JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Online RP-HPLC-DPPH Screening Method for Detection of Radical-Scavenging Phytochemicals from Flowers of *Acacia confusa*

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Acacia confusa is traditionally used as a medicinal plant in Taiwan. In this study, phytochemicals and antioxidant activities of extracts from flowers of *A. confusa* were investigated for the first time. In addition, a rapid screening method, online RP-HPLC-DPPH system, for individual antioxidants in complex matrices was developed. Accordingly, six antioxidants including gallic acid (**1**), myricetin 3-rhamnoside (**2**), quercetin 3-rhamnoside (**3**), kaempferol 3-rhamnoside (**4**), europetin 3-rhamnoside (**5**), and rhamnetin 3-rhamnoside (**6**) were detected using the developed screening method. Of these, compounds **2**, **3**, and **5** were found to be major bioactive phytochemicals, and their contents were determined as 11.3, 6.7, and 8.7 mg/g of crude extract, respectively. By comparison with quercetin, a well-known antioxidant, these compounds had the order of compound **2** > compound **5** > quercetin > compound **3** for DPPH radical-scavenging activity. Their IC_{50} values were 3.0, 3.2, 4.5, and 7.4 μM, respectively. Moreover, the same order was observed for superoxide radical-scavenging activity, and their IC_{50} values were 2.6, 2.7, 4.3, and 5.3 μM, respectively. However, for lipid peroxidation, quercetin, an aglycon, showed the best inhibitory activity. The IC_{50} values of quercetin, compound **2**, compound **5**, and compound **3** were 46.7, 88.5, 90.7, and 124.6 μM, respectively. These results indicated that a rhamnoside at the C3 position of flavonoids had a negative effect on radical-scavenging activity and antilipid peroxidation. In contrast, the number of hydroxyl groups on the B-ring exhibited a positive relationship with their inhibitory activities.

KEYWORDS: *Acacia confusa*; flower extract; radical-scavenging activity; online RP-HPLC-DPPH; antioxidant

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 (*1*). ROS have been found to play an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease (*2*). Thus, recent studies have investigated the potential of plant products as antioxidants against various diseases induced by free radicals (*3*). In addition, it has been determined that the antioxidant effect of plant products is mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins, and phenolic diterpenes (*4*).

Acacia confusa Merr. (Leguminosae), a native species in Taiwan, is widely distributed on the hills and lowlands of Taiwan, and it is traditionally used as a medicinal plant (*5*). An aqueous extract of *A. confusa* leaves was used in Taiwan for wound healing and anti-blood-stasis (*6*). The crude extracts of *A. confusa* heartwood, leaf, and bark contain a wide variety of phenolic compounds (*5, 7*−*10*). Recent studies have shown that some phenolic compounds have anticancer, anticarcinogenic, or antimutagenic activities (*11, 12*), and these bioactivities of phenolic compounds might be related to their antioxidant properties (*11*). In addition, many dietary phenolic constituents derived from plants are more effective antioxidants in vitro than vitamin C or vitamin E and, thus, might contribute significantly to the protective effects in vivo (*13*). Our previous study has found that the crude extracts of *A. confusa* bark and heartwood exhibited a high DPPH (1,1-dipheny1-2-picrylhydrazyl) radical-scavenging activity, and therefore it might be a good candidate for further development as antioxidant remedies (*5, 7, 8*). However, to the best of our knowledge there is no prior report on the antioxidant activities of *A. confusa* flowers. In this study, we investigated the antioxidant activities of the flower extract

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10.1021/jf072314c CCC: $40.75
Published on Web 12/29/2007

