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Article

Comparison of Tyrosinase Inhibitory Effect of the Natural Antioxidants from *Cedrela sinensis*

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DPPH and ABTS radical scavenging, and tyrosinase inhibitory activities of bioactive compounds 1-4 isolated from *Cedrela sinensis* leaf were assessed. Structures of isolated compounds were established by UV, one- and two-dimensional NMRs, and mass spectroscopic methods.

Key words: *Cedrela sinensis, antioxidant, DPPH radical scavenging activity, ABTS radical scavenging activity, tyrosinase inhibitory activity*

Cedrela sinensis (Meliaceae) is a broadleaf tree which has been widely cultivated in Korea and China.[1,2] It has been used to treat enteritis, dysentery, and skin itch in oriental medicine.[3,4] In addition, Koreans have been consuming the young leaves as herb salad during early spring. Many flavonoids, limonoids, and phenolic compounds have been isolated from this plant.[4-6] It is well known that flavonoids and phenolic compounds have antioxidant activity. Antioxidants, inhibitors of lipid peroxidation, are important not only for food protection but also for the defense of living cells against oxidative damages by the scavenging of free radicals.[7] Moreover, free radicals, produced by ultraviolet light, are absorbed and scavenged by melanin as a defense mechanism.[8] Biosynthesis of melanin in animals is normally initiated through the oxidation of tyrosine into DOPA by tyrosinase (monophenoloxidase, EC 1.14.18.1),[9] which is the rate-limiting enzyme in this pathway. The production of abnormal pigmentation such as melasma, freckles, senile lentigines, and other forms of melanin hyperpigmentation could be a serious aesthetic problem.[8]

In this paper, we report isolation and structural elucidation of the bioactive compounds from the leaves of *C. sinensis*. Their antioxidant activities were determined by measuring the scavenging effect on DPPH and ABTS radicals. Tyrosinase inhibition was investigated by measuring the inhibitory effect on mushroom tyrosinase.

Materials and Methods

Plant material. *C. sinensis* leaves were collected from Oksan-ri, Munsan, Gyeongsangnam-do, Korea. The scientific name was determined by Prof. Myong Gi Chung of Gyeongsang National University. A voucher specimen (*S. W. Hwang & M. S. Yang 023*) was deposited at the herbarium of the university.

General experimental procedures. Silica gel (MERCK, Germany) was used for isolation. All other chemicals used in this study were of analytical grade. UV spectra were measured using a Beckman DU650 spectrophotometer. ^{1}H-, ^{13}C-, and 2D-NMR data were obtained on a Bruker AM 500 (^{1}H-NMR at 500 MHz, ^{13}C-NMR at 125 MHz) spectrometer. Molecular weight was measured using an EI-MS (JEOL JMS-700) spectrometer.

Extraction and isolation procedures. The air-dried leaves (2.5 kg) of *C. sinensis* were extracted with MeOH (10 × 3) at room temperature for 72 hr. The MeOH solution was combined, concentrated, and dried under reduced pressure at temperature not higher than 45°C. The MeOH extract (320 g) was successively partitioned with $CHCl_3$, EtOAC, and H_2O fractions. The $CHCl_3$ extract (95 g) was chromatographed on a silica gel (1 kg; 70-230 mesh) column eluted with $CHCl_3$-Acetone (99/1 → 1/1) gradient, yielding 15 fractions (F1 through F15). Fraction F3 (12 g) was combined and applied to a silica gel column and eluted with hexane-EtOAc mixtures of increasing polarity (49/1 → 1/1) to give five major subfractions (F3-1 through F3-5). F3-1 (1.82 g) was purified by repeated column chromatography on silica gel with hexane-ether (99/1 → 1/1) to yield compound **1** (300 mg). Fraction F8 (3 g) was combined and applied to a silica gel column and eluted with $CHCl_3$-Acetone mixtures of increasing polarity (49/1 → 1/1) to give four major subfractions (F8-1 through F8-4). From subfraction F8-3, compound **2** (35 mg) was isolated by preparative TLC with $CHCl_3$-Acetone (9 : 1). Fraction F10 (10.6 g) was also applied to a silica gel column, eluted with

