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Antioxidant Activity and Total Phenolic Content of *Callistemon citrinus* Extracts

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Abstract Ethanol crude extracts of wood, bark, leaf, and fruit of *Callistemon citrinus* were compared for their antioxidant activities based on DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity and reducing power. Bark extract showed the most potent radical-scavenging activity and reducing power, showing 94.1 and 0.64% at 25 and 100 μg/mL, respectively. Total phenolic content of the bark extracts (275.0 mg GAE/g) was higher than those of others. Further fractionation of the bark extract using hexane, CH_2Cl_2, and EtOAc showed EtOAc fraction had the highest antioxidant activity (IC_{50} 6.7 μg/mL) and reducing power (0.82 at 100 μg/mL), with total phenolic content of 611.1 mg GAE/g. Total phenolic contents correlated with antioxidant activity (R^2 = 0.7061) and reducing power (R^2 = 0.7399).

Key words: *Callistemon citrinus*, antioxidant activity, radical scavenging, reducing power, total phenolic contents

Introduction

Antioxidants are used widely in food, drug, and cosmetic fields. They help to maintain the quality of many food products by delaying or inhibiting the oxidation of molecules including lipids by inhibiting the initiation or propagation of oxidative chain reaction (1). Oxidation is defined as the transfer of electrons from one atom to another, and its occurrence in living organisms is known to cause damages to DNA, protein, and lipids (2), resulting in cancer, cardiovascular disease, decline of immune system, and brain dysfunction.

Most of the antioxidants utilized are synthetic butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (3). However, their use has begun to be restricted due to their toxicity and carcinogenesis (4, 5). Therefore, the search for natural antioxidants, especially of plant origin, has greatly increased in recent years, because natural antioxidants can protect the human body from free radicals and delay the progress of many chronic diseases (6). Some studies have suggested that antioxidant compounds can be found in wood, bark, stem, leaf, fruit, root, flower, and seed of many plants (7, 8).

Callistemon citrinus (Myrtaceae), commonly known as lemon bottlebrush, is a shrub with a distinct citrus aroma. Its leaves are narrow, lance-shaped, and leathery. Bright red, plump, bottle-brush-shaped flowers composed mostly of stamens bloom off and on throughout hot weather. The ethanolic leaf extract of *C. citrinus* was found to have a strong anti-mycobacterium tuberculosis activity (9). In addition, leptospermone, a compound isolated as a component of the steam-volatile oils of *C. citrinus*, showed a moderately active herbicidal activity and produced unique bleaching symptoms on susceptible weed species (10). From the leaves and flowers of the water-distilled essential oil of *C. citrinus*, 1,8-cineole, α-pinene, and α-terpineol were identified based on their chemical compositions by GC, GC/MS, and GC/FTIR (11). In a previous study (12), we reported the antibacterial activities of plant extracts including *C. citrinus*, *Acer nipponicum*, and *Fraxinus griffithii* against *Streptococcus mutans* from woody plants.

We report, for the first time, the antioxidant activities and total phenolic contents of the wood, bark, leaf, and fruit of this plant. Solvent fractions were evaluated to determine their contribution, and the antioxidant activity was correlated with total phenolic contents.

Materials and Methods

Materials The wood, bark, leaf, and fruit of *C. citrinus* were collected from Jeju Province, Korea during June, 2002. A voucher specimen was deposited at the Korea Forest Research Institute, Suwon, Korea.

Extraction and fractionation Dried parts of *C. citrinus* were finely ground, extracted twice with ethanol (EtOH), and evaporated to give crude extracts. The crude extract of bark, which showed high antioxidant activity, was successively partitioned with organic solvents, *n*-hexane, methylene chloride (CH_2Cl_2), and ethyl acetate (EtOAc) (Fig. 1).

Total phenolic contents Total phenolic contents were measured according to the method of Cheung *et al.* (13). Each sample (1 mL) was mixed with Folin and Ciocalteu's phenol reagent (1 mL, Sigma chemical Co., St. Louis, USA). After 3 min, 1 mL of saturated Na_2CO_3 was added to the mixture, whose volume was made up to 10 mL by adding distilled water. After the reaction mixture was kept in the dark for 90 min, absorbance was measured at 725 nm. A calibration curve was constructed with different concentrations of gallic acid (0.01-0.1 mM; Wako pure chemical Industries) as a standard. Total phenolic contents were expressed as gallic acid equivalents (mg GAE/g extract).