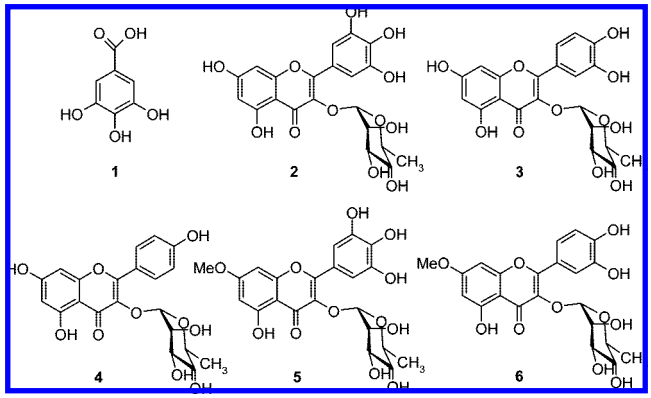

Figure 1. Phytochemicals isolated from *A. confusa* flowers: **1**, gallic acid; **2**, myricetin 3-rhamnoside; **3**, quercetin 3-rhamnoside; **4**, kaempferol 3-rhamnoside; **5**, europetin 3-rhamnoside; **6**, rhamnetin 3-rhamnoside.

of *A. confusa* for the first time and detected the active compounds by a rapid screening method, namely, the online RP-HPLC-DPPH method. Furthermore, the structure–activity relationships of these phytochemicals were also investigated.

MATERIALS AND METHODS

Chemicals. 1,1-Dipheny1-2-picrylhydrazyl (DPPH), Folin−Ciocalteu reagent, quercetin, nitroblue tetrazolium chloride (NBT), and (+)-catechin were all purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals and solvents used in this experiment were of the highest quality available.

Extraction and Purification of Antioxidative Compounds from Flowers of *A. confusa*. Fresh flowers of *A. confusa* were sampled from the experimental forest of National Taiwan University in Nan-Tou County. The species was identified by Sheng-You Lu of the Taiwan Forestry Research Institute, and a voucher specimen (AC001) was deposited at the School of Forestry and Resource Conservation, National Taiwan University. The dried samples (1.07 kg) were soaked in methanol at room temperature for 7 days. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized. The resulting crude extract (177.2 g) was fractionated successively with dichloromethane (CH_2Cl_2), butanol (BuOH), and water to yield soluble fractions of CH_2Cl_2 (44.7 g), BuOH (40.2 g), and H_2O (90.8 g). The antioxidative phytochemicals from the BuOH fraction were separated and purified by semipreparative HPLC on a model PU-980 pump (Jasco, Japan) equipped with a MD-910 photodiode array detector (Jasco, Japan) and a 250 × 10.0 mm i.d., 5 μm Luna RP-18 column (Phenomenex, Torrance, CA). The mobile phase was solvent A (100% acetonitrile) and solvent B (ultrapure water). Elution conditions were 0–5 min of 10% A to B, 5–10 min of 10–55% A to B (linear gradient), 10–30 min of 55% A to B, and 30–40 min of 55–100% A to B (linear gradient) at a flow rate of 4 mL/min. ESIMS data were collected using a Finnigan MAT-95S mass spectrometer, and NMR spectra were recorded by a Bruker Avance 500 MHz FT-NMR spectrometer. The structures of antioxidative compounds **1**–**6** (as shown in **Figure 1**) were identified by ESIMS and NMR, and all spectral data were consistent with the literature (*14*–*17*).

DPPH Radical-Scavenging Activity (DPPH Assay). The scavenging activity of DPPH free radical by *A. confusa* flower extract and its derived soluble fractions was determined according to the method reported by Chang et al. (*7*). Fifty microliters of a flower extract of *A. confusa* in methanol, yielding a series of extract concentrations of 1, 5, 10, and 50 μg/mL, respectively, in each reaction, was mixed with 1000 μL of 0.1 mM DPPH−ethanol solution and 450 μL of 50 mM Tris-HCl buffer (pH 7.4). Methanol (50 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 or quercetin was used as the positive control. The inhibition ratio was calculated using the equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

