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Free radical-scavenging activity of Taiwanese native plants

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Summary

The 70% aqueous acetone extracts of ten Taiwanese native plants were evaluated by various antioxidant assays, including 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl (˙OH) radicals, and reducing power assay. In the present study, extracts of *Acer buerferianum* var. *formosanum, Cleyera japonica* var. *morii, Cyclobalanopsis stenophylla* var. *stenophylloides*, and *Machilus zuihoensis* exhibited stronger activity against DPPH radicals, and their IC_{50} values ranged from 5.4 to 8.3 µg/ml. The ten selected extracts effectively inhibited the formation of ˙OH generated in the Fenton reaction system. Among the extracts whose reducing power activities were determined, *A. buerferianum* var. *formosanum, C. japonica* var. *morii, C. stenophylla* var. *stenophylloides, Eriobotrya deflex*, and *M. zuihoensis* showed high activity. The results indicate the 70% aqueous acetone extracts of *A. buerferianum* var. *formosanum, C. japonica* var. *morii, C. stenophylla* var. *stenophylloides*, and *M. zuihoensis* with great potency in these assay systems and may be candidates for the development of natural antioxidants.

Key words: Taiwanese native plants, 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl radical (˙OH), reducing power

Introduction

Reactive oxygen free radical species (ROS) are greatly reactive molecules, and include the hydroxyl radical (˙OH), the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and peroxyl ($ROO^{\bullet}$), which consequently generate metabolic products that attack lipids in cell membranes or DNA. Lipid peroxidation occuring in cell membranes or DNA which involves a series of free radical chain reaction processes is associated with several types of biological damage, DNA damage, carcinogenesis, and cellular degeneration related to aging. Cells are protected by their endogenous scavenging systems or by other substances (Halliwell et al., 1990). Cells are impaired by an imbalance between ROS-generating and -scavenging systems. Thus ROS play an important role in the pathogenesis of clinical human diseases including neurodegenerative disorders, cardiovascular diseases, and mutagenesis (Kawanishi, 2001).

In recent years, the possible toxicity of synthetic chemical antioxidants has been criticized. Thus, recent studies have investigated the potential of plant products to serve as antioxidants to protect against various diseases induced by free radicals. Plant products including phenolics, flavonoids, tannins, proanthocyanidins, and various plant or herbal extracts have been reported to be radical scavengers and inhibitors of lipid peroxidation (Xie et al., 1993; Formica and Regelson, 1995). Therefore, in view of the importance of these substances to health, phenolics have been proposed as

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health-promoting products in prophylactic medicines. Taiwan is located in southeastern Asia, and contains abundant plant species. The aim of this study was to screen plant material extracts of Taiwanese origin for their phenolic contents in order to find potential new sources of natural antioxidants. By using the scavenging activities of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl (·OH) radicals as well as a reducing power assay, a systematic survey of free radical-scavenging activity of selected Taiwanese native plants was undertaken, and results are discussed.

Meterials and Methods

Plant materials

All plant materials were collected from the Taiwan Endemic Species Research Institute (TESRI) garden, in Nantou County, central Taiwan (Table 1). Herbarium voucher specimens were deposited at the Graduate Institute of Pharmacognosy Science, Taipei Medical University, Taiwan, and identified by Chih-Hui Chen at the TESRI garden.

Preparation of plant extracts

Dried leaves of plants were pulverized and extracted with 70% acetone twice. After filtering, the combined filtrates were concentrated under reduced pressure. The final residues were freeze-dried and stored in a closed container until use. The yields of plant extracts were calculated by the following formula:

Yield (%) = (mass of the extract/mass of the dried raw plant material) × 100%.

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), deoxyribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), $FeCl_2$, $FeCl_3$, potassium ferricyanide (K_3FeCN_6), and Folin-Ciocalteu reagent were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of analytic grade.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity

The DPPH radical-scavenging effect was measured according to the method of Kang and Saltveit (2002). This method measures hydrogen atom- or electron-donating activity. DPPH is a stable free radical of a purple color which is reduced to yellow-colored diphenylpicrylhydrazine. Each tested sample was mixed with 160 µM DPPH radical in a methanol solution. After a 20-min incubation period at room temperature in the dark, the absorbance was read at 517 nm. The inhibitory percentage of DPPH was calculated according to the following equation:

Scavenging activity (%) = [(absorbance of the control absorbance of the sample)/absorbance of the control] × 100%.

