kuo, and a voucher specimen (voucher No. 4672) was deposited at the Herbarium of the China Medical University, Taichung, Taiwan.

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2.3. Extraction, isolation, and identification

The dried samples (male flower: 5.0 kg; female flower: 9.8 kg) were chopped into small pieces and extracted with methanol (40 l and 100 l for male and female flowers, respectively) by soaking for one week each at room temperature (25 °C) two times. The methanolic extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilised. The yields of crude extracts from male and female flowers were 8% and 13.2%, respectively. The resulting crude extract from the male flower of *B. laxiflora* (400.5 g) was fractionated successively with ethyl acetate (EtOAc), *n*-butanol (BuOH), and water to yield soluble fractions of EtOAc (205.1 g), BuOH (110.6 g), and water (50.0 g). The EtOAc soluble fraction from the male flower of *B. laxiflora* (150.0 g) was loaded into a chromatography column (Geduran Si-60, Merck, Darmstadt, Germany) and eluted with gradient EtOAc/*n*-hexane and MeOH/EtOAc solvent systems, and 10 subfractions (EA1–EA10) were obtained. The antioxidative phytochemicals from the EA8 were separated and purified by semipreparative HPLC using a PU-2080 pump (Jasco, Japan) equipped with a MD-2010 multiwavelength detector (Jasco, Japan) and a 250 × 10.0 mm i.d., 4-μm Synergi Polar-RP column (Phenomenex, Torrance, CA). The mobile phase was solvent **A**, 100% MeOH; and solvent **B**, ultrapure water. Elution condition was 0–80 min of 20–100% **A**–**B** (linear gradient) at a flow rate of 4 ml/min. The structures of compounds **1**–**5** were identified by MS (Finnigan MAT-95S, Germany) and NMR (Varian Unity Inova-600, USA), and all spectral data were consistent with the literature (Baderschneider & Winterhalter, 2001; Jiang et al., 2001; Villegas & Kojima, 1985; Yoshida, Ahmed, Memon, & Okusa, 1991).

2.4. DPPH radical scavenging activity (DPPH assay)

The DPPH free radical scavenging activity of the extracts from male and female flowers of *B. laxiflora* was determined according to the method reported by Gyamfi, Yonamine, and Aniya (1999). 10 μl of the test samples in methanol, yielding a series of extract concentrations of 1, 5, 10, 50, and 100 μg/ml, respectively, in each reaction, were mixed with 200 μl of 0.1 mM DPPH–ethanol solution and 90 μl of 50 mM Tris–HCl buffer (pH 7.4). Methanol (10 μl) alone was used as the control of this experiment. After 30 min of incubation at room temperature, the reduction in DPPH free radicals was measured by reading the absorbance at 517 nm. (+)-Catechin, a well-known antioxidant, was used as the positive control. Three replicates were made for each test sample. The inhibition ratio was calculated according to the following equation: % inhibition = [(absorbance of control − absorbance of sample)/absorbance of control] × 100.

2.5. Superoxide radical scavenging assay (NBT assay)

Measurement of superoxide radical scavenging activity was carried out according to the method of Kirby and Schmidt (1997). First, 20 μl of 15 mM Na_2EDTA in buffer (50 mM KH_2PO_4/KOH, pH 7.4), 50 μl of 0.6 mM nitroblue tetrazolium chloride (NBT) in buffer, 30 μl of 3 mM hypoxanthine in 50 mM KOH, 5 μl of the test samples in methanol and 145 μl of buffer were mixed in 96-well microplates. The reaction was started by adding 50 μl of xanthine oxidase in the buffer (1 unit in 10 ml buffer) to the mixture. The reaction mixture was incubated at room temperature, and the absorbance at 570 nm was determined every 1 min up to 9 min using the ELISA reader (Labsystems Multiskan, USA). (+)-Catechin was used as the positive control. Three replicates were made for each test sample. The inhibition ratio was calculated according to the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