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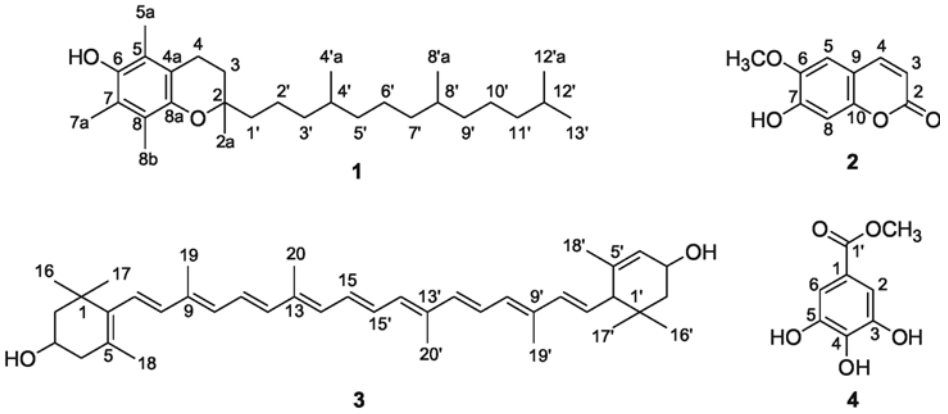

Fig. 1. Chemical structures of the isolated compounds.

$CHCl_3$-MeOH (24 : 1) to the ratio of increasing MeOH (1 : 1) to give five subfractions (F10-1 through F10-5). F10-4 (3.5 g) was purified by repeated column chromatographed on silica gel with $CHCl_3$-Acetone (24/1 → 1/1) to yield Compound **3** (143 mg). The EtOAc soluble fraction (50 g) was also column chromatography on silica gel (500 g; 70-230 mesh) with CH_2Cl_2-EtOH (19/1 → 1/1) gradient, yielding 12 fractions (E-F1 through E-F12). From subfraction E-F5 (4.6 g), compound **4** (200 mg) was isolated by recrystallization with petroleum ether.

DPPH radical scavenging activity. Antioxidant activities of the isolated compounds and the standards were assessed on the basis of the radical scavenging effect of the stable DPPH free radicals.[10] The sample solution (100 μ*l*) was added to an ethanolic solution (900 μ*l*) of DPPH radicals (0.15 mM DPPH). After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm (Beckman DU650).

ABTS radical scavenging activity. The total antioxidant activity was measured based on the ability of the antioxidant to scavenge the radical cation 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\cdot+}$) through spectrophotometeric analysis. ABTS (7 mM) and potassium persulfate (2.45 mM) were reacted for 12 h at dark at room temperature to produce ABTS cation radicals. The ABTS solution was diluted with PBS to an $A_{734} = 0.7$. The reaction was initiated by adding 900 μ*l* of ABTS to 100 μ*l* of sample solution. The absorbance of each solution was determined at 734 nm (Beckman DU650).

Tyrosinase inhibitory activity. To a 96-well microtitre plate was added 150 μ*l* of 0.1 M phosphate buffer (pH 6.5), 25 μ*l* of 1.5 mM L-tyrosine solution, and 7 μ*l* of 2,100 unit/ml mushroom tyrosinase (Sigma, 0.05 mM phosphate buffer pH 6.5). After incubation at 30°C for 10min, the amount of DOPA produced in the reaction mixture was determined as the optical density at 490 nm. The inhibitory activity of the sample was expressed as the concentration at which 50% of the enzyme activity is inhibited (IC_{50}).

Results and Discussion

Identification of active constituents. The structure of each compound was identified by the following evidence and comparing the corresponding NMR spectral data with those in the literature.[11-14]

