Research paper

Antioxidant Activity of Constituents from the Methanolic Extract of *Acacia confusa* Leaves

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【Summary】

In this study, the antioxidant activities of the leaf extract of *Acacia confusa* and constituents isolated from its ethyl acetate (EtOAc) fraction were investigated for the first time using various in vitro assays. Ten antioxidant compounds, namely, 3,4,5-trihydroxybenzoic acid methyl ester (**1**), 5,7,3',4'-tetrahydroxyl-flavone (**2**), myricetin 3-*O*-(3''-*O*-galloyl)-α-rhamnopyranoside (**3**), myricetin 3-*O*-(3''-*O*-galloyl)- α-rhamnopyranoside 7-methyl ether (**4**), myricetin 3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether (**5**), myricetin-3-*O*-β-glucopyranoside (**6**), myricetin-3-*O*-α-rhamnopyranoside (**7**), myricetin 3-*O*-(2''-*O*-galloyl)-α- rhamnopyranoside (**8**), quercetin-3-*O*-α-rhamnopyranoside (**9**), and europetin-3-*O*-α- rhamnopyranoside (**10**) were isolated and identified from the leaf extract. In addition, their 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, superoxide radical-scavenging activity, and trolox equivalent antioxidant capacity (TEAC) were compared with catechin (as the positive control). As to the DPPH-scavenging activity, all compounds except for **1** and **2** displayed greater antioxidant capacities than catechin (EC_{50} = 10.3 μM), and all of their TEAC values were higher than that of catechin (3.49 mM of trolox) except for **1**, **6**, **9**, and **10**. In addition, all compounds except for **1**, **2**, **5,** and **6**, also expressed as high a superoxide radical scavenging activity as catechin (EC_{50} = 7.4 μM).

Key words: *Acacia confusa*, antioxidant activity, constituents, leaves.

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研究報告

相思樹葉子之抗氧化活性成分

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摘 要

本研究首次利用不同in vitro試驗對相思樹葉子抽出物及其乙酸乙酯可溶部中分離得到的成分進行抗氧化活性體外試驗。由葉子抽出物中共分離鑑定出10種化合物，包括：3,4,5-trihydroxybenzoic acid methyl ester (**1**), 5,7,3',4'-tetrahydroxyl-flavone (**2**), myricetin 3-*O*-(3''-*O*-galloyl)-α-rhamnopyranoside (**3**), myricetin 3-*O*-(3''-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether (**4**), myricetin 3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether (**5**), myricetin-3-*O*-β-glucopyranoside (**6**), myricetin-3-*O*-α-rhamnopyranoside (**7**), myricetin 3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside (**8**), quercetin-3-*O*-α-rhamnopyranoside (**9**)和europetin-3-*O*-α-rhamnopyranoside (**10**)，此外，將10種化合物進行DPPH自由基、超氧自由基捕捉試驗及總抗氧化能力評估，並與標準品兒茶素做比較。在DPPH自由基捕捉試驗，除了化合物**1**與**2**，其他化合物之清除能力均較兒茶素(EC_{50} = 10.3 μM)強。總抗氧化能力方面，除了化合物**1**、**6**、**9**和**10**的總抗氧化能力稍差，其他化合物均較兒茶素(TEAC = 3.49 mM)優異。另外，清除超氧自由基能力方面，除了化合物**1**、**2**、**5**及**6**較兒茶素(EC_{50} = 7.4 μM)差，其它化合物則與兒茶素相當。綜合上述結果，相思樹葉子抽出物頗具潛力開發為抗氧化之保健用品。

關鍵詞：相思樹、抗氧化活性、成分、葉子。

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INTRODUCTION

Acacia confusa Merr. (Leguminosae) is an erect tree widely distributed in the hills and lowlands of Taiwan. It was once extensively planted to support a local charcoal industry. An aqueous extract of its leaves can be used to heal wounds and anti-blood stasis (Kan 1978). In previous studies, the heartwood, bark, twig, and flower extracts of *A. confusa* displayed excellent antioxidant activities such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity, superoxide radical-scavenging activity, and inhibition of lipid peroxidation due to their high phenolic contents (Chang et al. 2001, Wu et al. 2005, Huang et al. 2007, Tung et al. 2007). As of now, however, the potential antioxidant activities of the leaf extracts of *A. confusa* and their application have not been studied in detail.

