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ARTICLE

Antioxidant Activities and Xanthine Oxidase Inhibitory Effects of Phenolic Phytochemicals from *Acacia confusa* Twigs and Branches

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This study investigates bioactivities and active phytochemicals of the extracts of twigs and branches from the indigenous Taiwanese tree *Acacia confusa* Merr. The bioassay-guided fractionation yielded 8 potent antioxidative phytochemicals. Catechins are the major components in the extract of 5 cm branch bark, including catechin, catechin-3-*O*-rhamnopyranoside, *epi*-catechin, and quercetin-3-*O*-rhamnopyranoside, while flavonol glycosides are the major components in twig extract, namely, myricetin-3-*O*-glucopyranoside, myricetin-3-*O*-rhamnopyranoside, quercetin-3-*O*-rhamnopyranoside, myricetin-3-*O*-(2″-*O*-galloyl)-α-rhamnopyranoside, and luteolin. Of the test compounds, myricetin-3-*O*-(2″-*O*-galloyl)-α-rhamnopyranoside exhibited the highest antioxidant activity against DPPH radicals, showing a 2.8-fold lower IC_{50} value of 5.3 μM related to that of quercetin. On the other hand, catechins apparently had no XOD inhibitory effect apart from their good antioxidant activities, while luteolin exhibited excellent activity against XOD with an IC_{50} value of 11.6 μM. These results may provide useful information for future studies on the applications of *A. confusa* to be used as a source for natural health products.

KEYWORDS: ***Acacia confusa*; antioxidant activity; branch; catechin; luteolin; twig; xanthine oxidase**

INTRODUCTION

Oxidation is the basis of aerobic life and of human metabolism, so radicals are produced either naturally or by biological dysfunction. Reactive oxygen species (ROS) are the most common radicals affecting human health, and there has been extensive research on the hazards of the overproduction of ROS: lipid peroxidation, protein aggregation, and DNA damage, causing various pathologies and degenerative disorders (*1*). The use of herbal treatments in Chinese medicine has a history of several thousand years. Since numerous positive effects of phenolics from plant sources have been confirmed, extraction of active phenolic compounds from plants is receiving increasing attention. Due to their redox properties, phenolics can act as reducing agents, hydrogen donors, and singlet oxygen quenchers, making them effective for the neutralization or scavenging of ROS (*2*).

In addition to their antioxidant capacity, some phenolic compounds can reduce oxidative stress via inhibition or activation of key regulating enzymes such as xanthine oxidase, phospholipase and nitric oxide synthase (*3*). Xanthine oxidase has been reported as a very important enzyme that increases during oxidative stress. Granger et al. (*4*) first suggested that, during ischemia−reperfusion, superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) production are enhanced due to increased conversion of xanthine dehydrogenase to xanthine oxidase, and this hypothesis has been confirmed by Rasmussen et al. (*5*). Such highly reactive radicals may generate more ROS in vivo. Thus therapeutic use of inhibitors of xanthine oxidase has been proposed in the prevention of ischemia−reperfusion injury.

Flavonoids may provide a remedy for radical-mediated diseases (*6*). These low molecular phenolic substances are found abundantly in several *Acacia* species belonging to the Leguminosae family. *Acacia confusa* Merr., a native species widely distributed over the hills and lowlands of Taiwan, has been traditionally used as a medicinal plant for wound healing and increasing blood circulation (*7*). According to previous studies, the extracts of *A. confusa* heartwood and stem bark contain a wide variety of phenolic compounds and show excellent antioxidant activities (*8*, *9*). Moreover, chemical studies on *A. confusa* showed that distributions of phenolic compounds vary between different plant parts: flavone aglycons in the heartwood (*10*), phenolic acids and proanthocyanidin dimers in the stem bark (*9*, *11*) and flavanoid glycosides in the leaf and flower (*12*, *13*). These results indicate that the plant contains many phenolic compounds in whole plants, and thus might be a good candidate for further development as an antioxidant or xanthine oxidase inhibitory remedy. However, to the best of our knowledge there has been no prior report on the antioxidant activities or xanthine oxidase inhibitory actions of ethanolic extracts from *A. confusa* twigs and branches. Accordingly, this study investigates the active compounds of *A. confusa* twigs and branches. Furthermore, the content, antioxidant activities, xanthine oxidase inhibitory effects, and structure−activity relationships of these phytochemicals from twigs and branches were also investigated.