Reducing power activity

Reducing powers were measured by $K_3Fe(CN)_6$-$FeCl_3$ (Yen and Chen, 1995). The test sample was mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide (K_3FeCN_6). The mixture was incubated at 37 °C for 20 min. An equal volume of 10% trichloroacetic acid was added to the mixture, then it was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and 0.1% $FeCl_3$ at a ratio of 1:1:2. After standing for 10 min, the absorbance was measured at 700 nm.

Deoxyribose (DR) assay

The deoxyribose method for determining the rate of reaction of the hydroxyl radical with an antioxidant was performed as described by Halliwell et al. (1987). The reaction mixtures contained the following reagents at their final concentrations: 2.8 mM deoxyribose, 20 mM potassium phosphate buffer (pH 7.4), 100 µM $FeCl_3$, 780 µM EDTA, 1 mM H_2O_2, and 100 µM ascorbic acid. Solutions of $FeCl_3$, H_2O_2, and ascorbic acid were prepared immediately before use. After incubating at 37 °C for 1 h, equal volumes of 1% 2-thiobarbi-

Table 1. Ethnobotanical data of Taiwanese native plants studied.

Botanical name; Family	Voucher specimen
1. *Acer buerferianum* Miq. var. *formosanum* (Hayata) Sasaki; Aceraceae	3544
2. *Cinnamomum insulari-montanum* Hayata; Lauraceae	3549
3. *Cleyera japonica* Thunb. var. *morii* (Yamamoto) Masamune; Theaceae	3559
4. *Cyclobalanopsis stenophylla* (Makino) Liao var. *stenophylloides* (Hayata) Liao; Fagaceae	3711
5. *Eriobotrya deflex* (Hemsl.) Nakai; Rosaceae	3550
6. *Machilus zuihoensis* Hayata; Lauraceae	3551
7. *Malus docmeri* (Bois) Chev. C. R. Ac. Sc.; Rosaceae	3547
8. *Pyracantha koidzumii* (Hayata) Rehder; Rosaceae	3545
9. *Pyrus taiwanensis* Iketani & Ohashi; Rosaceae	3546
10. *Styrax formosana* Matsum.; Styracaceae	3560

turic acid (TBA) and 10% trichloroacetic acid (TCA) were added to the reactants and boiled for 5 min. Then the reactants were cooled and centrifuged at 13,000 × *g* for 5 min, and the absorbance was determined spectrophotometrically at 532 nm.

Determination of total phenolics

The amount of total phenolics in extracts was determined according to a modified Folin-Ciocalteu method (Kujala et al., 2000). A 250-µl aliquot of sample solution (2.5 mg/ml) was mixed with 250 µl of 1 N Folin-Ciocalteu reagent, 500 µl of a 20% sodium carbonate (Na_2CO_3) solution, and 4 ml water. After a 25-min incubation at room temperature, the reaction mixture was centrifuged at 5000 rpm for 10 min. The supernatant was measured at 730 nm using a spectrophotometer. The amount of total phenolics was expressed as gallic acid equivalent (GAE) in milligrams per gram dry plant extract.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) of each triplicate test.

Results and Discussion

Ten Taiwanese native plants were extracted with 70% aqueous acetone, and their yields ranged from 7.3% to 32.1% (Table 2). The antioxidant activities of these ex-

Table 2. The phenolics and IC_{50} values of Taiwanese native plants against DPPH and OH free radicals.