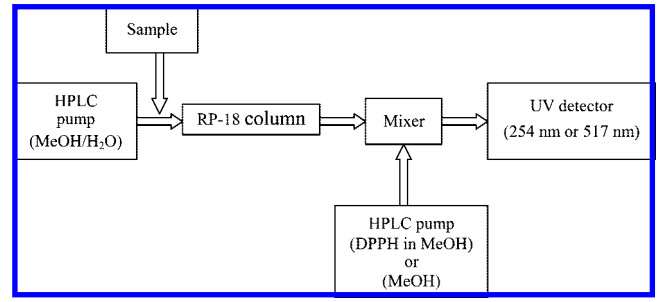

Figure 2. Instrumental setup for the HPLC-DPPH online detection of radical-scavenging compounds.

Superoxide Radical-Scavenging Activity (NBT Assay). Measurement of superoxide radical-scavenging activity was carried out using the method described by Kirby and Schmidt (*18*). 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 NBT in buffer, 30 μL of 3 mM hypoxanthine in 50 mM KOH, 5 μL of test samples in methanol, and 145 μL of buffer were mixed in 96-well microplates (Falcon). The reaction was started by adding 50 μL of xanthine oxidase solution in buffer (1 unit in 10 mL of buffer) to the mixture. The reaction mixture was incubated at room temperature, and the absorbance at 570 nm was determined every 20 s up to 5 min using a plate reader (Labsystems multiskan MS, Finland). The control was 5 μL of methanol instead of the sample solution. Quercetin was used as the positive control. The inhibition ratio was calculated using the equation: % inhibition = [(rate of control − rate of sample reaction)/rate of control] × 100.

Colorimetric Assay for Antilipid Peroxidation Activity. This assay was determined according to the method reported by Duh et al. (*19*). Lecithin (300 mg) was sonicated in an ultrasonic cleaner in 30 mL of phosphate buffer (50 mM, pH 7.4) for 2 h at ice-cold temperature. Then 500 μL of sonicated solution (10 mg of liposome/mL of phosphate buffer) was incubated with the test samples (100 μL) in the presence of 1 mM $FeCl_3$ (200 μL) and 1 mM ascorbic acid (200 μL) at 37 °C for 1 h. The reaction was terminated by the addition of 500 μL of trichloroacetic acid (20% w/v) and 500 μL of thiobarbituric acid (1% w/v); then the solution was heated at 100 °C. After 15 min, the color of the malondialdehyde (MDA)−TBA complex was measured at 532 nm. Quercetin was used as the positive control. The inhibition ratio was calculated using the equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

Determination of Total Phenolics. Total phenolic contents were determined according to the Folin−Ciocalteu method (*20*) using gallic acid as the standard. The flower extract (5 mg) of *A. confusa* 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 a 2−5 min period, 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 gram of sample.

Online DPPH Radical-Scavenging Analysis. The best antioxidant activity of the extract (BuOH fraction) of *A. confusa* flowers was further monitored by the online RP-HPLC-DPPH method. The instrumental setup was depicted in **Figure 2**. The BuOH fraction (stock concentration = 17 mg/mL) was monitored by analytic HPLC on a model PU-980 instrument (Jasco, Japan) with a 250 × 4.6 mm i.d., 5 μm Luna RP-18 column (Phenomenex, Torrance, CA). The mobile phase was solvent A (100% MeOH) and solvent B (ultrapure water). Elution conditions were 0–60 min of 10–70% A to B (linear gradient) and 60–65 min of 70–100% A to B (linear gradient) at a flow rate of 1.0 mL/min using a detector, Jasco MD-910 photo diode array at 254 nm wavelength. As for online DPPH radical-scavenging analysis, the flow of DPPH reagent (50 mg/L in methanol) was set to be 0.5 mL/min, and the induced bleaching was detected photometrically as a negative peak at 517 nm.