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MATERIALS AND METHODS

Chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (+)-catechin, disodium ethylene diamine tetraacetic acid (Na_2EDTA), Folin−Ciocalteu reagent, potassium phosphate monobasic (KH_2PO_4) and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). Allopurinol, gallic acid, potassium persulfate, potassium hydroxide, quercetin and sodium carbonate were purchased from Acros Chemicals (Morris Plains, NJ). The other chemicals and solvents were of analytical grade.

Plant Materials. *Acacia confusa* twigs and branches were sampled from the experimental forest of National Taiwan University in Nan-Tou County, and the species was confirmed by Mr. Sheng-You Lu of the Taiwan Forestry Research Institute. The voucher specimen (AC004) was deposited at the School of Forestry and Resource Conservation, National Taiwan University.

Extraction and Isolation. After air-drying at ambient temperature (25 °C), the dried twigs (1.3 kg) were separated from the branches (3.2 kg). The branches were divided into small branches (∼2 cm diameter) and large branches (∼5 cm diameter), and the branches were further separated into bark and wood parts. Specimens from twigs and branches were cut into small pieces respectively and extracted in 95% ethanol at ambient temperature for 7 days. The extracts were decanted and filtered under vacuum, concentrated in a rotary evaporator and then lyophilized. In total, 7 extracts, including small branch extract (2 cm branch extract, 2B), 2 cm branch bark extract (2BB), 2 cm branch wood extract (2BW), large branch extract (5 cm branch extract, 5B), 5 cm branch bark (5BB), 5 cm branch wood (5BW) and twig extract (TW) were obtained. Following an in vitro bioactivity-guided fractionation procedure, fractionation was done by liquid−liquid partition successively with *n*-hexane (not used for branch), EtOAc, *n*-butanol (BuOH), and water to yield four soluble fractions: *n*-hexane (TW-Hex, 5.6 g), EtOAc (TW-EA, 4.9 g), BuOH (TW-Bu, 13.6 g), and H_2O (TW-W, 3.9 g). 5BB was then obtained in three soluble fractions: EtOAc (5BB-EA, 50.9 g), BuOH (5BB-Bu, 87.4 g), and H_2O (5BB-W, 19.7 g). The 5BB-EA was further divided into fifteen subfractions (EA1−EA15) by chromatography with a Lichroprep RP-18 gel (Merck, Darmstadt, Germany) column eluted with MeOH/H_2O (gradient elution was performed by changing the ratio (v/v) from 10/90 to 100/0). Purification was based on an antioxidant activity-guided fractionation procedure with semipreparative HPLC, which was 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). 5BB-EA used solvent A, 100% methanol; and solvent B, ultrapure water as the mobile phase; elution conditions were 0−5 min of 25% A to B (isocratic), 5−28 min of 25−65% A to B (linear gradient), 28−30 min of 65−100% A to B (linear gradient) at a flow rate of 4 mL/min. The mobile phase of TW-EA was 0−3 min of 35% A to B (isocratic), 3−30 min of 35−80% A to B (linear gradient), 30−35 min of 80−100% A to B (linear gradient). The structures of compounds were all analyzed by mass spectrometer (Finnigan MAT-95S) and NMR spectrometer (Bruker Avance 500 MHz FT).

Total Phenolic Contents. Total phenolic content was determined using the Folin−Ciocalteu's reagent (*14*). 500 μL of samples (0.08 mg/mL) were introduced into test tubes followed by 500 μL of 1 N Folin−Ciocalteu's reagent and 1 mL of sodium carbonate (20% w/v). The tubes were vortexed and incubated at room temperature for 10 min, then centrifuged (150*g*) for 8 min. The absorption at 730 nm was measured. The total phenol contents were expressed as gallic acid equivalents (GAE) in milligrams per gram sample (mg of GAE/g).

Total Proanthocyanidin Contents. A modified vanillin−H_2SO_4 assay (*15*) was adopted for quantification of the total proanthocyanidin content of the twig and branch ethanolic extracts. The total volume of reaction medium was 300 μL, comprising 50 μL of sample (1 mg/mL), 125 μL of vanillin reagent (1%, w/v) and 125 μL of H_2SO_4 reagent (10%, v/v) both dissolved in methanol. The absorbance at 500 nm was measured after 15 min. The calibration curve was performed with (+)-catechin and expressed as (+)-catechin equivalents (CE) in milligrams per gram sample (mg of CE/g).