2.6. Reducing power assay

This assay was determined according to the method reported by Oyaizu (1986) with slight modifications, using (+)-catechin as the standard. Briefly, 1 ml of reaction mixture, containing 500 μl of the test samples in 500 μl of phosphate buffer (0.2 M, pH 6.6), was incubated with 500 μl of potassium ferricyanide (1%, w/v) at 50 °C for 20 min. The reaction was terminated by adding trichloroacetic acid (10%, w/v), and then the mixture was centrifuged at 12,000*g* for 10 min. The supernatant solution (500 μl) was mixed with distilled water (500 μl) and 100 μl of ferric chloride (0.1%, w/v) solution, then the optical density (OD) was measured at 700 nm. The reducing power ability was expressed as (+)-catechin equivalents (CE) in milligrams per gramme sample.

2.7. Determination of total phenolics

Total phenolic content was determined according to the Folin–Ciocalteu method (Quettier-Deleu et al., 2000) using gallic acid as the standard. The extract (5 mg) was dissolved in 5 ml of methanol/water (50:50 v/v). The extract solution (500 μl) was mixed with 500 μl of 50% Folin–Ciocalteu reagent. The mixture was kept for 5 min, which was followed by the addition of 1.0 ml of 20% Na_2CO_3. After 10 min of incubation at room temperature, the mixture was centrifuged for 8 min (150*g*), and the absorbance of the supernatant was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gramme of sample.

2.8. Online HPLC–DPPH method

The extract with the best antioxidant activity (EA8 subfraction from the male flower of *B. laxiflora*) was further monitored by online HPLC–DPPH method. The EA8 subfraction (stock concentration = 20 mg/ml) was monitored by HPLC on a model PU-2080 instrument (Jasco, Japan) with a 250 × 10.0 mm i.d., 4-μm Synergi Polar-RP column (Phenomenex, Torrance, CA). The mobile phase was solvent **A**, 100% MeOH; and solvent **B**, ultrapure water. Elution condition was 0–80 min of 20–100% **A** to **B**, at a flow rate of 4 ml/min, using a detector, Jasco MD-2010 multiwavelength at 280 nm wavelength. As for online DPPH radical scavenging analysis, the flow of DPPH reagent (50 mg/l in MeOH) was set to be 2 ml/min, and the induced bleaching was detected photometrically as a negative peak at 517 nm.

2.9. Statistical analyses

All results were expressed as mean ± SD ($n = 3$). The significance of difference was calculated by Scheffe's test, and values <0.05 were considered to be significant.

3. Results and discussion

3.1. Radical scavenging activity of the extracts from male and female flowers of B. laxiflora

In order to compare the extracts from male and female flowers of *B. laxiflora*, the DPPH and NBT assays were used. As shown in Table 1, the extract of the male flower has a better DPPH radical scavenging activity, and its IC_{50} value (the concentration required to inhibit radical formation by 50%) was 6.0 μg/ml. In the superoxide radical scavenging assay, the extract of the male flower was more effective than that of the female flower, and their IC_{50} values were 5.4 and 17.0 μg/ml, respectively. These results indicate that radical scavenging activity of crude extract from the male flower

Table 1
DPPH radical and superoxide radical scavenging activities of methanolic extracts from male and female flowers of *B. laxiflora*.

Extracts	IC$_{50}$ (μg/ml)	
	DPPH radical	Superoxide radical
Male flower	6.0[b]	5.4[a]
Female flower	6.4[b]	17.0[c]
(+)-Catechin	2.7[a]	9.0[b]

Different letters within a column indicate significant difference at $P < 0.05$.

was more effective than that from the female flower. Thus, the crude extract and its derived soluble fractions from the male flower of *B. laxiflora* were further investigated in the study.