Results gained from our preliminary study on the antioxidant performance of the methanolic extract of *A. confusa* leaves revealed that the crude extract, and EtOAc-soluble and BuOH-soluble fractions possessed high total phenolic and total flavonoid contents. These high levels indicate great effectiveness in scavenging DPPH free radicals and superoxide radicals, inhibiting lipid peroxidation, and reducing peroxidated substrates; they also exhibit a high trolox equivalent antioxidant capacity (Lin et al. 2008). Among them, the EtOAc-soluble fraction displayed the best antioxidant performance. Its scavenging action of the superoxide radical

(EC_{50} = 2.5 μg mL^{-1}) was more effective than that of catechin (EC_{50} = 7.0 μg mL^{-1}), and its 3 subfractions were also proven to display the best antioxidant activities in DPPH free radical- and superoxide radical-scavenging assays. It is recommended that further research on the antioxidant performance of the constituents from the EtOAc-soluble fraction be carried out.

In this continuing study, column chromatogryphy (CC) and high-performance liquid chromatography (HPLC) were used to separate and purify antioxidant compounds from the EtOAc-soluble fraction of the methanolic extract of *A. confusa* leaves. The chemical structures of compounds were identified by comparing their spectroscopic data with those reported in the literature. In addition, the antioxidant activities of the isolated compounds were further evaluated using various in vitro assays, including DPPH radical-scavenging activity, superoxide radical-scavenging activity, and trolox equivalent antioxidant capacity assay.

MATERIALS AND METHODS

Preparation of the plant extract

Leaves of a 35-year-old *A. confusa* were sampled at the beginning of June 2006 from the Fu-Chou Mountain Park in Taipei, Taiwan. Samples (air-dried weight of 4.0 kg) were soaked in 95% methanol at ambient temperature for 7 d. The extract was decanted, filtered under a vacuum, concentrated in a rotary evaporator, and then lyophilized to yield the crude extract (460.0 g) of *A. confusa* leaves.

Liquid-liquid partitioning and column chromatography

The crude extract (352.0 g) was successively extracted with ethyl acetate (EtOAc), butanol (BuOH), and water to yield the EtOAc (126.1 g), BuOH (90.7 g), water-soluble (76.4 g), and water-insoluble fractions (14.6 g). The EtOAc-soluble fraction was divided into 8 subfractions (Et1~Et8) by chromatography with a Lichroprep Si-60 gel (Merck, Darmstadt, Germany) column eluted with EtOAc/*n*-hexane (gradient elution was performed by changing from 10/90% to 100/0%), acetone/EtOAc (50/50% and 100/0%), and MeOH/acetone (50/50% and 100/0%). The Et6, Et7, and Et8 subfractions were re-chromatographed on a Lichroprep RP-18 gel (Merck) column eluted with MeOH/H_2O (gradient elution was performed by changing from 10/90% to 100/0%) and yielded the Et6-1~Et6-9, Et7-1~Et7-20, and Et8-1~Et8-46 subfractions, respectively.

Isolation of compounds

Ten compounds (Fig. 1) were isolated and purified from the Et6-3 (compounds **1** and **2**), Et7-7 (compounds **3**, **4**, and **5**), Et8-10 (compounds **6** and **7**), and Et8-13 (compounds **8**, **9**, and **10**) subfractions by semipreparative HPLC on a model L-7100 instrument (Hitachi) with a 250×10.0-mm inside diameter, 5-μm RP-18 column (Ribar® Fertigsaule).

Identification of compounds

All spectral data of the 10 compounds obtained in this study including 3,4,5-trihydroxybenzoic acid methyl ester (**1**), 5,7,3',4'-tetrahydroxyl-flavone (**2**), myricetin 3-*O*-(3''-*O*-galloyl)-α-rhamnopyranoside (**3**), myricetin 3-*O*-(3''-*O*-galloyl) -α-rhamnopyranoside 7-methyl ether (**4**), myricetin 3-*O*-(2''-*O*-galloyl)-α- rhamnopyranoside 7-methyl ether (**5**), myricetin-3-*O*-β-glucopyranoside (**6**), myricetin -3-*O*-α-rhamnopyranoside (**7**), myricetin 3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside (**8**), quercetin-3-*O*-α-rhamnopyranoside (**9**), and europetin-