Total Flavonoid Contents. The $AlCl_3$ method (*16*) was used for determination of the total flavonois content of the extracts. The sample solution (150 μL) was mixed with 150 μL of the $AlCl_3$ solution (2%, w/v). The absorbance was read at 450 nm after 10 min of incubation. The calibration curve was performed with quercetin and expressed as quercetin equivalents (QE) in milligrams per gram sample (mg of QE/g).

1,1-Diphenyl-2-picrylhydrazyl Assay (DPPH Assay). The DPPH radical scavenging activity of *A. confusa* twigs and branches extracts was examined according to the method reported by Chang et al. (*8*). Briefly, 10 μL of test samples or compounds in methanol (final concentrations were 1, 5, 10, 25, 50, and 100 μg/mL, respectively) was mixed with 90 μL of 50 mM Tris-HCl buffer (pH 7.4) and 200 μL of 0.1 mM DPPH−ethanol solution. After 30 min of incubation at ambient temperature, the reduction of the DPPH free radical was measured by reading the absorbance at 517 nm using the ELISA reader. (+)-Catechin was used as a 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.

Superoxide Radical-Scavenging Assay (NBT Assay). Superoxide radical scavenging activity followed the method reported by Chang et al. (*8*). Briefly, 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 or compounds in methanol (final concentrations were 1, 5, 10, 50, and 100 μg/mL) and 145 μL of buffer were mixed in 96-well microplates. The reaction was started by adding 50 μL of xanthine oxidase in buffer (1 unit in 10 mL of buffer) to the mixture. The reaction mixture was incubated at ambient temperature, and the absorbance at 570 nm was determined every 20 s up to 8 min using the ELISA reader (Labsystems Multiskan, USA). Quercetin was used as positive control. Three replicates were made for each test sample. The percent inhibition ratio (%) was calculated according to the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

Total Antioxidant Capacity by Trolox Equivalent Antioxidant Capacity (TEAC) Assay. TEAC was determined following the procedure described by Re et al. (*17*) with slight modifications, using Trolox as a standard. The ABTS radical cation was generated by mixing ABTS stock solution (7 mM in water) with 2.45 mM potassium persulfate. This mixture was kept at ambient temperature for 12−16 h until the reaction was complete and the absorbance was stable. The $ABTS^{\bullet+}$ solution was diluted with water to give an absorbance value of 0.700 ± 0.020 at 730 nm. The sample solution (15 μL) was mixed with 1485 μL of the $ABTS^{\bullet+}$ solution. After 6 min of incubation at ambient temperature, the absorbance value of the mixture was measured at 730 nm in a Jasco V-550 UV−vis spectrophotometer. Quercetin, a well-known antioxidant, was used as a positive control. Three replicates were made for each test sample. The TEAC of the sample was expressed as Trolox equivalent in millimolars per 500 μg/mL (extracts) or 1 mM (compounds).

Reducing Power Assay. The assay was done according to the method of Oyaizu (*18*) with minor modifications. First, 1 mL of reaction mixture, containing 500 μL of the test samples or compounds in 500 μL of phosphate buffer (0.2 M, pH 6.6), then incubated with 500 μL of potassium ferricyanide (1%, w/v) at 50 °C for 20 min. After terminated the reaction by adding trichloroacetic acid (10%, w/v), the mixture was centrifuged at 3000 rpm for 10 min. Take 500 μL of supernatant solution added to distilled water (500 μL) and 100 μL of ferric chloride (0.1%, w/v) solution, and then measure the optical density (OD) at 700 nm. Three replicates were made for each test sample. Increased OD value of the reaction mixture indicates increased reducing power.

Determination of XOD-Inhibitory Activity. Measurement of XOD-inhibitory activity was carried out according to the method of Kong et al. (*19*) with slight modifications. First, 798 μL of 0.1 unit of xanthin oxidase (XOD) in buffer (200 mM sodium pyrophosphate/HCl, pH 7.5) and 2 μL of the test extracts or compounds in DMSO were mixed at 37 °C for 5 min. The reaction was started by adding 200 μL of 0.6 mM xanthine in double-distilled water to the mixture. The reaction mixture was incubated at ambient temperature, and the absorbance at 295 nm was determined every 1 min up to 8 min. Allopurinol was used as a positive control. Three replicates were made for each test sample. The percent inhibition ratio (%) was calculated according to the following equation: % inhibition = [(rate of control reaction − rate of sample reaction)/rate of control reaction] × 100.

