Antioxidant Activity of Extracts from *Acacia confusa* Bark and Heartwood

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The antioxidant activity of extracts from bark and heartwood of *Acacia confusa* was evaluated by various antioxidant assays, including free radical and superoxide radical scavenging assays and lipid peroxidation assay as well as hydroxyl radical-induced DNA strand scission assay. In addition, an ex vivo antioxidant assay using a flow cytometric technique was also employed in this study. The results indicate that both bark and heartwood extracts clearly have strong antioxidant effects. Similar inhibitory activities for each test sample were found for both 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical generation and lipid peroxidation. As for the superoxide radical scavenging activity, the heartwood extract was more effective than the bark extract. Furthermore, the heartwood extract protected ΦX174 supercoiled DNA against strand scission induced by ultraviolet photolysis of H_2O_2, and it reduced the amounts of intracellular hydrogen peroxide, a reactive oxygen species, when it was co-incubated with human promyelocytic leukemia (HL-60) cells under oxidative stress.

Keywords: *Acacia confusa; antioxidant activity; lipid peroxidation; reactive oxygen species; flow cytometry*

INTRODUCTION

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen, and hydrogen peroxide are often generated as byproducts of biological reactions or from exogenous factors (*1*). In vivo, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and intercellular signaling, or synthesis of biologically important compounds (*2*). However, ROS may also be very damaging; they can attack lipids in cell membranes and also attack DNA, inducing oxidations that cause membrane damage such as membrane lipid peroxidation and a decrease in membrane fluidity, and also cause DNA mutation leading to cancer (*3*, *4*). A potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases (*5*). Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert antioxidant actions (*6*−*10*).

Acacia confusa Merr. (Leguminosae), a species indigenous to Taiwan, is widely distributed on the hills and lowlands of Taiwan. It represents a traditionally important commodity used for feedstock, charcoal-making, and construction material, as well as being particularly significant for the conservation of soil and water (*11*). It has been reported that *A. confusa* is an allelopathic plant, and the leaves contain bioactive agents, for example, myricetin 3-*O*-(2″-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether, and myricetin 3-*O*-(3″-*O*-galloyl)-α-rhamnopyranoside 7-methyl ether, which exhibit antihatch activity against brine shrimp (*12*). However, the potential health benefits of *A. confusa* bark and heartwood extracts have not been studied to date. The objectives of this study were to evaluate the antioxidant activities of *A. confusa* extracts and to compare the activity of bark and heartwood extracts using a variety of in vitro and ex vivo assay systems.

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MATERIALS AND METHODS

Chemicals. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), hypoxanthine, xanthine oxidase, nitroblue tetrazolium chloride (NBT), 2-thiobarbituric acid (TBA), ΦX174 RF1 supercoiled DNA, Folin−Ciocalteu reagent, and (+)-catechin were purchased from Sigma Chemical Co. (St. Louis, MO). The other chemicals and solvents used in this experiment were of the highest quality available.

Preparation of Plant Extracts. Bark and heartwood of *A. confusa* were sampled from the experimental forest of National Taiwan University in Nan-Tou county. The dried samples were cut into small pieces and soaked in 70% ethanol at room temperature for 7 days. The extract was decanted, filtered under vacuum, concentrated in a rotary evaporator, and then lyophilized, and the resulting powder extract was used in the present study.

DPPH Assay. The scavenging activity of DPPH free radical by *A. confusa* plant extracts was done according to the method reported by Gyamfi et al. (*8*). Fifty microliters of the bark or heartwood 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) only was used as control of this experiment. After 30 min of incubation at room temperature, the reduction of the DPPH free radical was measured by reading the absorbance at 517 nm. L-Ascorbic acid and (+)-catechin were used as positive controls. The inhibition ratio

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(percent) was calculated from the following equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

NBT (Superoxide Scavenging) Assay. Measurement of superoxide radical scavenging activity was carried out on the basis of the method described by Kirby and Schmidt (*13*) with slight modifications. Twenty microliters 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 (final concentrations were 1, 5, 10, and 50 μg/mL, respectively), 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 25 °C, and the absorbance at 570 nm was determined every 20 s up to 5 min using a plate reader (Labsystems Multiskan MS). The control was 5 μL of methanol instead of the sample solution. (+)-Catechin was used as a positive control. The inhibition ratio (percent) was calculated from the following equation: % inhibition = [(rate of control − rate of sample reaction)/rate of control] × 100.