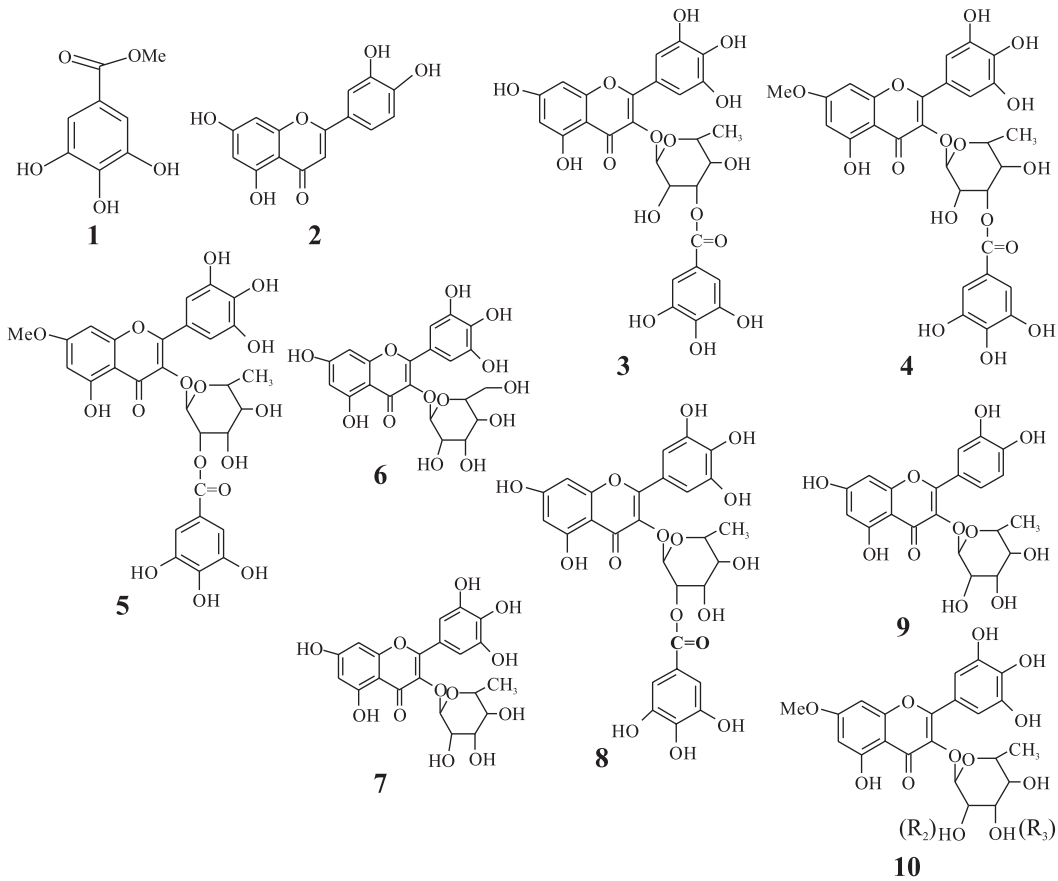

Fig. 1. Chemical structures of compounds (1-10). (1):3,4,5-Trihydroxybenzoic acid methyl ester, (2):5,7,3',4'-tetrahydroxyl-flavone, (3):myricetin 3-*O*-(3''-*O*-galloyl)-α-rhamnopyranoside, (4):myricetin 3-*O*-(3''-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether, (5):myricetin 3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether, (6):myricetin-3-glucopyranoside; (7):myricetin-3-rhamnopyranoside, (8):myricetin 3-*O*-(2''-*O*-galloyl)-α-rhamnopyranoside, (9):quercetin-3-rhamnopyranoside, (10):europetin-3-rhamnopyranoside.

3-*O*-α-rhamnopyranoside (**10**), were in good agreement with published results (Sun et al. 1991, Lee et al. 2000, Flamini et al. 2001, Furusawa et al. 2003, Kazuma et al. 2003, Chung et al. 2004).