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

RESULTS AND DISCUSSION

Antioxidant Activities and Total Phenolic Contents of *A. confusa* Twig and Branch Extracts. The DPPH radical scavenging activity, superoxide radical scavenging activity and total phenolic contents of 7 ethanolic extracts from *A. confusa* twigs and branches are shown in **Table 1**. The concentration (IC_{50}) required to inhibit 50% radical-scavenging effect was determined from the results of a series of concentrations tested. A lower IC_{50} value corresponds to a larger scavenging activity. The 5 cm branch extract (5B) had better performance in both DPPH radical and superoxide radical scavenging activity than 2 cm branch (2B) and twig extracts (TW), which can be ranked as 5B > 2B > TW. Moreover, extract of 5B in the bark part (5BB) showed even better activities than in the wood part (5BW). The IC_{50} value of 5BB were 7.4 and 5.8 μg/mL in the DPPH radical and superoxide radical scavenging activities, a 3.3- and 1.7-fold lower value of IC_{50} related to that of 5BW, respectively. A similar antioxidant trend was also found in 2BB and 2BW. These results imply that 5BB was a potent antioxidant source. Comparison with the results of crude extracts from leaves and flowers of *A. confusa* with the IC_{50} values of 17.6 and 62.9 μg/mL against DPPH radical (*12*, *13*) demonstrated that the antioxidant activities decreased in the order of: 5BB > leaves > twigs > flowers. The extract of 5BB from *A. confusa* was a better potential source of antioxidants than extracts from its leaves and flowers.

Table 1. DPPH Radical Scavenging Activity, Superoxide Radical Scavenging Activity and Total Phenolic Contents of *A. confusa* Twig and Branch Extracts

	IC_{50} (μg/mL)		
specimen	DPPH radical[a]	superoxide radical	TPC (mg of GAE/g)
TW	24.0 ± 1.5 b	31.8 ± 1.6 a	121.4 ± 1.8 f
2B	19.7 ± 0.5 c	19.6 ± 1.3 b	243.2 ± 3.9 d
2BB	15.1 ± 0.2 d	5.8 ± 0.3 d	362.2 ± 12.8 b
2BW	40.8 ± 3.2 a	12.8 ± 0.9 c	123.0 ± 4.0 f
5B	11.5 ± 0.3 e	9.6 ± 0.6 c	348.2 ± 1.3 c
5BB	7.4 ± 0.1 f	5.8 ± 0.4 d	416.9 ± 1.9 a
5BW	24.8 ± 3.0 b	10.4 ± 0.1 c	177.0 ± 5.3 e
quercetin	2.8 ± 0.4	4.4 ± 0.8	

[a] Different letters in the table are significantly different at the level of $p < 0.05$ according to Scheffe's test.

Table 2. Total Phenolic Contents, Total Proanthocyanidin Contents and Total Flavonoid Contents of *A. confusa* Twig, Branch Bark and Wood Extracts

specimen	TPC[a] (mg of GAE/g)	TPAC (mg of CE/g)	TFC (mg of QE/g)	TPA/TP[b] (%)	TF/TP[c] (%)
5BB	416.9 ± 1.9 a	128.4 ± 7.6 a	0.9 ± 0.0 c	30.8	0.2
5BW	177.0 ± 5.3 b	30.4 ± 0.4 b	2.1 ± 0.0 b	17.2	1.2
TW	121.6 ± 4.6 c	nd	7.7 ± 0.3 a		6.3

[a] Different letters in the table are significantly different at the level of $p < 0.05$ according to Scheffe's test; nd, not detected. [b] TPA/TP, total proanthocyanidins/total phenolics. [c] TF/TP, total flavonoids/total phenolics.

Various Phenolic Contents of Extracts from Twigs, 5 cm Branch Bark and Wood. As shown in **Table 2**, the proanthocyanidin (128.4 mg of CE/g) was the most abundant phenolic in 5BB compared to that of 5BW (30.4 mg of CE/g), while it cannot even be detected in TW. Adversely, the flavonoid contents were much less in 5BB (0.9 mg of QE/g) and 5BW (2.1 mg of QE/g), while they were slightly higher in TW (7.7 mg of QE/g). Furthermore, we calculated the ratio of total proanthocyanidins to total phenolics (TPA/TP) and total flavonoids to total phenolics (TF/TP), respectively. The proanthocyanidins accounted for 30.8% of the total phenolics in 5BB, which is about 2 times higher than 5BW (17.2%). On the other hand, the total flavonoids accounted for 6.3% of the total phenolics in TW. Similar findings have been reported by Luximon-Ramma et al. (*20*), who found that proanthocyanidins were the dominant phenolics and contributed to the antioxidant activities of vegetative plant parts