3.2. DPPH radical scavenging activity of the crude extract and its derived soluble fractions from the male flower of B. laxiflora

DPPH· method is one of the most popular methods in natural antioxidant studies (Moon & Shibamoto, 2009). Accordingly, as shown in Fig. 1A, the DPPH radical scavenging activity of methanolic extract and its derived soluble fractions from the male flower of *B. laxiflora*, including the soluble fractions of EtOAc, BuOH, and water, were shown in a dose-dependent manner. Of these, the EtOAc fraction exhibited the strongest activity. Meanwhile, except for the water soluble fraction, all extracts showed a good inhibitory activity against the DPPH radical. The IC$_{50}$ values of the crude extract, EtOAc fraction, BuOH fraction, and water fraction were 6.0, 3.0, 6.4, and 15.7 μg/ml, respectively. As for (+)-catechin, a well-known antioxidant compound used as the reference control in this study, its IC$_{50}$ value was 2.7 μg/ml. On the other hand, the flowers of *Matricaria chamomilla* and *Jasminum sambac* are two common species used for herbal teas on market, and it is claimed their flower extracts usually exhibit good antioxidant activities (Huang, Tung, Lin, & Chang, 2007; McKay & Blumberg, 2006). According to the results reported by Huang et al. (2007), the flower extracts of *M. chamomilla* and *J. sambac* were found to possess significant DPPH radical scavenging activity with IC$_{50}$ values of 62.8 and >100 μg/ml, respectively. Comparing these two flower extracts, the DPPH radical scavenging activity of crude extract from the male flower of *B. laxiflora* was stronger that that of *M. chamomilla*m and *J. sambac*. These results imply that there are abundant antioxidative phytochemicals present in the male flower of *B. laxiflora*, especially in the EtOAc fraction.

3.3. Superoxide radical scavenging activity of the crude extract and its derived soluble fractions from the male flower of B. laxiflora

Superoxide radicals are known to be very harmful to cellular components as a precursor of many ROS, for example, when in presence of metal ions, the superoxide radical would further produce a highly reactive hydroxyl radical (MacDonald-Wicks, Wood, & Garg, 2006). Additionally, the superoxide radical is biologically important because it can be decomposed to form a stronger oxidative species such as singlet oxygen and hydroxyl radicals (Wu & Tung et al., 2008). As shown in Fig. 1B, the superoxide radical scavenging activity of the crude extract and its derived soluble fractions from the male flower of *B. laxiflora* were shown in a dose-dependent manner. And the IC$_{50}$ values of crude extract, EtOAc fraction, BuOH fraction, water fraction, and (+)-catechin were 5.4, 4.1, 5.8, 20.4, and 9.0 μg/ml, respectively. Accordingly, the results of NBT assay were similar to the DPPH· assay; the EtOAc fraction exhibited the highest radical scavenging activity, whilst the water soluble fraction was the weakest. In addition, this result also showed that, with the exception of the water fraction, all the tested samples exhibited stronger superoxide radical scavenging activity than (+)-catechin, a well-known antioxidant.

3.4. Reducing power of the crude extract and its derived soluble fractions from the male flower of B. laxiflora

Many studies have demonstrated that the reducing power in plant extracts was highly correlated with their antioxidant activities (Chang, Yen, Huang, & Duh, 2002). In general, the reducing properties are associated with the presence of reductones, which have been shown to exert antioxidant action by donating a hydrogen atom (Wu & Tung et al., 2008). Fig. 2 shows the reducing power

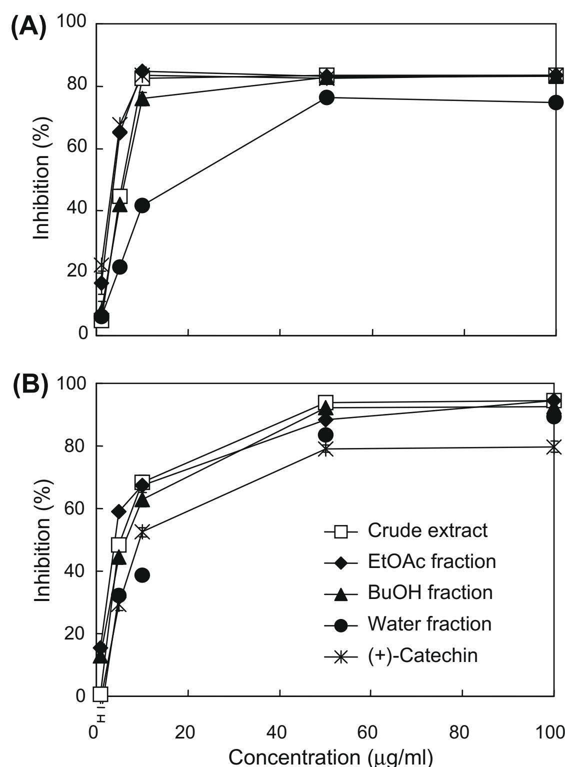

Fig. 1. (A) DPPH radical and (B) superoxide radical scavenging activities of the extract and its derived soluble fractions from the male flower of *B. laxiflora*. Results are mean ± SD ($n = 3$).