DPPH free radical-scavenging activity

The scavenging activities of the 10 compounds against the DPPH radical were modified and measured according to the method of Chang et al. (2001). Various concentrations of each compound (10 μL) were added to 90 μL of 50 mM Tris-HCl buffer (pH 7.4) and then mixed with 200 μL of 0.1 mM DPPH in ethanol for 30 min while being protected from light at ambient temperature. Methanol (10 μL) alone was used as the control in this experiment. Reduction of the DPPH radical was measured by reading the absorbance at 517 nm. (+)-Catechin was used as the positive control. The inhibition percent was calculated by the following equation: % inhibition

= [(absorbance of the control－absorbance of the test sample) / absorbance of the control] ×100, and the DPPH radical-scavenging activity of compounds was expressed as the effective concentration which produced 50% inhibition (EC_{50}). All analyses were run in triplicate.

Superoxide radical-scavenging activity (NBT assay)

The method was adapted and modified from Kirby and Schmidt (1997). Various concentrations of each compound (5 μL) were mixed with 20 μL of 15 mM Na_2EDTA in buffer (50 mM KH_2PO_4/KOH (pH 7.4), in deionized water), 50 μL of 0.6 mM NBT in buffer, 30 μL of 3 mM hypoxanthine in 50 mM KOH, and 145 μL buffer, and 50 μL xanthine oxidase solution in buffer (1 unit in 10 mL buffer) in 96-well microplates, then the plate reader took readings at 570 nm every 20 s for 5 min. The control consisted of 5 μL of buffer solutions instead of the compound solution. Results were expressed as the percentage inhibition relative to the control, calculated by the following equation: [(rate of the control / rate of the sample reaction) / rate of the control]×100.

TEAC assay

The TEAC assay was based on the method reported by Re et al. (1999). The ABTS radical cation ($ABTS^+$) was diluted in potassium persulfate (2.45 mM) to obtain an optical density at 734 nm of about 0.700 units of absorbance. 1485 μL of the $ABTS^+$ solution was placed in a plastic cuvette, 15 μL of each compound solution was added, and the absorbance was read after exactly 6 min. Distilled water was used for the control. TEAC values were expressed as mM of trolox for 0.5 mM of a compound. All analyses were run in triplicate.

RESULTS AND DISCUSSION

In this study, compounds **1**~**10** were isolated from the EtOAc subfractions. Their antioxidant performances were evaluated by DPPH radical-scavenging activity, superoxide radical-scavenging activity, and TEAC assay, and also compared with catechin which is a well-known antioxidant.

Antioxidative activities of the isolated compounds

In the DPPH assay, the EC_{50} values of the compounds (Table 1) against the DPPH radical were ranked in the following ascending order: **8** (3.9 μM) < **3** (4.7 μM) < **4** (5.0 μM) < **5** (6.1 μM) < **10** (6.2 μM) < **7** (7.0 μM) < **6** (8.3 μM) < **9** (8.4 μM) < **2** (11.2 μM) < **1** (14.2 μM). Except for compounds **1** and **2**, all test compounds displayed significantly higher inhibitory activity against the DPPH radical than catechin (10.3 μM), meaning that those compounds have greater hydrogen atom- or electron-donating activity than catechin.

In addition**,** when comparing the EC_{50} values of the superoxide radical-scavenging activity between the 10 compounds and catechin (Table 1), compound **10** (6.2 μM) had the greatest activity compared to that of catechin (7.4 μM), followed by **3** (7.7 μM), **9** (8.0 μM), and **8** (8.7 μM). Compounds **4** (10.6 μM), **7** (14.3 μM), **2** (29.7 μM), **6** (30.3 μM), **5** (30.9 μM), and **1** (116.3 μM) had lower inhibition capacity against the superoxide radical than catechin.