The total phenolic contents were also higher in the bark parts of branches than in the wood parts, the same as the antioxidant activities. Extracts of both 2BB and 5BB had higher phenolic contents with 362.2 and 416.9 mg of GAE/g, which were about 2−3 times higher than its wood parts (123.0 and 177.0 mg of GAE/g, respectively). As shown in **Table 1**, the extract of 5BB exhibited the best antioxidant activities, and it had the highest phenolic contents (416.9 mg of GAE/g) among the extracts from twigs and branches. It is clear that branches with larger diameters had higher phenolic contents which were especially distributed in the bark part, and so 5BB had better antioxidant performance.

Table 3. DPPH Radical Scavenging Activity, Superoxide Radical Scavenging Activity, XOD-Inhibitory Activity, Trolox Equivalent Antioxidant Capacity and Reducing Power of *A. confusa* 5 cm Branch Bark Extract and Its Soluble Fractions

	IC_{50} (μg/mL)				
specimen	DPPH radical[a]	superoxide radical	XOD inhibition	TEAC (mmol of TE/g)	reducing power (μmol of TE/g)
5BB	7.4 ± 0.2 b	5.8 ± 0.7 c	110.0 ± 3.2 a	32.7 ± 0.4 b	240.2 ± 4.8 b
5BB-EA	6.7 ± 0.1 c	4.6 ± 1.3 d	89.2 ± 1.4 b	33.1 ± 1.3 b	272.9 ± 0.5 a
5BB-BuOH	9.1 ± 0.2 a	8.7 ± 0.9 a	90.8 ± 0.5 b	26.0 ± 0.7 c	196.5 ± 3.2 c
5BB-H_2O	7.7 ± 1.2 b	7.1 ± 1.4 b	>400	41.5 ± 5.0 a	274.0 ± 8.2 a
quercetin	2.8 ± 0.4	4.4 ± 0.8		117.9 ± 9.3	382.4 ± 2.8

[a] Different letters in the table are significantly different at the level of $p < 0.05$ according to Scheffe's test.

Table 4. DPPH Radical Scavenging Activity, Superoxide Radical Scavenging Activity, XOD-Inhibitory Activity, Trolox Equivalent Antioxidant Capacity and Reducing Power of *A. confusa* Twig Extract and Its Soluble Fractions

	IC_{50} (μg/mL)				
specimen	DPPH radical[a]	superoxide radical	XOD inhibition	TEAC (mmol of TE/g)	reducing power (μmol of TE/g)
TW	24.0 ± 1.5 d	31.8 ± 1.6 c	113.0 ± 0.4 b	6.4 ± 0.2 b	77.2 ± 0.8 b
TW-Hex	50.2 ± 2.8 b	nd	333.9 ± 1.7 a	1.9 ± 0.3 c	9.3 ± 1.0 e
TW-EA	7.3 ± 0.1 e	6.2 ± 0.7 d	26.6 ± 4.1 c	22.4 ± 0.3 a	142.1 ± 5.1 a
TW-BuOH	37.9 ± 1.3 c	41.0 ± 2.2 b	>400	6.2 ± 0.2 b	50.7 ± 3.3 c
TW-H_2O	63.1 ± 2.7 a	64.3 ± 3.8 a	>400	0.7 ± 0.1 d	12.5 ± 0.7 d
quercetin	2.8 ± 0.4	4.4 ± 0.8	3.9 ± 0.7	117.9 ± 9.3	382.4 ± 2.8

[a] Different letters in the table are significantly different at the level of $p < 0.05$ according to Scheffe's test; nd, not detected.

such as branches, while the antioxidant activities of reproductive plant parts (example: twigs) were mostly influenced by flavonoids.

Antioxidant Activities and XOD-Inhibitory Effects of Fractions from Twigs and 5 cm Branch Bark. As shown in **Tables 3** and **4**, among all soluble fractions, the two EtOAc fractions of 5BB and TW have the best antioxidant activities. The IC_{50} values of the EtOAc fraction derived from 5BB were 6.7 and 4.6 μg/mL against DPPH radical and superoxide radical, and the EtOAc fraction from TW were 7.3 and 6.2 μg/mL, respectively. Furthermore, quercetin had IC_{50} values of 2.8 and 4.4 μg/mL, respectively. By comparison with this well-known antioxidant compound, the DPPH radical scavenging and superoxide radical scavenging activities of both EtOAc fractions from TW and 5BB were similar to it. These results imply that there is an abundance of antioxidative phytochemicals present in EtOAc fractions of TW and 5BB.