α-Tocopherol (1): Red Oil; ^{1}H-NMR (500 MHz, $CDCl_3$) δ; 0.84 - 0.87 (H-12', 13', 4'a and 8', s, 12H), 1.06 (H-3' and H-5', m, 4H), 1.14 (H-11', m, 2H), 1.22 (H-2a, s, 3H), 1.25 (H-7' and 9', m, 4H), 1.37 - 1.40 (H-4' and 8', m, 4H), 1.51-1.55 (H-2', 12' and 1', m, 5H), 1.77 (H-3, m, 2H), 2.10 (H-5a and 8b, s, 6H), 2.11 (H-7a, s, 3H), 2.59 (H-4, t, J = 6.7 Hz, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ; 11.27 (C-5a), 11.77 (C-8b), 12.20 (C-7a), 19.67 (C-8'a), 19.76 (C-4'a), 20.78 (C-4), 21.06 (C-2'), 22.63 (C-13'), 22.73 (C-12'), 23.81 (C-2a), 24.46 (C-6'), 24.82 (C-10'), 27.99 (C-12'), 31.60 (C-3), 32.72 (C-8'), 32.82 (C-4'), 37.31 (C-9'), 37.45 (C-7'), 37.48 (C-5'), 37.51 (C-3'), 39.41 (C-11'), 39.87 (C-1'), 74.51 (C-2), 117.34 (C-4a), 118.53 (C-5), 121.09 (C-7), 122.61 (C-8), 144.57 (C-6), 145.58 (C-8a).

Scopoletin (2): Pale yellow powder; ^{1}H-NMR (500 MHz, CD_3OD) δ; 3.88 (6-OCH_3, s, 3H), 6.13 (H-3, d, J = 9.5 Hz, 1H), 6.70 (H-5, s, 1H), 7.0 (H-8, s, 1H), 7.8 (H-4, d, J = 9.5 Hz, 1H); ^{13}C-NMR (125 MHz, CD_3OD) δ; 57.0 (6-OCH_3), 104.6 (C-5), 109.9 (C-8), 111.6 (C-3), 112.0 (C-10), 146.6 (C-4), 148.3 (C-6), 152.4 (C-9), 155.9 (C-7), 164.8 (C-2).

Lutein (3): Red powder; ^{1}H-NMR(500 MHz, $CDCl_3$) δ; 0.85 (H-16', s, 3H), 1.00(H-17', s, 3H), 1.07 (H-16 and 17, s, 6H), 1.38 (H_{ax}-2', dd, J = 5.3, 13.9 Hz, 1H), 1.48 (H_{ax}-2, m, 1H), 1.63 (H-18', s, 3H), 1.73 (H-18, s, 3H), 1.84 (H_{eq}-2, m, 1H), 1.83 (H_{eq}-2', dd, J = 6.2, 13.2 Hz, 1H), 1.91 (H-19', s, 3H), 1.96 (H-19, 20 and 20', s, 9H), 2.05 (H_{ax}-4, dd, J = 10.5, 16.2 Hz, 1H), 2.38 (H_{eq}-4, dd, J = 5.0, 18.1 Hz, 1H), 2.39 (H-6', d, J = 9.2 Hz), 4.00 (H-3, m, 1H), 4.25 (H-3', s, 1H), 5.43 (H-7', dd, J = 9.2, 15.5 Hz, 1H), 5.54 (H-4', s, 1H), 6.09 (H-7, d, J = 16.2 Hz, 1H), 6.13 (H-10, d, J = 12 Hz, 1H), 6.14 (H-8, d, J = 16 Hz, 1H), 6.15 (H-10', d, J = 11.5 Hz, 1H), 6.16 (H-8', d, J = 15.5 Hz, 1H), 6.25 (H-14 and 14', m, 2H), 6.34 (H-12',

Table 1. Scavenging effects of the isolated compounds on DPPH and ABTS radicals

Compounds	IC_{50} (μM)[a]	
	DPPH	ABTS
1	25.5 ± 0.4	16.0 ± 0.2
2	> 200	7.0 ± 0.3
3	82.8 ± 0.3	> 200
4	10.8 ± 0.3	2.8 ± 0.2
BHA	35.9 ± 0.5	
Trolox		25.2 ± 0.4

[a]Mean ± S. D. of triplicate determinations.

d, *J* = 14.9 Hz, 1H), 6.36 (H-12, d, *J* = 14.9 Hz, 1H), 6.63 (H-11 and 11', m, 2H), 6.64 (H-15 and 15', m, 2H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ; 12.76 (C-19'), 12.82 (C-20), 12.82 (C-20'), 13.11 (C-19), 21.62 (C-18), 22.86 (C-18'), 24.32 (C-16'), 28.75 (C-16), 29.52 (C-17'), 30.28 (C-17), 33.48 (C-1'), 37.14 (C-1), 42.59 (C-4), 44.72 (C-2'), 48.48 (C-2), 55.02 (C-6'), 65.12 (C-3), 65.91 (C-3'), 124.51 (C-4), 124.89 (C-11), 125.01 (C-11'), 125.57 (C-7), 126.18 (C-5), 128.71 (C-7'), 130.09 (C-15), 130.14 (C-15'), 130.78 (C-10'), 131.34 (C-10), 132.54 (C-14 and 14'), 135.13 (C-9), 135.68 (C-9'), 136.41 (C-13), 136.53 (C-13'), 137.57 (C-12), 137.61 (C-12'), 137.79 (C-6), 137.85 (C-5'), 138.04 (C-8), 138.53 (C-8').