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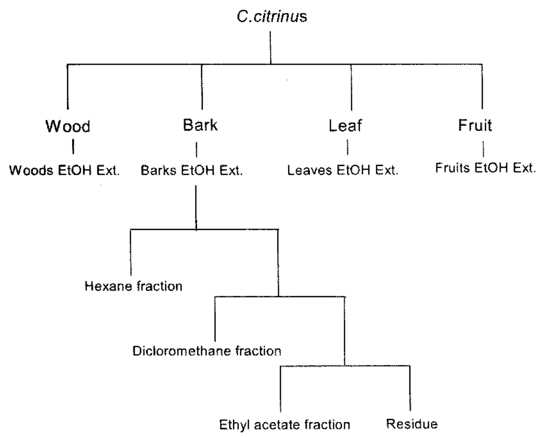


Fig. 1. Flow diagram of fractionation of *C. citrinus*.

Antioxidant activity The antioxidant activity was measured by the DPPH method according to the procedure of Park *et al.* (14). MeOH solution (4 mL) of samples at various concentrations were added to a solution of DPPH in MeOH (4.5×10^{-4} M, 1 mL). Subsequently, the reaction mixtures were shaken vigorously and left standing for 30 min at room temperature. Remaining parts of DPPH were determined by colorimetry (8452A Diode Array Spectrophotometer, Hewlett Packard Co., USA) at 520 nm. The mixture of 4 mL MeOH and a solution of 1 mL DPPH was used as the control. The mean values were obtained from triplicate experiments.

Reducing power The reducing power was determined according to the method of Oyaizu (15). Extracts (100 and 50 μg/mL) in distilled water (2.5 mL) were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL potassium ferricyanide (10 mg/mL). The mixture was then incubated at 50°C for 20 min, added with 2.5 mL trichloroacetic acid (100 mg/mL), and centrifuged at 4000 rev/min for 10 min. The upper layer (5 mL) was mixed with 5 mL distilled water and 1 mL ferric chloride (1 mg/mL), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power.

Results and Discussion

Extraction yield The yields (%) of the *C. citrinus* wood, bark, leaf, and fruit extracts were 3.5, 11.6, 14.7, and 11.8% (w/w), respectively (Fig. 2).

Total phenolic contents Total phenolic contents of the *C. citrinus* extracts were in the decreasing order of bark extract (275.0 mg GAE/g) > wood extract (140.2 mg GAE/g) > fruit extract (134.4 mg GAE/g) > leaf extract (20.7 mg GAE/g) (Fig. 3).

Total phenolic contents of the bark extracts were in the decreasing order of EtOAc fraction (611.1 mg GAE/g) > residue (472.9 mg GAE/g) > CH_2Cl_2 fraction (70.8 mg GAE/g) > hexane fraction (14.2 mg GAE/g) (Fig. 4). These results suggest that higher extraction yields of phenolic compounds were obtained with increasing polarity of the solvent (16).

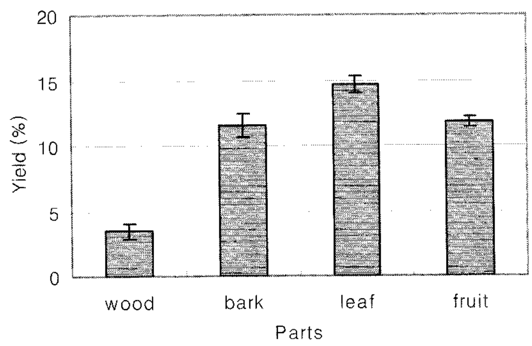


Fig. 2. Yield of the extracts from the wood, bark, leaf and fruit of *C. citrinus*. All values are mean±SD (n=3).

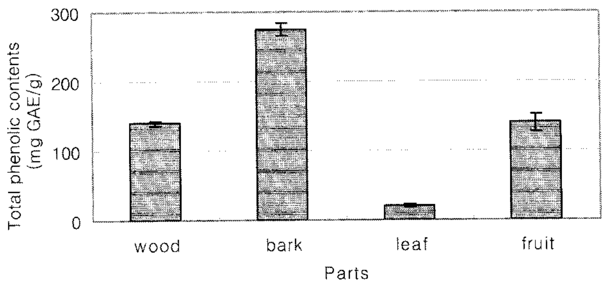


Fig. 3. Total phenolic contents of the extracts from the wood, bark, leaf and fruit of *C. citrinus*. All values are mean±SD (n=3).