Botanical name	Yield (%)	Total phenolics (mg of GAE/g)	IC_{50} value	
			DPPH radical (µg/ml)	OH radical (µg/ml)
1. *Acer buerferianum* var. *formosanum*	15.5	27.8	8.3	0.6
2. *Cinnamomum insulari-montanum*	10.2	3.0	24.9	1.1
3. *Cleyera japonica* var. *morii*	32.1	50.3	5.4	0.5
4. *Cyclobalanopsis stenophylla* var. *tenophylloides*	31.0	33.5	5.4	0.2
5. *Eriobotrya deflex*	8.8	29.6	16.9	0.8
6. *Machilus zuihoensis*	7.3	51.7	7.9	0.8
7. *Malus docmeri*	16.5	2.2	23.1	0.7
8. *Pyracantha koidzumii*	14.8	2.6	21.2	0.5
9. *Pyrus taiwanensis*	8.9	2.6	13.6	0.6
10. *Styrax formosana*	13.5	2.7	31.5	0.3

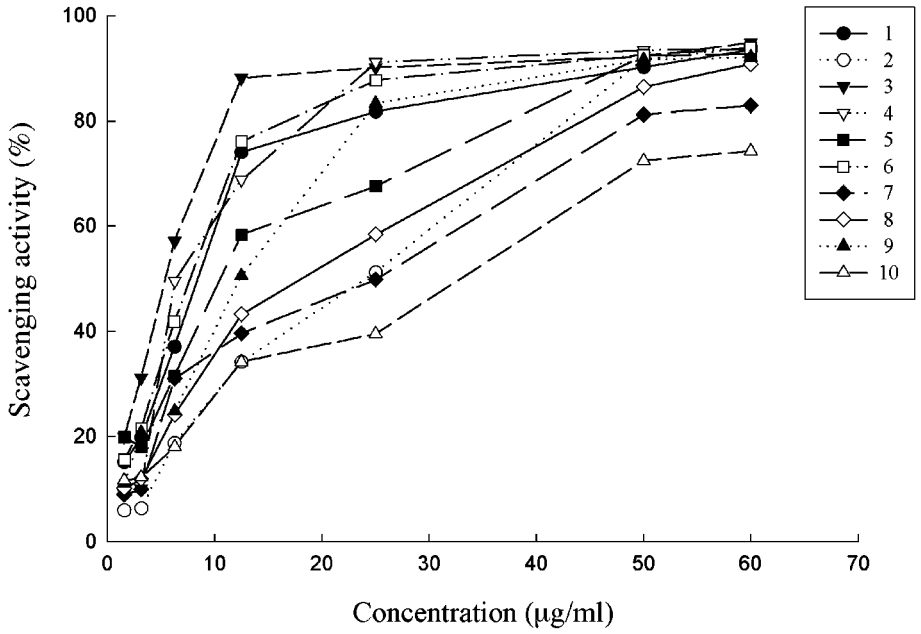

Fig. 1. Scavenging activity of Taiwanese native plants on the DPPH radical.

tracts were investigated by DPPH, hydroxyl radical-scavenging activities, and reducing power activity in this study.

DPPH reactivity is one popular method for screening the free radical-scavenging ability of compounds or the antioxidant activity of plant extracts. Figure 1 shows the dose-response curve for DPPH radical-scavenging activity of the extracts of ten Taiwanese native plants, and the IC_{50} values were calculated and are presented in Table 2. Four out of ten plants, including *Acer buerferianum* var. *formosanum*, *Cleyera japonica* var. *morii*, *Cyclobalanopsis stenophylla* var. *stenophylloides*, and *Machilus zuihoensis*, were exhibited stronger activities and their IC_{50} values ranged from 5.4 to 8.3 μg/ml. The results imply that the radical scavenging activities of the four plants may be attributed to