Assay of Lipid Peroxidation Using Mouse Brain Homogenates. The brains of young adult male Balb/c mice were dissected and homogenized with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1/10 homogenate. The homogenate was centrifuged at 12000*g* for 15 min at 4 °C, and the supernatant was used for in vitro lipid peroxidation assay. A 1 mL aliquot of the supernatant was incubated with the test samples (final extract concentrations in the assays were 1, 5, 10, and 50 μg/mL, respectively) in the presence of 10 μM $FeSO_4$ and 0.1 mM ascorbic acid at 37 °C for 1 h. The reaction was terminated by the addition of 1.0 mL of trichloroacetic acid (TCA; 28%, w/v) and 1.5 mL of TBA (1%, w/v), followed by heating at 100 °C for 15 min. After centrifugation to remove precipitated protein, the absorbance of the malondialdehyde (MDA)−TBA complex was measured at 532 nm (*9*). (+)-Catechin was used as a positive control. The inhibition ratio (percent) was calculated from the following equation: % inhibition = [(absorbance of control − absorbance of test sample)/absorbance of control] × 100.

Assay of Hydroxyl Radical-Induced DNA Strand Scission. The assay was done according to the method of Keum et al. (*14*) with minor modifications. The reaction mixture (30 μL) contained 10 mM Tris-HCl−1 mM EDTA buffer (pH 8.0), ΦX174 RF1 DNA (0.3 μg), and H_2O_2 (0.04 M). Various amounts of the test extract samples dissolved in 10 μL of ethanol (final concentrations of the plant extract in each assay were 1, 10, 100, 500, and 1000 μg/mL, respectively) were added prior to H_2O_2 addition. Hydroxyl radicals were generated by irradiation of the reaction mixtures at a distance of 5 cm with a 12 W UV lamp (Spectroline, Spectronics Co.). After incubation at room temperature for 20 min, the reaction was terminated by the addition of a loading buffer (0.25% bromophenol blue tracking dye and 40% sucrose), and the mixtures were then analyzed by 0.8% submarine agarose gel electrophoresis (70 eV, 1 h). The gels were stained with ethidium bromide, destained in water, and photographed on a transilluminator.

Cellular Assay of Antioxidant Activity. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA), a peroxide-sensitive dye, was used for the evaluation of oxidative stress in cells based on the method reported by Simizu et al. (*15*). In this study, HL-60 cells (human promyelocytic leukemia cell line) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS), 100 units/mL penicillin, and streptomycin in an incubator at 37 °C, 5% CO_2, 95% air, and humidity. The cell suspensions (1.5 mL at the concentration of 3×10^5 cells/well) were seeded in six-well plates (Falcon) and incubated with various dosages of test samples (final concentrations were 50, 100, and 250 μg/mL, respectively) for 20 min. Then cells were co-incubated with 25 μM DCFH-DA in the absence or presence of 40 μM hydrogen peroxide in darkness at 37 °C for 30 min. After incubation, cells were collected and washed once with ice-cold phosphate-buffered saline (PBS), resuspended in 1 mL of the same PBS, and placed on ice in darkness until flow cytometry was carried out. The amounts of intracellular hydrogen peroxide were detected by Coulter EPICS XL flow cytometer (Beckman/Coulter). At least 10000 cells were analyzed for each test, and the observed fluorescence reflects the intracellular hydrogen peroxide level.

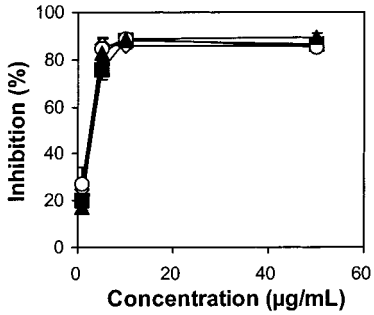

Figure 1. Free-radical scavenging activity of *A. confusa* extracts measured using the DPPH assay: (◇) heartwood extract; (■) bark extract; (○) (+)-catechin; (▲) ascorbic acid. Results are mean ± SD ($n = 3$).

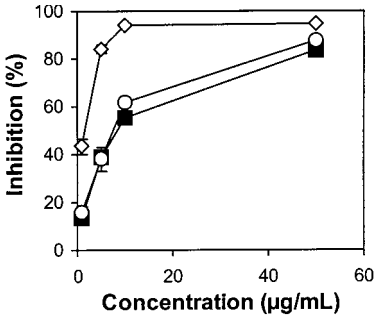

Figure 2. Superoxide scavenging activity of *A. confusa* extracts measured using the NBT assay: (◇) heartwood extract; (■) bark extract; (○) (+)-catechin. Results are mean ± SD ($n = 3$).