Methyl gallate (4): Crystall; ^{1}H-NMR (500 MHz, CD_3OD) δ; 3.82 (OCH_3, s, 3H), 7.07(H-2 and 6, s, 2H); ^{13}C-NMR (125 MHz, CD_3OD) δ; 51.3 (OCH_3), 109.1(C-2 and 6), 120.5 (C-1), 138.7 (C-4), 145.5 (C-3 and 5), 168.0 (C-1').

DPPH radical scavenging activity. The compounds isolated from *C. sinensis* were tested for their DPPH radical scavenging activity. A synthetic antioxidant (BHA) was used as the positive control. Most tested samples were dose-dependent scavengers of DPPH radicals (Table 1). The IC_{50} values of α-tocopherol (**1**), lutein (**3**), methyl gallate (**4**), and BHA were 25.5, 82.8, 10.8, and 35.9 μM, respectively. Activity of methyl gallate was about three times higher than that of BHA. Lutein showed high activity. α-Tocopherol is very important as a potent antioxidant. Accordingly, the isolated α-tocopherol from *C. sinensis* also exhibited high activity. On the other hand, even at 200 μM scopoletin showed no obvious hydrogen donating ability.

ABTS radical scavenging activity. The isolated compounds were tested for their ability to ABTS radical scavenging activities. Trolox was used as the positive control. The compounds were dose-dependent scavengers of ABTS radicals (Table 1). The IC_{50} values of following α-tocopherol (**1**), scopoletin (**2**), methyl gallate (**4**), and trolox were 16.0, 7.0, 2.8, and 25.2 μM, respectively. Methyl gallate showed the highest activity, about nine times more potent than that of trolox. α-Tocopherol and scopoletin also showed more pronounced activities than trolox. However, at 200 μM lutein showed no obvious radical scavenging activity.

Methyl gallate (**4**) showed high DPPH and ABTS radical scavenging activities, whereas other compounds exhibited pronounced activities on either DPPH or ABTS radicals, suggesting that the four isolated compounds from *C. sinensis* could be useful as natural antioxidants.

Table 2. Inhibitory effects of the isolated compounds on the mushroom tyrosinase

Compounds	IC_{50} (μM)[a]
1	75.5 ± 1.5
2	53.1 ± 1.3
3	103.2 ± 1.5
4	21.6 ± 1.2
Arbutin	48.1 ± 1.5

[a]Mean ± S. D. of triplicate determinations.

Tyrosinase inhibitory activity. Inhibitory effects of the isolated compounds on mushroom tyrosinase activity were examined using L-tyrosine as the substrate. Bioassay was performed with arbutin as the positive control. The tyrosinase activity in the mushroom tyrosinase solution was inhibited by all tested agents in a dose-dependent manner. The IC_{50} values of α-tocopherol (**1**), scopoletin (**2**), lutein (**3**), methyl gallate (**4**), and arbutin were 75.5, 53.1, 103, 21.6, and 48.1 μM, respectively (Table 2), with methyl gallate exhibiting superior activity of about two times more potent than that of arbutin. Scopoletin showed activity similar to that of arbutin, whereas activities of α-tocopherol and lutein were lower than that of arbutin.

Presence of the hydroxyl group in all these compounds made it possible to study their structure-enzyme inhibitory activity relationships. Previous studies[15,16] showed that OH group(s) at the aromatic rings are important for the tyrosinase inhibitory activity.

These results suggest that the four bioactive compounds, α-tocopherol, scopoletin, lutein, and methyl gallate, isolated from the leaves of *C. sinensis* have potential as new biological drugs and that *C. sinensis* may be a useful herb, with antioxidant and skin aging-preventive activities.

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