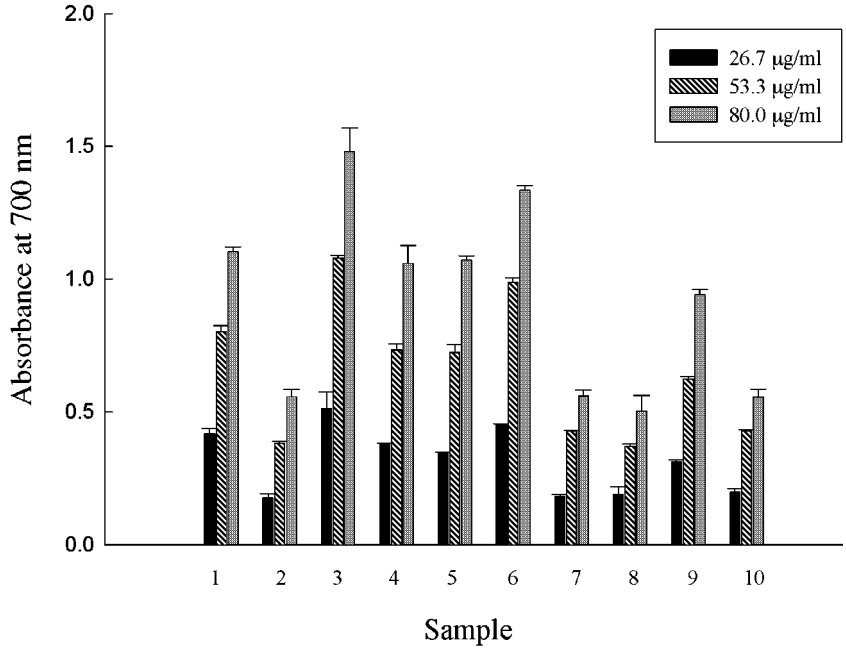

Fig. 3. Deoxyribose assay of Taiwanese native plants.

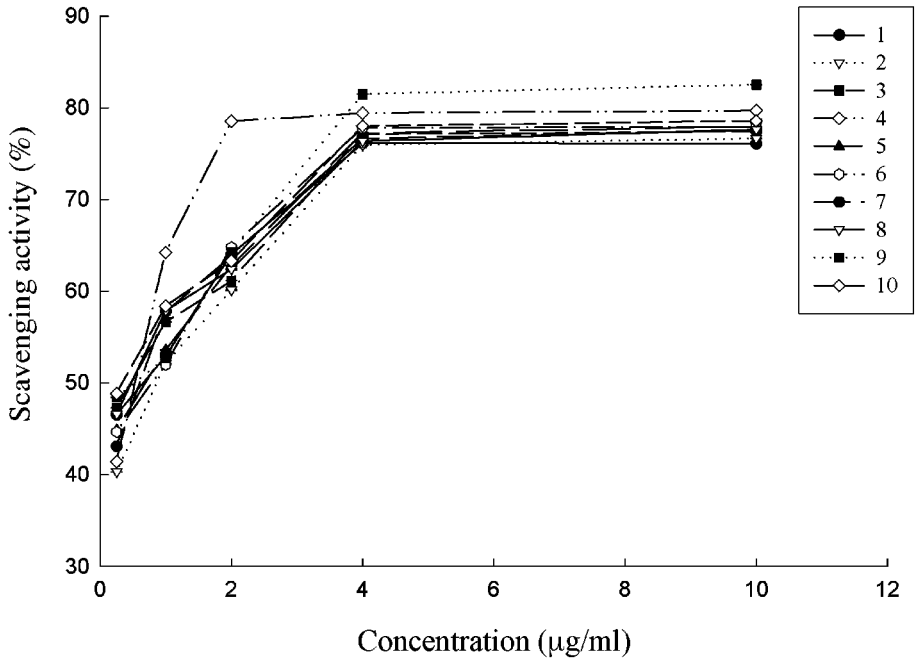

Fig. 2. Reducing power activity of Taiwanese native plants.

their stronger proton-donating abilities. In addition to their H-donating ability (Hou et al., 2001), the DPPH scavenging activity was also related to the structure of the active substances in plant extracts (Sawai and Moon, 2000).

In the reducing power assay, the antioxidant activity of samples was measured by their ability to reduce the Fe^{3+}/ferricyanide complex by formimg ferrous products. Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increased absorbance at 700 nm indicates a stronger reducing power. When these tested extracts (with a concentration of 80 μg/ml) were respectively added to the reaction mixture, *A. buerferianum* var. *formosanum*, *C. japonica* var. *morii*, *C. stenophylla* var. *stenophylloides*, *E. deflex*, and *M. zuihoensis* showed greater reducing powers relative to the other plant extracts (Fig. 2), and the absorbances at 700 nm were found to be greater than 1. They also showed a dose-dependent effect. The five extracts may contain more active materials which can donate electrons and react with free radicals, and then convert them into more-stable metabolites and terminate the radical chain reaction (Yen and Chen, 1995).