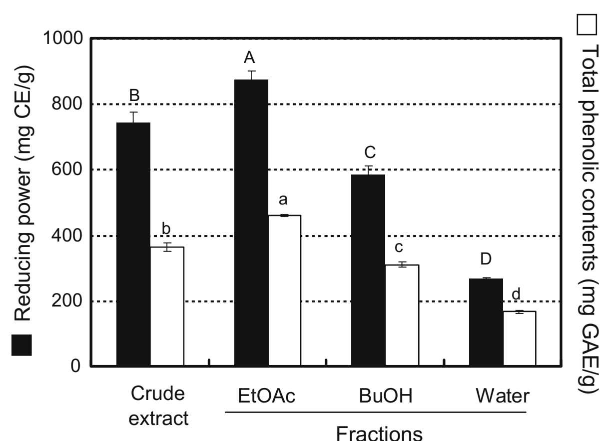

Fig. 2. Reducing power and total phenolic contents of the extract and its derived soluble fractions from the male flower of *B. laxiflora*. Results are mean ± SD ($n = 3$). The bars marked by different letters are significantly different at the level of $P < 0.05$ according to the Scheffe test.

of the crude extract and its derived fractions calculated as (+)-catechin equivalent (CE). As shown in Fig. 2, the reducing power of the EtOAc fraction (875.7 mg/g) was higher than that of crude extract (742.9 mg/g), BuOH fraction (585.9 mg/g), and water fraction (267.6 mg/g). Additionally, according to the above results, the reducing power of the extracts from the male flower of *B. laxiflora* had a direct correlation to its antioxidant activities, and a similar result was obtained by another author (Kumaran & Karunakaran, 2006).

3.5. Total phenolic contents of the crude extract and its derived soluble fractions from the male flower of B. laxiflora

Phenolic compounds are rich in plants and vegetables, and also are the most abundant secondary metabolites in plants. Additionally, phenolic compounds have been found in many food and their derivatives, such as wine, tea, olive oil, cereals, legumes, fruits, and juices (Velioglu, Mazza, Gao, & Oomah, 1998). It is well accepted that phenolic compounds have many biological activities, such as antiinflammatory, anticarcinogenic, antiatherogenic, and cardioprotective effects (Tagliazucchi, Verzelloni, & Conte, 2005). Fig. 2 shows the content of total phenolics in the crude extract and its derived fractions calculated as gallic acid equivalent (GAE). Results reveal that the total phenolic contents of EtOAc fraction were much higher than the other fractions. The total phenolic content decreased in the following order: EtOAc fraction (460.0 mg GAE/g) > crude extract (363.6 mg GAE/g) > BuOH fraction (310.2 mg GAE/g) > water fraction (165.6 mg GAE/g). According to the aforementioned antioxidant results, there was a high correlation between the total phenolic content and antioxidant activities, including DPPH radical scavenging activity, superoxide radical scavenging activity, and reducing power. Similar results were also found in many plant extracts (Kujala, Loponen, Klika, & Pihlaja, 2000; Wu, Huang, Tung & Cheng, 2008; Wu & Tung et al., 2008). On the other hand, according to the results reported by Huang et al. (2007), the total phenolic contents of male flower extract of *B. laxiflora* were higher (ca. 10-fold) than those of *M. chamomilla* (41.5 mg/g) or *J. sambac* (32.4 mg/g). These results indicated that antioxidant activities of *B. laxiflora* were comparable with those of flowers available on market, and it is proposed here that the phytochemicals from the male flower extract of *B. laxiflora* may play an important role in the free radical scavenging activity.