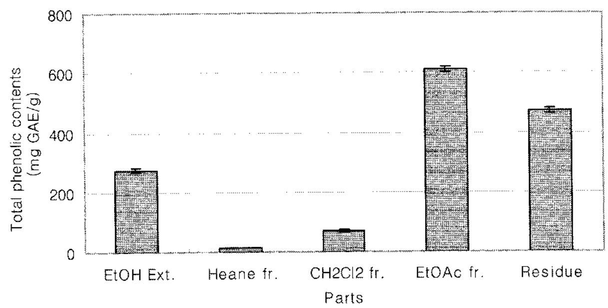


Fig. 4. Total phenolic contents of solvent fractions from the bark of *C. citrinus*. All values are mean±SD (n=3).

Antioxidant activity Free radicals are chemical fragments that cause oxidation, and antioxidants act as free radical scavengers, among which DPPH is a purple compound having a characteristic absorption at 520 nm in methanol. For this reason, DPPH was used to measure the antioxidant activity.

The free radical-scavenging activities and IC_{50} (50% decrease of DPPH radical) values of the crude extracts of *C. citrinus* wood, bark, leaf, and fruit are shown in Table 1. The scavenging activities against DPPH radical were in the decreasing order of bark extract > fruit extract > wood extract > leaf extract. The IC_{50} data revealed that bark ethanolic extracts showing lowest IC_{50} (8.8 μg/mL) is the most active and has higher antioxidant activity than the reference compound, BHT (IC_{50} = 17.4 μg/mL).

Table 2 shows free radical-scavenging activities of solvent fractions of the *C. citrinus* bark. The DPPH radical-scavenging activity was in decreasing order of EtOAc fraction > residue > CH_2Cl_2 fraction > hexane fraction,

Table 1. Free radical scavenging activities of extracts from the wood, bark, leaf and fruit of *C. citrinus*

Parts	Free radical scavenging activity (%)				IC_{50} (μg/mL)[1]
	50[2]	25	10	5	
wood	95.7±2.10	90.7±2.72	47.3±3.76	18.0±1.66	12.4
bark	96.9±2.16	94.1±1.40	58.3±3.25	23.7±1.07	8.8
leaf	92.2±1.37	75.9±2.42	22.6±2.61	15.2±1.61	17.7
fruit	96.3±1.06	92.9±2.33	56.0±1.70	21.2±1.28	9.5
quercetin	98.1±1.44	97.8±0.64	93.7±2.55	65.3±1.95	2.3
BHT	83.9±3.67	65.4±2.02	35.2±0.57	20.5±0.75	17.4
α-tocopherol	95.1±1.31	87.5±2.26	66.3±4.97	32.3±2.36	7.6

[1]The values indicate 50% decrease of DPPH radical and are the means of triplicate data.
[2]μg/mL.

Table 2. Free radical scavenging activities of solvent fractions of the bark of *C. citrinus*

Fractions	Free radical scavenging activity (%)				IC_{50} (μg/mL)[1]
	50[2]	25	10	5	
hexane	17.3±1.71	14.9±0.87	12.7±1.80	11.3±0.86	>50
CH_2Cl_2	86.2±2.21	60.5±7.05	12.6±2.14	8.6±1.57	21.7
EtOAc	88.0±2.04	84.3±4.78	74.1±1.67	38.1±2.07	6.7
residue	88.9±1.00	88.1±1.45	59.7±2.26	32.2±4.49	8.2

[1]The values indicate 50% decrease of DPPH radical and are the means of triplicate data.
[2]μg/mL

among which EtOAc fraction showed the highest antioxidant activity with IC_{50} value of 6.7 μg/mL. Hexane and CH_2Cl_2 fractions showed poor activities when compared with other fractions and reference antioxidants such as BHT and α-tocopherol. These results suggest that the antioxidant activity of bark ethanolic extract of *C. citrinus* is partially attributable to the EtOAc fraction.

Reducing power Reducing power has been found to be associated with the antioxidant activity (17) and thus may serve as a significant indicator of the potential antioxidant activity (18). The reducing powers of the ethanolic extracts of *C. citrinus* wood, bark, leaf, and fruit were 0.57, 0.64, 0.36, and 0.63 at 100 μg/mL, and 0.29, 0.34, 0.17, and 0.33 at 50 μg/mL, respectively (Fig. 5). The reducing power of the bark ethanolic extract was higher than that of the reference compound, α-tocopherol (0.26 at 100 μg/mL), and the leaf exhibited lowest reducing power.

The crude extract of bark, which had highest reducing power, was successively partitioned with organic solvents, *n*-hexane, CH_2Cl_2, and EtOAc, and their reducing powers were measured (Fig. 6). As observed with free radical-scavenging activities, the reducing powers of the solvent fractions were in the order of EtOAc fraction > residue > CH_2Cl_2 fraction > hexane fraction; the reducing power of EtOAc fraction (0.82 at 100 μg/mL) was higher than those of other solvent fractions and references, BHT (0.60 at 100 μg/mL) and α-tocopherol (0.26 at 100 μg/mL).