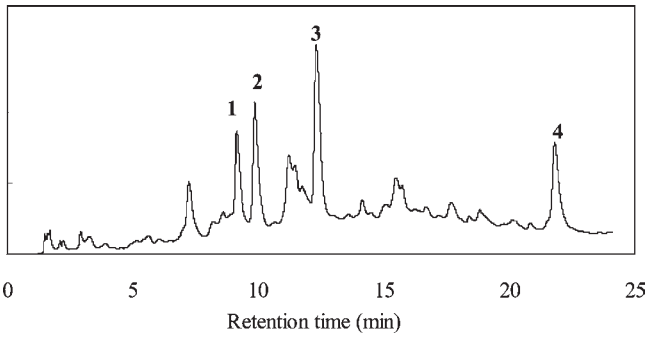

Figure 1. HPLC profile of extracts from the EtOAc soluble fraction of *A. confusa* branches.

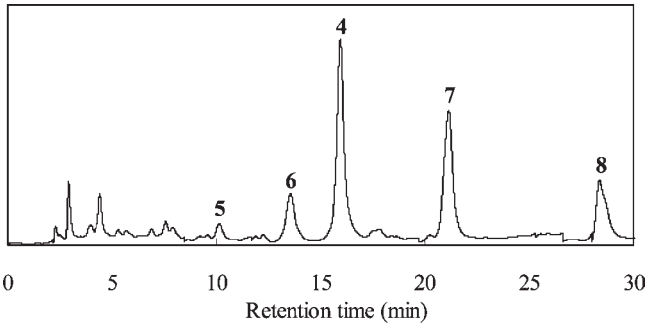

Figure 2. HPLC profile of extracts from the EtOAc soluble fraction of *A. confusa* twigs.

In terms of the xanthine oxidase inhibitory effect, both 5BB and TW extracts showed no significant inhibitory effects (IC_{50} values were 110.0 and 113.0 μg/mL, separately). Only TW-EA showed better inhibitory effect with IC_{50} value of 26.6 μg/mL. Thus, the TW-EA was further investigated in this study for their phytochemical characteristics.

Isolation and Identification of Phytochemicals from *A. confusa* Twig and Branch Extracts. According to the above results, the EtOAc fraction showed excellent DPPH radical scavenging and superoxide radical scavenging activities, as well as potential xanthine oxidase inhibitory effect. The phytochemicals of EtOAc fraction were purified and determined by using HPLC, MS and 1D- and 2D-NMR spectroscopy, the spectral data obtained from the MS and NMR analyses are consistent with those reported in the literature (*21*−*26*). **Figures 1** and **2** are HPLC profiles of EtOAc fractions from extracts of branches and twigs, and **Figure 3** shows the chemical structures of eight phytochemicals (**1**−**8**), including catechin (**1**), catechin-3-*O*-rhamnopyranoside (**2**), *epi*-catechin (**3**), quercetin-3-*O*-rhamnopyranoside (**4**), myricetin-3-*O*-glucopyranoside (**5**), myricetin-3-*O*-rhamnopyranoside (**6**), myricetin-3-(2″-*O*-galloyl)-*O*-rhamnopyranoside (**7**), and luteolin (**8**). Phytochemicals **1**−**4** were the major bioactive compounds from 5BB extract, and their contents were determined to be 8.2,