3.6. Screening, quantification, and determination of major antioxidants from the male flower of B. laxiflora

In previous antioxidant assays, we found the EtOAc soluble fraction from the male flower of *B. laxiflora* had a powerful antioxidant activity and it might be a good candidate to be developed as a novel natural antioxidant. Based on a bioactivity-guided isolation principle, the EtOAc soluble fraction was further derived into 10 subfractions by column chromatography. Table 2 shows the elution solvent, collected weight, and DPPH radical scavenging activity for these 10 subfractions. Of these, the EA8 was eluted out with 100% of EtOAc and exhibited the strongest inhibitory activity against DPPH radical (IC_{50} = 3.0 μg/ml).

It is well-known that the online HPLC–DPPH method can be used for a rapid assessment of pure antioxidant compounds in complex mixtures, particularly plant extracts. The more rapid the absorbance decreases, the more the potent antioxidant activity of the compound will be in terms of hydrogen-donating ability (Wu & Tung et al., 2008). Therefore, in order to rapidly screen the active phytochemicals from the EA8, the online HPLC–DPPH method was used in the present study. Fig. 3A shows the combined UV (positive signals) and DPPH· quenching (negative signals) chromatograms of the EtOAc fraction from the male flower of *B. laxiflora*. As can be seen, amongst several eluted phytochemicals, 1-*O*-(*E*)-caffeoyl-*β*-D-glucopyranose (**1**), 1-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranose (**2**), caffeic acid (**3**), 1,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (**4**), and 1-*O*-(*E*)-caffeoyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose (**5**) (Fig. 3B) showed the significant hydrogen-donating capacity (negative peak) towards the DPPH radical at the applied concentration, and their contents were determined to be 10.8, 2.0, 0.5, 2.4, and 9.5 mg per gramme of crude extract, respectively (Table 3). This result reveals that the method can be applied for a quick screening of antioxidant compounds or more precisely radical scavenging activity of phytochemicals. Thus, it is

Table 2
Mobile phase, yield, and DPPH radical scavenging activity of EtOAc soluble fraction from the male flower of *B. laxiflora*.

Subfractions	Mobile phase[a] (v/v)	Yields (wt.%)	DPPH radical (IC_{50}, μg/ml)
EA1	5/95 (E/H)	4.2	>100
EA2	10/90 (E/H)	1.6	>100
EA3	25/75 (E/H)	2.3	>100
EA4	25/75 (E/H)	0.5	85.3
EA5	25/75 (E/H)	0.5	7.9
EA6	50/50 (E/H)	12.7	3.6
EA7	75/25 (E/H)	28.9	3.2
EA8	100/0 (E/H)	18.9	3.0
EA9	10/90 (M/E)	16.9	4.1
EA10	30/70 (M/E)	5.7	4.5

[a] E: EtOAc; H: *n*-hexane; M: methanol.