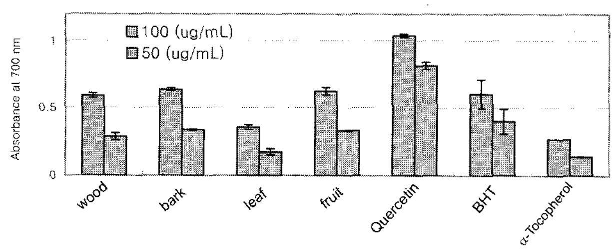


Fig. 5. Reducing power of extracts from the wood, bark, leaf and fruit of *C. citrinus*. All values are mean±SD (n=3).

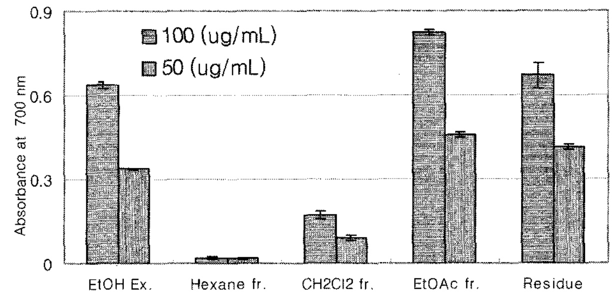
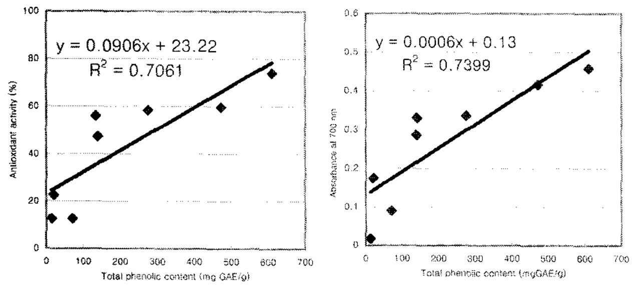


Fig. 6. Reducing power of solvent fractions from the bark of *C. citrinus*. All values are mean±SD (n=3).

Relation between antioxidant activity and total phenolic contents EtOAc fraction, which had highest phenolic content, also had highest antioxidant activity and reducing power among the four solvent fractions partitioned from the bark ethanolic extracts of *C. citrinus*. The antioxidant activity and reducing power increased with the increase in the contents of total phenolics. Pyo *et al.* (19) also reported that polyphenols played an important role in the DPPH radical-scavenging activity. They determined the antioxidant activity, total phenolic contents, and phenolic composition of Swiss chart extract, and found a positive linear correlation between radical-scavenging activity and total phenolic content. Yen *et al.* (20) discovered that polyphenols are the most abundant group of compounds in tea leaf and appear to be responsible for antioxidant activity. The increase in the antioxidant activity with the increase of total phenolics may be the result of polyphenols exerting their antioxidant action by donating hydrogen atoms to the free radicals (21).



A B

Fig. 7. Correlation between antioxidant activity (at 10 μg/mL) with total phenolic content (A), and reducing power (at 50 μg/mL) with total phenolic content (B).

A linear correlation (R^2 = 0.7061) was shown between antioxidant activity (at 10 μg/mL) and total phenolic contents of each extract and its solvent fractions (Fig. 7A). A high correlation (R^2 = 0.7399) was also found between reducing power (at 50 μg/mL) and total phenolic contents in all bark extracts and their solvent fractions (Fig. 7B). Because polyphenolic substances are generally known to be closely related to antioxidant activity and reducing power (22), the antioxidant and reducing powers of the extracts and solvent fractions of the *C. citrinus* bark could be mainly due to its phenolic contents. In the present study, about 70% of polyphenolics appeared to contribute to the antioxidant activity and reducing power. Other substances that could have caused the antioxidant activity may be non-phenolic secondary metabolites, such as volatile oils, carotenoids, and vitamins.

Results of our study show that *C. citrinus* bark ethanol extract has stronger antioxidant activity and reducing power than BHT, a reference compound with an antioxidant potential. Moreover, the antioxidant activity and reducing power of the *C. citrinus* extracts appeared to be concentration dependent, and the bark extract showed the highest antioxidant activity. Among the four fractions (hexane, CH_2Cl_2, EtOAc, and residue fractions) of *C. citrinus* bark, EtOAc fraction showed the highest antioxidant activity, suggesting this fraction to be the major contributor to the antioxidant activities of *C. citrinus*.

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