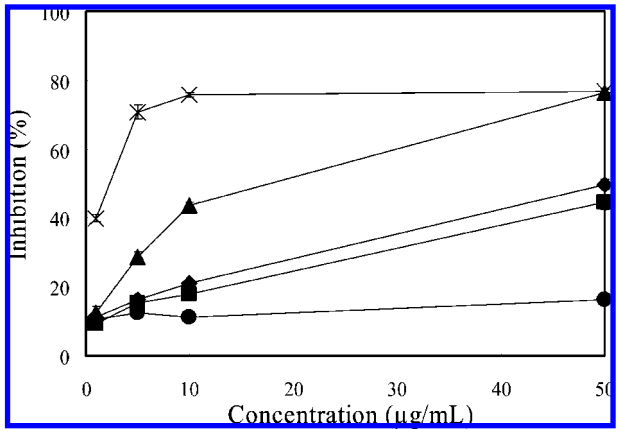

Figure 3. Free radical-scavenging activity of methanolic extracts from flowers of *A. confusa* measured by DPPH assay: (◆) crude extract, (■) dichloromethane fraction, (▲) butanol fraction, (●) water fraction, and (×) (+)-catechin. Results are means ± SD ($n = 3$).

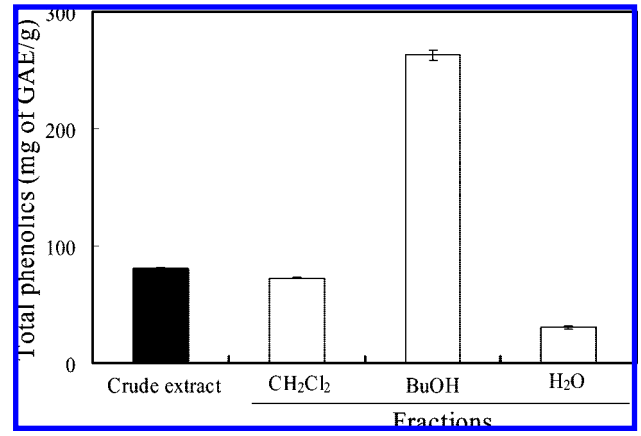

Figure 4. Total phenolic content from flowers of *A. confusa* measured using the Folin−Ciocalteu method. Results are means ± SD ($n = 3$).

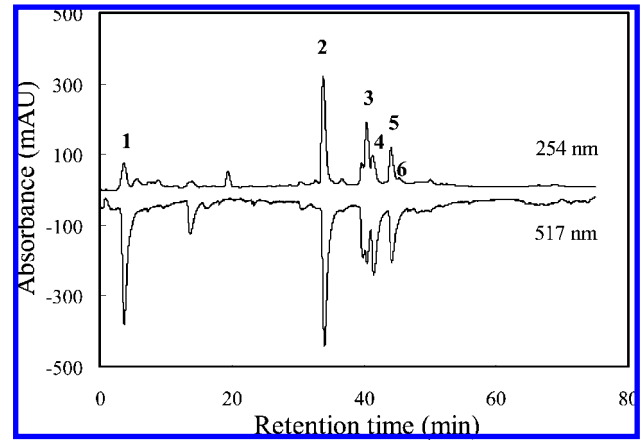

Figure 5. UV and DPPH radical quenching chromatograms of the BuOH fraction extracted from flowers of *A. confusa*.