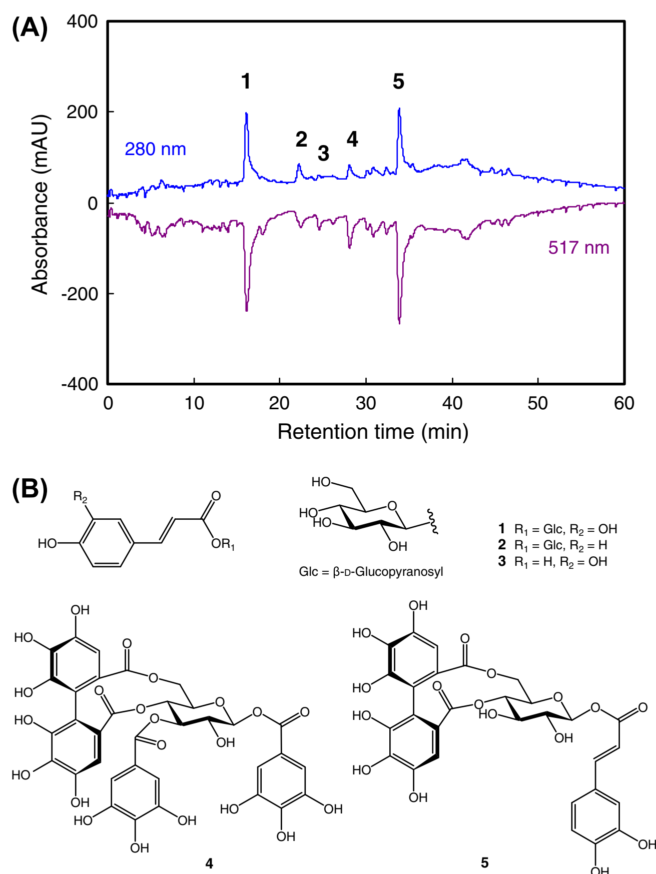

Fig. 3. (A) UV and DPPH radical quenching chromatograms of EA 8 subfraction from the male flower of *B. laxiflora*. (B) Isolated and identified phytochemicals. **1**, 1-*O*-(*E*)-caffeoyl-*β*-D-glucopyranose; **2**, 1-*O*-(*E*)-*p*-coumaroyl-*β*-D-glucopyranose; **3**, caffeic acid; **4**, 1,3-di-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose, and **5**, 1-*O*-(*E*)-caffeoyl-4,6-(*S*)-hexahydroxydiphenoyl-*β*-D-glucopyranose.

Table 3
Antioxidant activities and contents of major phytochemicals from the male flower of *B. laxiflora*.

Phytochemicals	Contents (mg/g of methanolic extract)	IC_{50} (μM)	
		DPPH radical	Superoxide radical
1	10.8	8.0^{b}	34.9^{e}
2	2.0	25.6^{e}	18.8^{c}
3	0.5	13.7^{d}	18.1^{c}
4	2.4	3.2^{a}	9.4^{b}
5	9.5	2.9^{a}	2.8^{a}
(+)-Catechin	–	11.3^{c}	27.2^{d}

Different letters within a column indicate significant difference at $P < 0.05$.

no longer necessary to isolate and purify non-target phytochemicals, leading to very significant reductions in cost and faster results.

On the other hand, to determine the antioxidant activities of these five phytochemicals, DPPH· and NBT assays were performed. (+)-Catechin was used as a positive control. As shown in Table 3, compared with (+)-catechin, these compounds had an order of **5** ⩾ **4** > **1** > (+)-catechin > **3** > **2** for DPPH radical scavenging activity. Their IC_{50} values were 2.9, 3.2, 8.0, 11.3, 13.7, and 25.6 μM, respectively. This result demonstrated that compounds **1**, **4**, and **5** exhibited greater DPPH radical scavenging activity than (+)-catechin. In addition, the decreasing superoxide radical scavenging activity order of phytochemicals in NBT assay can be ranked as **5** > **4** > **3** ⩾ **2** > (+)-catechin > **1** (Table 3). In other words, except for compound **1**, all compounds were more effective than (+)-catechin (IC_{50} = 27.2 μM). Furthermore, amongst five phytochemicals, compounds **4** and **5** (both belong to hydrolysable tannins) were the two best antioxidants. The DPPH radical and superoxide radical scavenging activities of these two phytochemicals were totally higher than those of (+)-catechin. This result also implied that the hexahydroxydiphenoyl (HHDP) moiety played an important role for enhancing antioxidant activities.

4. Conclusions

It is well-known that ROS have a high correlation with several diseases such as ageing, atherosclerosis, inflammatory injury, cancer, and cardiovascular disease. This study demonstrated for the first time that, amongst the extracts from male and female flowers of *B. laxiflora*, the EtOAc soluble fraction of the male flower exhibited the highest antioxidant activities. On the other hand, the results confirm the feasibility of assessing radical scavenging activity of specific phytochemicals using the online HPLC–DPPH method. This technique could allow rapid detection of natural antioxidants in complex matrices with only a simple operation. Accordingly, five specific and excellent antioxidants were detected and identified. These results imply that the extracts or the derived phytochemicals from *B. laxiflora* flowers have great potential to prevent diseases caused by the overproduction of radicals, and they may be suitable for the treatment of degenerative diseases.

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