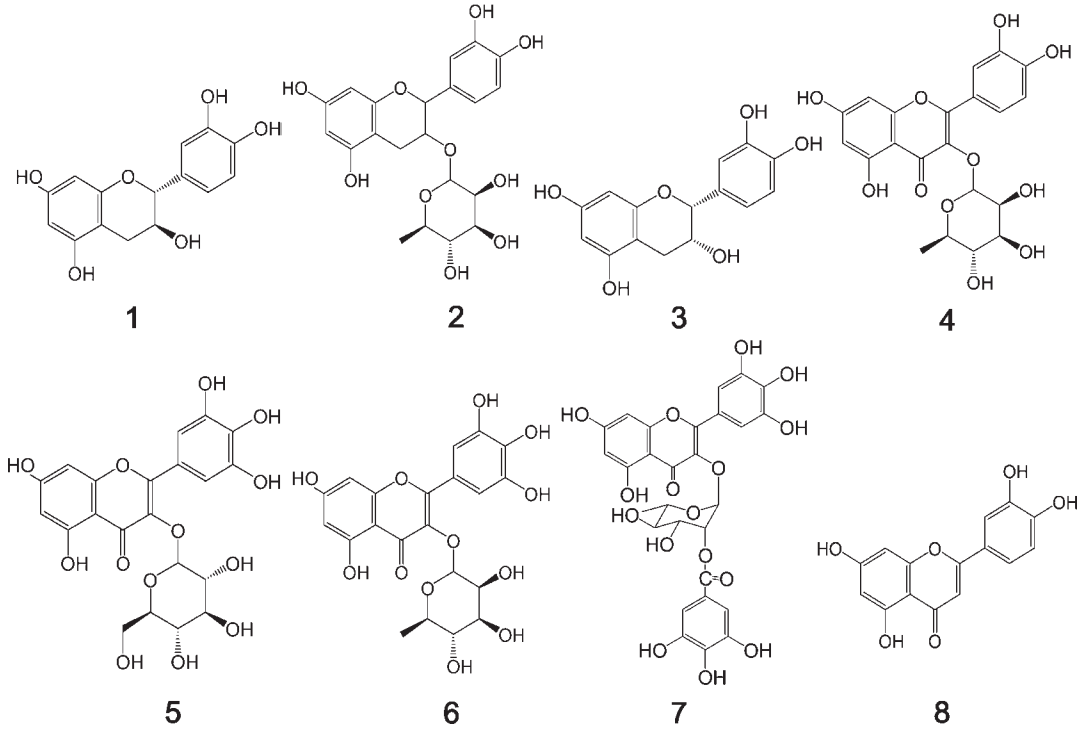

Figure 3. Chemical structures of phytocompounds isolated from the EtOAc soluble fraction of *A. confusa* twigs and branches. **1**, catechin; **2**, catechin-3-*O*-rhamnopyranoside; **3**, *epi*-catechin; **4**, quercetin-3-*O*-rhamnopyranoside; **5**, myricetin-3-*O*-glucopyranoside; **6**, myricetin-3-*O*-rhamnopyranoside; **7**, myricetin-3-(2″-*O*-galloyl)-*O*-rhamnopyranoside; **8**, luteolin.

Table 5. DPPH Radical Scavenging, Superoxide Radical Scavenging Activity, XOD-Inhibitory Activity, Reducing Power and Contents of Phytochemicals from EtOAc Soluble Fraction of *A. confusa* Twig and Branch Bark Extract

	IC_{50} (μM)				
photochemical	DPPH radical	superoxide radical	XOD inhibition	reducing power (μmol of TE/g)	content (mg/g of extract)
(−)-catechin (**1**)	17.2	15.5	>200	309.2	8.2 ± 0.1
catechin-3-*O*-rhamnopyranoside (**2**)	27.5	24.6	>200	154.6	11.3 ± 0.4
epi-catechin (**3**)	19.8	18.6	>200	185.5	19.5 ± 0.4
quercetin-3-*O*-rhamnopyranoside (**4**)	22.7	19.9	37.7	267.7	4.5 ± 0.2[a] (12.0 ± 0.6)[b]
myricetin-3-*O*-glucopyranoside (**5**)	14.8	8.3	>200	363.3	
myricetin-3-*O*-rhamnopyranoside (**6**)	15.3	7.7	>200	420.6	4.7 ± 0.7
myricetin-3-(2″-*O*-galloyl)-*O*-rhamnopyranoside (**7**)	5.3	3.9	117.3	535.4	10.8 ± 1.4
luteolin (**8**)	13.5	9.2	11.6	305.9	9.7 ± 0.2
quercetin (positive control)	15.0	6.8	9.3	382.4	
myricetin (positive control)	9.9	3.4	12.9	573.6	

[a] Content was determined from branches. [b] Content was determined from twigs. Allopurinol used as reference compound with IC_{50} value of 5.6 μM.

11.3, 19.5, and 4.5 per gram of 5BB crude extract, respectively (**Table 5**). Phytochemicals **4**−**8** were isolated from TW, and the contents of compounds **4** and **6**−**8** were determined to be 12.0, 4.7, 10.8, and 9.7 per gram of TW crude extract, respectively.