The protection of lipids against free radical reactions can be evaluated by the Fenton reaction using the deoxyribose assay (Hu and Kitts, 2001; Lai et al., 2001). In this system, a mixture of Fe^{3+}-EDTA, hydrogen peroxide (H_2O_2), and ascorbic acid were used to generate hydroxyl radicals ($^{\cdot}OH$). The response degrades the sugar deoxyribose into fragments which under heating with thiobarbituric acid at a low pH, are detected because they generate a pink chromogen. This capability to reduce Fe^{3+} and stimulate deoxyribose degradation was also adopted as one of the pro-oxidant properties of actual or proposed antioxidants (Hsieh and Yen, 2000; Matsingou, 2001). The data in Fig. 3 show that the ten extracts could effectively inhibit the formation of $^{\cdot}OH$ generated in a concentration-dependent manner. The IC_{50} values were calculated and are presented in Table 2; they ranged from 0.3 to 1.1 μg/ml. In this assay, the tested samples inhibit color formation may be not only by reacting with hydroxyl radical but also by chelating iron (Li and Xie, 2000). Although the maximum scavenging activity of hydroxyl radicals in this system was about 80% for the ten extracts, they possessed a concentration-dependent effect at lower concentrations (0.25 to 4 μg/ml) as shown in Fig. 3. This phenomenon might be because the tested extracts contain compounds which can chelate iron in high concentrations. Whatever, the deoxyribose assay is a convenient method to determine the reaction of water-soluble compounds with the hydroxyl radical (Li and Xie, 2000).

According to the above results, the crude extracts of *A. buerferianum* var. *formosanum*, *C. japonica* var. *morii*, *C. stenophylla* var. *stenophylloides*, and *M. zuihoensis* showed excellent *in vitro* antioxidant activity in this study. By using the Folin-Ciocalteu method to determine the total phenolics, it was shown that the four extracts contain abundant phenolics (Table 2). The total phenolic contents were expressed as gallic acid equivalents (GAEs) per gram of dried plant extract; the amount of four plant extracts ranged from 27.8 to 51.7 mg GAE/g dry material. Thus, the total phenolics may play a role in the antioxidant activity. In current epidemiological studies, phenolic compounds of the plant kingdom have been reported to have multiple biological effects, including antioxidant (Duthie and Crozier, 2000; Eastwood, 2001), antiviral (De Clercq, 2000), and antibacterial activities (Alcaraz et al., 2000) as well as tumor cell growth inhibition (Ito et al., 2000; Rafi et al., 2000). Free radicals are one of the causes of several diseases, such as aging diseases, cardiovascular diseases, atherosclerosis (Kendler, 1995), and cancers (Meydani, 2001; Rice-Evans, 2001; Feiz and Mobarhan, 2002). In addition to the four active extracts of *A. buerferianum* var. *formosanum*, *C. japonica* var. *morii*, *C. stenophylla* var. *stenophylloides*, and *M. zuihoensis*, *E. deflex* was also containing rich phenolics and effective in the reducing power test but not so effective in the DPPH system. This may be attributed to the compounds of plant extracts possessing small differences in their proton- and electron-donating abilities. However, *C. insulari-montanum*, *M. docmeri*, *P. koidzumii*, *P. taiwanensis*, and *S. formosana* did not show significant correlations between the deoxyribose assay and other assay systems. Thus, the hydroxyl radical scavenging activity of these four plant extracts could not be predicted on the basis of phenolic compounds.

In the present study, we demonstrate that Taiwanese native plants contain phenolic compounds which can serve as natural sources to develop free radical scavengers. Natural antioxidants may by responsible for the protective effects against the risk of many physiological and pathological processes. Because there are few reports about Taiwanese native plants, we are interested in the antioxidant properties of these native plants. Therefore, it is suggested that further work should focus on the isolation and identification of the radical-scavenging components using bio-organic chemical methods to study these active extracts.

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