Determination of Total Phenolics. Total phenolics content was determined according to the Folin−Ciocalteu method (*16*), using gallic acid as standards. The bark or heartwood extract (5 mg) of *A. confusa* was dissolved in 5 mL of methanol/water (50:50, v/v). The plant extracts solution (500 μL) was mixed with 500 μL of 50% Folin−Ciocalteu reagent. The mixture was allowed to stand 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 sample.

Statistical Analyses. All results are expressed as mean ± SD ($n = 3$). The significance of difference was calculated by Student's *t* test, and values <0.05 were considered to be significant.

RESULTS

The free radical scavenging activity of *A. confusa* extracts was assessed by DPPH assay. As shown in Figure 1, both bark and heartwood ethanolic extracts from *A. confusa* demonstrated a significant inhibitory activity against the DPPH radical. The complete inhibition of DPPH radical by test samples was observed at a range of 5−10 μg/mL, and at the same dosage there was a similar free radical scavenging activity for L-ascorbic acid and (+)-catechin, both of which are well-known antioxidant compounds.

Figure 2 shows the superoxide scavenging activity of the samples compared to that of (+)-catechin. The

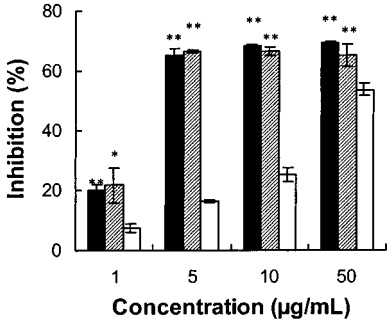

Figure 3. Effects of *A. confusa* extracts on both ferric ion and ascorbic acid induced lipid peroxidation on mouse brain homogenates: (black bars) heartwood extract; (striped bars) bark extract; (white bars) (+)-catechin. Results are mean ± SD (n = 3). * and ** represent $p < 0.05$ and $p < 0.01$, respectively, when compared with the data obtained from (+)-catechin-treated mouse brain homogenates.

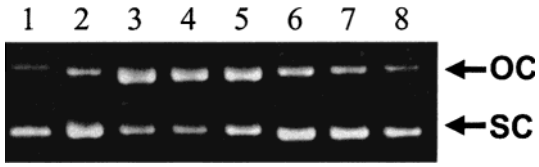

Figure 4. Protection effect of heartwood extract of *A. confusa* on DNA strand scission induced by H_2O_2 and UV. ΦX174 supercoiled DNA was exposed to UV alone (lane 2), UV plus H_2O_2 (lane 3) or plus H_2O_2 in the presence of final concentrations of 1 μg/mL (lane 4), 10 μg/mL (lane 5), 100 μg/mL (lane 6), 500 μg/mL (lane 7), and 1000 μg/mL (lane 8) of heartwood extract. Lane 1 represents native ΦX174 supercoiled DNA without any treatment. Arrows indicate distinct forms of bacteriophage DNA: OC (open circular); SC (supercoiled).

heartwood extract of *A. confusa* had a higher inhibitory activity against superoxide radicals induced by hypoxanthine−xanthine oxidase than both the bark extract and (+)-catechin at all measured concentrations ($p < 0.05$). Almost all superoxide radicals were inhibited by the addition of 10 μg/mL heartwood extract. Even with dosages as low as 5 μg/mL, the heartwood extract was capable of scavenging >80% of the present superoxide radicals, whereas bark extract or (+)-catechin scavenged ~40%. These results reveal that the ethanolic extracts of *A. confusa* heartwood have a stronger superoxide radical scavenging activity.

The *A. confusa* extracts not only exhibited excellent free radical and superoxide radical scavenging activities but were also potent in suppressing lipid peroxidation on mouse brain homogenates. According to the results shown in Figure 3, both bark and heartwood extracts had better inhibitory effects than (+)-catechin at all concentrations. More than 60% of the inhibitory activity of lipid peroxidation was observed at the concentration of 5 μg/mL bark extract or heartwood extract.

Furthermore, to illustrate the protective effect of *A. confusa* extracts on DNA strand scission, in this study the ΦX174 RF1 DNA cleavage by hydroxyl radical generated by UV photolysis of H_2O_2 was measured by agarose gel electrophoresis. Figure 4 shows that UV illumination alone did not cause DNA strand cleavage (lane 2), whereas treatment of supercoiled DNA with UV plus H_2O_2 led to conversion of the DNA to open circular form (lane 3). However, the heartwood extract of *A. confusa* exhibits a dose-dependent protection of DNA under oxidative stress (lanes 4−8). When the DNA was incubated with 100 μg/mL of heartwood extract, the prevention of DNA strand scission was clearly observed (lane 6). Moreover, nearly complete protection by heartwood extract was found at a dose of 1000 μg/mL (lane 8).