Antioxidant Activities of Phytochemicals from *A. confusa* Twig and Branch Extracts. As shown in **Table 5**, compounds **5**, **7**, and **8** had better activity against DPPH with IC_{50} values of 14.8, 5.3, and 13.5 μM than quercetin (IC_{50} value was 15.0 μM). The activities of these phytochemicals against superoxide radical can be ranked in the decreasing order **7** > **6** > **5** > **8** > **1** > **3** > **4** > **2**. Phytochemicals **7** exhibited excellent superoxide scavenging activity, and their IC_{50} values were even below quercetin (IC_{50} value was 6.8 μM).

As for reducing power, the CE values ((+)-catechin equivalent in millimolars per molar sample) of 8 phytochemicals were obtained using reducing power assay. These phytochemicals can be ranked as **7** > **6** > **5** > **1** > **8** > **4** > **3** > **2**, indicating that compound **7** had the best reducing power, even better than quercetin.

These results demonstrate that flavonoids containing the 2,3 double bond in the C ring and the 4-oxo functional group have better antioxidant activities. In contrast, flavans have a saturated heterocyclic C ring, and the consequent lack of conjugation between the A and B rings means that electrons are less able to be delocalized from the B ring to the A ring, thus lowering their antioxidant activities. The DPPH assay showed that compounds **4**, **5**, **6**, **7**, and **8** have better activities than compounds **1**, **2**, and **3**. In addition, myricetin had higher radical scavenging activity than quercetin, showing that the degree of hydroxylation on the B ring is important to the antioxidant activity. The glycosylation of flavonoids reduces their activity when compared to the corresponding aglycons, and different glycosides have no significant effects on their activity, such as compounds **5** and **6** have similar activity. In addition, galloyl function on the glycoside would promote the activity, as compound **7** is better than compound **6**. According to Rice-Evans et al. (*27*), galloyl increased the proton donated-hydroxyl functions to the molecule, contributing to higher antioxidant activity.

XOD-Inhibitory Activities of Phytochemicals from *A. confusa* Twig and Branch Extracts. The XOD-inhibitory activity of phytochemicals from *A. confusa* twigs and branches extracts are shown in **Table 5**. Though catechins had excellent antioxidant activities, they showed no XOD-inhibitory activity. These results are in accordance with Cos et al. (*28*), who reported that catechins can be classified as strong antioxidants without XOD-inhibitory activity. These flavan structures are saturated on the C ring, with lack of conjugation between the A ring and the B ring. Furthermore, the nonplanar structures make it even more difficult for molecules to react with the active site of xanthine oxidase (*29*). Of all the phytochemicals, luteolin showed the best XOD-inhibitory activity, with an IC_{50} value of 11.6 μM. Compared with quercetin and myricetin (IC_{50} values were 9.3 and 12.9 μM, respectively), XOD-inhibitory activity is in the decreasing order quercetin > luteolin > myricetin. It can be found that the hydroxyl group on the C-3 of the C ring could increase XOD inhibition, while more hydroxyl groups on the B ring decreased the inhibition. Meanwhile, having glycoside attached would significantly decrease the inhibition, such as quercetin-3-*O*-rhamnopyranoside (**4**) decreased to IC_{50} value of 37.7 μM compared with quercetin (IC_{50} value of 9.3 μM). Myricetin containing a sugar moiety decreased the inhibition to a nondetectable level, although myricetin-3-(2″-*O*-galloyl)-*O*-rhamnopyranoside (**7**) had moderate inhibition with IC_{50} value of 117.3 μM, which might be due to its galloyl group (*30*).

In conclusion, *Acacia confusa* is used as a medicinal plant in Taiwan, and the activities of extracts from its twigs and branches against free radicals and xanthine oxidase (XOD) are reported here for the first time. The activities of extracts from various diameters of twigs and branches were also compared and discussed. The antioxidant test revealed a correlation between diameter and antioxidant activities, with the extract from 5 cm branch bark exhibiting the best activity. Eight compounds were identified, and a significant difference in the type of phenolics was observed between the extracts of twigs and 5 cm branch bark. Accordingly, only the EtOAc fraction from twig extract showed effective inhibition on XOD. These results indicate that extracts from *A. confusa* twigs and 5 cm branch bark have great potential for preventing diseases caused by the overproduction of radicals and the XOD activity. These results can be useful as a starting point for further applications of the twigs and branches of *A. confusa* in pharmaceutical preparations after performing clinical in vivo research.

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