The TEAC assay is used to measure the potential antioxidant capacity of samples compared to trolox, a hydrophilic vitamin E analog with high antioxidant activity. In the TEAC assay (Table 1), the TEAC values of all compounds except **1** (2.37 mM), **6** (2.89 mM), **9** (2.61 mM), and **10** (2.40 mM), were higher than that of catechin (3.49 mM). The

Table 1. Inhibition (EC_{50}) of DPPH free radicals and superoxide free radicals and results of the TEAC assay of compounds from the EtOAc fraction of *Acacia confusa* leaves

Compound	DPPH assay (μM)[1)]	NBT assay (μM)[2)]	TEAC assay (mM of trolox)[3)]
(**1**)	14.2±0.4	116.3	2.37±0.24
(**2**)	11.2±1.1	29.7	4.58±0.17
(**3**)	4.7±0.5	7.7	5.63±0.11
(**4**)	5.0±0.1	10.6	6.99±0.37
(**5**)	6.1±0.2	30.9	4.84±0.02
(**6**)	8.3±0.3	30.3	2.89±0.20
(**7**)	7.0±0.6	14.3	6.50±0.11
(**8**)	3.9±0.1	8.7	6.87±0.38
(**9**)	8.4±0.1	8.0	2.61±0.03
(**10**)	6.2±0.1	6.2	2.40±0.12
Catechin[4)]	10.3±0.6	7.4	3.49±0.11

[1)] Each value in the DPPH assay represents the mean±SD of 3 measurements.
[2)] Each value in the NBT assay represents the mean of 3 measurements.
[3)] Each value in the TEAC assay represents the mean±SD of 3 measurements.
[4)] Used as the positive control.

results obtained from the TEAC assay showed that these isolated compounds have as potent antioxidant capacities as trolox.

Structure-activity relationships of the 10 compounds

The structure-activity relationship (SAR) study of flavonoids by Pannala et al. (2001) demonstrated how the *ortho*-dihydroxy structure in the B ring and the 2,3 double bond in conjugation with the 4-oxo function in the C ring are essential for effective free radical-scavenging activities. An extra hydroxyl group on the B ring was also reported to increase the antioxidant activity (Pietta 2000). Because the 10 compounds (except for compound **1**) isolated from the leaves of *A. confusa* in this study possess the *O*-dihydroxy structure in the B ring and the 2,3 double bond in conjugation with the 4-oxo function in the C ring, and compounds **3**, **4**, **5**, **6**, **7**, **8**, and **10** have an additional hydroxyl group on the B ring, all of them exhibited as high antioxidant activities as catechin in the DPPH, NBT, and TEAC assays.

In addition, the SARs of the DPPH radical-scavenging activity and the TEAC assay for compounds **3**, **4**, **5**, **7**, **8**, and **10** revealed that gallic acid in the R_3 or R_2 position (as marked in Fig. 1, compound **10**) is a key for both antioxidant activities. In compounds **7** and **9**, the hydroxyl group bonded on C-5' in compound **7** was shown to have a positive influence on the DPPH radical-scavenging activity and TEAC assay. Comparing compounds **7** and **10**, the methoxyl group bonded on C-7 in compound **10** had a positive influence on the DPPH radical-scavenging activity and NBT assay, but resulted in a significantly smaller TEAC value.

Furthermore, there were interesting results of the DPPH, NBT, and TEAC assays for compounds **3**, **4**, **5**, and **8**, on differences from one another in terms of the a) methoxyl or hydroxyl group bonded on C-7, and b) the gallic acid bonded on C-2" or C-3" position. Among them, compound **5** (with a methoxyl group bonded on C-7 and gallic acid bonded on C-2") showed the least effectiveness in the DPPH, NBT, and TEAC assays, but there was

no significant effect for compound **4** (with a methoxyl group bonded on C-7 and gallic acid bonded on C-3") or **8** (with no methoxyl group bonded on C-7 and gallic acid bonded on C-2") alone.

CONCLUSIONS

Results gained from a previous study revealed that the heartwood, bark, twig, and flower extracts of *A*. *confusa* have great antioxidant capacities with high phenolic contents. In this study, the extract of *A*. *confusa* leaves exhibited strong antioxidant activities in the in vitro assays. In addition, the 10 compounds isolated from *A*. *confusa* leaves also showed excellent effectiveness in DPPH free radical-scavenging activity, superoxide free radical-scavenging activity, and the TEAC assay, and some constituents were found to be more effective than catechin. This indicates that the leaf extract of *A*. *confusa* or its derived phytocompounds have great potential to be used as a source of natural health products such as antioxidants. These results can be useful as starting points for further applications of *A*. *confusa* leaves or their constituents in pharmaceutical preparations after performing clinical in vivo research.

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