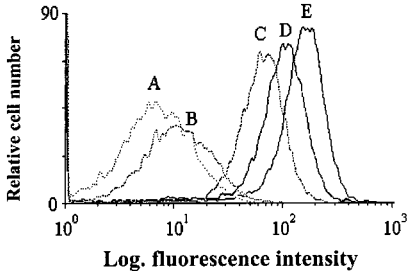

Figure 5. Antioxidant activity of heartwood extract of *A. confusa* measured by incubating HL-60 cells with hydrogen peroxide and different concentrations of extract: (A) cells were treated with solvent control, mean = 7; (B) cells were treated with 250 μg/mL extract before H_2O_2 was added, mean = 11; (C) cells were treated with 100 μg/mL extract before H_2O_2 was added, mean = 64; (D) cells were treated with 50 μg/mL extract before H_2O_2 was added, mean = 109; (E) cells were treated with H_2O_2 alone, mean = 154.

Table 1. Total Phenolic Content of Ethanolic Extracts from *A. confusa*[a]

extract	total phenolics[b] (mg of GAE/g)
bark	470.6 ± 43.9
heartwood	529.7 ± 14.4

[a] The experiments were measured according to the Folin−Ciocalteu method as detailed Materials and Methods. [b] Total phenolics are expressed as gallic acid equivalent (GAE). Each experiment was performed in triplicate, and the results are mean ± SD. Values in the table are significantly different ($p < 0.05$).

To examine whether the heartwood extract of *A. confusa* could actually inhibit the oxygen stress, HL-60 cells were preincubated with various dosages of extracts followed by treatment with H_2O_2. The intracellular hydrogen peroxide level was determined by using DCFH-DA and detected by flow cytometry. A 22-fold increase in fluorescence intensity was observed in HL-60 cells treated with 40 μM H_2O_2 for 20 min, as compared to untreated control cells (Figure 5, compare curves E and A). Under the same experimental conditions, the hydrogen peroxide level was reduced to 69.4% (Figure 5D), 28.8% (Figure 5C), and 2.7% (Figure 5B) in HL-60 cells following the treatment with 50, 100, and 250 μg/mL of heartwood extract, respectively. It is clear that the intracellular ROS in HL-60 cells were reduced after co-incubation with heartwood extract of *A. confusa*.

It is well-known that plant phenolics, in general, are the highly effective free radical scavengers and antioxidants. The content of total phenolics in the heartwood and bark extracts of *A. confusa* was determined spectrometrically according to the Folin−Ciocalteu method and calculated as gallic acid equivalents (GAE). Table 1 shows that the total phenolic content of heartwood extract (529.7 mg/g) was higher than that of the bark extract (470.6 mg/g). These results indicate that a higher antioxidant activity of the heartwood extract compared to the bark extract may be correlatable to the phenolic content of the respective plant extracts.

DISCUSSION

DPPH is a stable radical that has been used to evaluate the antioxidant activity of plant and microbial extracts (*17*). In the current study, DPPH scavenging

activity was found in both bark and heartwood ethanolic extracts from *A. confusa*. Compared to ascorbic acid and (+)-catechin, the radical scavenging activities of both test samples were similar to those of the reference compounds, and their IC_{50} values (the concentration required to inhibit radical formation by 50%) ranged from 1 to 5 μg/mL. This indicates that *A. confusa* extract has good potential as a source for natural antioxidants. In addition, the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (*18*, *19*). In this study, lipid peroxidation of mouse brain homogenates was induced by ferric ion plus ascorbic acid. At the concentration of 5 μg/mL, both heartwood and bark extracts significantly inhibited the lipid peroxidation in vitro (Figure 3). It is noteworthy that (+)-catechin, which has an IC_{50} value of $\sim$50 μg/mL, showed less effectiveness than *A. confusa* extracts at all tested concentrations. This indicates that a higher inhibitory activity against lipid peroxidation was present in *A. confusa* crude extracts relative to the monocomponent (+)-catechin.