RESULTS AND DISCUSSION

DPPH Radical-Scavenging Activity of *A. confusa* Flower Extract and Its Derived Soluble Fractions. In the present study, the free radical-scavenging activity of the *A. confusa* flower extract was assessed by DPPH assay. Accordingly, as shown in **Figure 3**, the DPPH radical-scavenging activity of the methanolic extract and its derived soluble fractions from flowers of *A. confusa*, including the soluble fractions of CH_2Cl_2, BuOH, and water, was shown in a dose-dependent manner. Of these, the BuOH fraction showed 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 (the concentration required to inhibit radical formation by 50%) of the crude extract, CH_2Cl_2 fraction, BuOH fraction, and water fraction were 62.9, 72.4, 13.9, and 358.1 μg/mL, respectively. As for (+)-catechin, a well-known antioxidant compound used as the reference control in this study, its IC_{50} value was 2.9 μg/mL. On the other hand, the flowers of *Matricaria chamomilla* and *Jasminum sambac* are two common species used for herbal teas on the market, and their flower extracts are usually claimed to be exhibiting good antioxidant activities (*21, 22*). According to the results reported by Huang et al. (*22*), 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. By comparison with these two flower extracts, the DPPH radical-scavenging activity of crude extract from *A. confusa* flower was similar to that of *M. chamomilla* but stronger than that of *J. sambac*. These results imply that there are abundant antioxidative phytochemicals present in the flower extract of *A. confusa*, especially in the BuOH fraction.

Total Phenolic Contents of *A. confusa* Flower Extract and Its Derived Soluble Fractions. Phenolic compounds are commonly found in the plant kingdom, and they have been reported to have multiple biological effects (*23, 24*). Correlation between the content of phenolic compounds and antioxidant activities has been described in many studies (*7, 25, 26*). The phenolic compounds are very important plant constituents because of their scavenging ability of free radicals. **Figure 4** shows the content of total phenolics in the crude extract and its derived fractions calculated as gallic acid equivalent (GAE) in milligrams per gram of sample. Apparently, the total phenolic content of the BuOH fraction (263.2 mg/g) was higher than that of the crude extract (80.5 mg/g), CH_2Cl_2 fraction (72.6 mg/g), and water fraction (30.7 mg/g). Furthermore, according to the results reported by Huang et al. (*22*), the total phenolic content of the flower extract of *A. confusa* is about twice as much as that of *M. chamomilla* (41.5 mg/g) or *J. sambac* (32.4 mg/g). Results obtained indicated that antioxidant activities of *A. confusa* flowers were comparable with those of flowers available on the market. These results suggested that the antioxidant activity of the flower extract may be correlated to its phenolic content, and it is proposed here that the phytochemicals from the flower extract of *A. confusa* may play an important role in the DPPH radical-scavenging activity. Moreover, similar results were found in many plant extracts (*20, 24, 27, 28*).

Online RP-HPLC-DPPH Method. The online RP-HPLC-DPPH method can be used for a rapid assessment of pure antioxidant compounds in complex mixtures, particularly plant extracts (*29*). The more rapidly the absorbance decreases, the more potent the antioxidant activity of the compound in terms of hydrogen-donating ability (*30*). Combined UV (positive signals) and DPPH$^{\bullet}$ quenching (negative signals) chromatograms under gradient conditions of the BuOH fraction from the flowers of *A. confusa* are presented in **Figure 5**. Several eluted phytochemicals in the BuOH fraction were detected and gave positive peaks on the UV detector (254 nm). Among them, gallic acid (**1**), myricetin 3-rhamnoside (**2**), quercetin 3-rhamnoside (**3**), kaempferol 3-rhamnoside (**4**), europetin 3-rhamnoside (**5**), and rhamnetin 3-rhamnoside (**6**) showed hydrogen-donating capacity (negative peak) toward the DPPH radical at the applied concentration. Results revealed that the method can be applied for a quick screening of antioxidant compounds or, more

Table 1. Contents and IC_{50} Values of Major Phytochemicals from Flowers of *A. confusa* in Inhibiting DPPH Radical, Superoxide Radical, and Lipid Peroxidation[a]

phytochemicals	IC_{50} (μM) DPPH radical	IC_{50} (μM) superoxide radical	IC_{50} (μM) lipid peroxidation	contents (mg/g of methanolic extract)
myricetin 3-rhamnoside (**2**)	3.0	2.6	88.5	11.3 ± 0.2
quercetin 3-rhamnoside (**3**)	7.4	5.3	124.6	6.7 ± 0.1
europetin 3-rhamnoside (**5**)	3.2	2.7	90.7	8.7 ± 0.3
quercetin (positive control)	4.5	4.3	46.7	

[a] Results are means ± SD ($n = 3$).

precisely, radical-scavenging activity of compounds. Thus, it is no longer necessary to isolate and purify nontarget phytochemicals, leading to very significant reductions in costs and faster results.