On the other hand, superoxide and hydroxyl radicals are the two most representative free radicals. In cellular oxidation reactions, superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents (*9*). Moreover, xanthine oxidase is one of the main enzymatic sources of those ROS in vivo. Hypoxanthine−xanthine oxidase generates superoxide radicals, which reduce NBT to yield blue formazan. The *A. confusa* crude extracts dose-dependently inhibited the NBT reduction induced by hypoxanthine−xanthine oxidase. The IC_{50} values of bark and heartwood extracts were in the ranges of 5−10 and 1−5 μg/mL, respectively. It is noteworthy that the bark extract from *A. confusa* inhibited both DPPH radical generation and lipid peroxidation but was less effective than heartwood extract on the superoxide radical scavenging. At a concentration of 50 μg/mL of bark extract, the inhibitory activity of superoxide radical was similar to that of 5 μg/mL of heartwood extract.

In addition, the damaging action of hydroxyl radicals is the strongest among free radicals. In biochemical systems, superoxide radical is converted by superoxide dismutase to hydrogen peroxide, which can subsequently generate extremely reactive hydroxyl radicals in the presence of certain transition metal ions such as iron and copper, especially iron (*20*), or by UV photolysis (*14*, *21*). In addition, hydroxyl radicals can attack DNA to cause strand scission. Thus, incubation of ΦX174 RF1 DNA with both H_2O_2 and UV resulted in complete conversion of supercoiled DNA to the open circular form. However, addition of heartwood ethanolic extract of *A. confusa* to the reaction mixture substantially diminished the DNA strand scission induced by both H_2O_2 and UV. There was almost complete protection by heartwood extract at a dose of 1000 μg/mL. Keum et al. (*14*) demonstrated the antioxidant and anti-tumor-promoting activities of the methanol extracts of heat-processed ginseng and also found that ginseng extracts could completely prevent DNA strand scission at the dose of 1000 μg/30 μL (33.3 mg/mL). These results indicate that the heartwood extract from *A. confusa* is an excellent DNA protector, which clearly provides antioxidant activity to prevent DNA damage.

According to the above results, the crude extracts of *A. confusa*, especially the heartwood ethanolic extract, show excellent antioxidant activity in vitro. To further evaluate the antioxidative properties of heartwood extract on living cells, a flow cytometric technique was employed. This technique is a powerful method for screening of new antioxidant molecules (*22*). In the present study, the HL-60 cells were preincubated with or without heartwood extract, followed by treatment with H_2O_2. A 22-fold increase in fluorescence intensity of dichlorofluorescein (DCF) was observed in HL-60 cells under oxidative stress. However, the intracellular hydrogen peroxide level was significantly decreased when the cells were co-incubated with heartwood extract of *A. confusa*. At a concentration of 250 μg/mL, the amount of intracellular hydrogen peroxide was reduced to 2.7%, indicating that the heartwood extract of *A. confusa* could decrease the amount of intracellular ROS when it was co-incubated with cells.

Phenolic compounds are commonly found in the plant kingdom, and they have been reported to have multiple biological effects, including antioxidant activity (*23*, *24*). In the current study, we examined the content of phenolic compounds for the heartwood and bark extracts of *A. confusa*. Our results showed that the heartwood extract contained a higher amount of phenolics than did the bark extract based on Folin−Ciocalteu assays (Table 1). We have also demonstrated that, overall, the antioxidant activity of the heartwood extract is more effective than that of the bark extract. These results suggested that the effectiveness of the antioxidant activity of both test plant extracts may be correlatable to their contents of phenolics, and it is proposed here that the phenolic compounds of the heartwood and bark extracts of *A. confusa* may play an important role in the observed antioxidant activities of the extracts. A similar finding has been demonstrated by Yen and Hsieh (*25*) that the plant extracts of *Eucommia ulmoides* (Du-zhong) with enriched phenolic compounds are correlated well with their antioxidant activities. Further studies of the compound compositions and specific compounds of the *A. confusa* plant extracts giving the antioxidant activity, using bio-organic chemistry, mass spectrometry, and NMR spectrometry experiments are in progress.

It is well-known that free radicals are one of the causes of several diseases, such as Parkinson's disease, coronary heart disease, and cancer (*4*, *26*, *27*). This study demonstrated that *A. confusa* extracts, especially heartwood extract, have excellent antioxidant activities. It is interesting and worthy to further investigate the potential effectiveness or usage of *A. confusa* in preventing diseases caused by the overproduction of radicals. The significance of natural antioxidants from *A. confusa* will be further characterized, and they will be evaluated for their bioavailability and potential toxicity in vivo. These points will be addressed in the coming series of studies of *A. confusa*.

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