Quantification and Antioxidant Activities of Major Active Compounds in the Flower Extract of *A. confusa*. According to the screening result of the online RP-HPLC-DPPH system, myricetin 3-rhamnoside (**2**), quercetin 3-rhamnoside (**3**), and europetin 3-rhamnoside (**5**) were found to be the three major bioactive phytochemicals in the BuOH fraction, and their contents were determined to be 11.3, 6.7, and 8.7 mg/g of methanolic crude extract, respectively (**Table 1**). To determine the antioxidant activities of these major active compounds, DPPH, NBT, and antilipid peroxidation assays were performed. Quercetin was used as the positive control. As shown in **Table 1**, the IC_{50} values for DPPH radical-scavenging activity of these major phytochemicals (compounds **2**, **3**, and **5**) were 3.0, 7.4, and 3.2 μM, respectively. This revealed that these flavonoid rhamnosides showed a significant inhibitory activity against the DPPH radical. Even the free radical-scavenging effect of compounds **2** and **5** was stronger than that of quercetin (4.5 μM), a well-known antioxidant. Similarly, these compounds showed the same order of compound **2** > compound **5** > quercetin > compound **3** for the superoxide radical-scavenging activity with IC_{50} values of 2.6, 2.7, 4.3, and 5.3 μM, respectively. However, for lipid peroxidation, of these, quercetin, an aglycon of compound **3**, showed the best inhibitory activity. The IC_{50} values of quercetin, compound **2**, compound **5**, and compound **3** were 46.7, 88.5, 90.7, and 124.6 μM, respectively. These results indicated that a rhamnoside at the C3 position of flavonoids exhibited a negative effect on antioxidant activities, especially for antilipid peroxidation. Moreover, comparison between quercetin 3-rhamnoside and myricetin 3-rhamnoside revealed that the number of hydroxyl groups on the B-ring was positively related to their inhibitory activities. With the same number of hydroxyl groups in the B-ring, however, the methoxyl group at the C7 position in the A-ring (europetin 3-rhamnoside) had a similar effect on the hydroxyl group at the same position (myricetin 3-rhamnoside).

In conclusion, phytochemicals, especially flavonoids and phenolic acids, are of current interest because of their important biological and pharmacological properties. To our knowledge, this is the first report to address respective bioactive compounds from the flowers of *A. confusa*. On the other hand, the results confirm the feasibility of assessing radical-scavenging activity of specific phytochemicals using the online RP-HPLC-DPPH method. This technique could allow a rapid detection of natural antioxidants in complex matrices with simple operation. Accordingly, six specific antioxidants, i.e., gallic acid, myricetin 3-rhamnoside, quercetin 3-rhamnoside, kaempferol 3-rhamnoside, europetin 3-rhamnoside, and rhamnetin 3-rhamnoside, were detected and identified from the flower extract of *A. confusa*. Of these, myricetin 3-rhamnoside, quercetin 3-rhamnoside, and europetin 3-rhamnoside were three major bioactive phytochemicals, which exhibited an excellent inhibitory activity against free radicals. Thus, these results showed that extracts from flowers of *A. confusa* might have good potential as a source for natural health products due to their antioxidant activities.

ACKNOWLEDGMENT

We thank Shou-Ling Huang (Department of Chemistry, National Taiwan University) for NMR spectral analyses.

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Received for review August 1, 2007. Revised manuscript received November 27, 2007. Accepted November 28, 2007. We thank the Forestry Bureau of the Council of Agriculture for financial support (93-00-5-10 and 94-00-5-04).

JF072314C