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Antioxidant Compounds from Twig of *Morus alba*

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Abstract – The MeOH extract of the twig of *Morus alba* L. (Moraceae) inhibited strong lipid peroxidation activity. In order to find out active principle from the plant, acivity-guided fractionation was performed and five antioxidant compounds were isolated. Their chemical structures were identified as 6-geranylapigenin (**1**), 6-geranylnorartocarpetin (**2**), resveratrol (**3**), oxyresveratrol (**4**) and quercetin (**5**) by physicochemical and spectrometric methods. Compounds **1-5** significantly inhibited lipid peroxidation in rat brain homogenate (IC_{50} values of 3.37, 3.74, 0.23, 0.29 and 0.06 μM, respectively).

Keywords – *Morus alba* L., Moraceae, 6-geranylapigenin, 6-geranylnorartocarpetin, resveratrol, oxyresveratrol, quercetin, lipid peroxidation inhibitory activity.

Introduction

In a search for effective antioxidants from plants, the MeOH extracts from approximately 200 Korean plants were investigated. Among them, the MeOH extract of the twig of *M. alba* showed the activity in the lipid peroxidation assay. In this paper, we discribe the isolation and identification of active constituents and evaluate their lipid peroxidation inhibitory activity.

Morus alba L. (Moraceae) is widely cultivated in Korea, China, and Japan. The root bark and leaf have been used as a blood pressure depressant in China. Besides, *M. alba* have been reported to exhibit a variety of biological activities such as antimicrobial (Nomura *et al.*, 1978), antifungal (Takasuki *et al.*, 1979, 1982), anti-HIV (Luo *et al.*, 1995), anti-allergic (Lee *et al.*, 1998), antioxidant (Kim *et al.*, 1999), hypoglycemic activity (Hikino *et al.*, 1985) *etc.*

The constituents of the root bark and leaf have been comparatively well studies. Mullberrofuran A-Q (Luo *et al.*, 1995), kuwanon D-H (Nomura *et al.*, 1980, 1981, 1988), morusin (Luo *et al.*, 1995), moracin C-H (Takasugi *et al.*, 1978, 1979), moracenin A (Oshima *et al*, 1980), albafura A-C (Takasugi *et al.*, 1982) have been reported. The four flavone derivatives mulberrin, mulberrochromene, cyclomulberrin, cyclomulberrochromene were isolated from the stem of this plant by Deshpande (Deshpande *et al.*, 1968) and the mulberranol, alboctalol were isolated from the stem bark by the same group (Deshpande *et al.*, 1976). Many reports on the constituents and biological activity of the root bark and leaf have been reported. But, there has no study on chemical constituents and biological activity of the twig of *M. alba* .

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Experimental

Plant Material – The twig of *M. alba* was collected in medicinal plant garden at Chungnam National University in October 2001. A voucher specimen (CNU 390) has been deposited in the herbarium of the College of Pharmacy, Chungnam National University.

Extraction, fractionation and isolation – The dried twig of *M. alba* (9.5 kg) was extracted twice with MeOH for 3 h by reflux. The MeOH extract (471 g) was suspended in water and then partitioned with hexane, EtOAc and BuOH, sequentially. Among the solvent fractions, the hexane and EtOAc fraction inhibited in the lipid peroxidation with an IC_{50} value of 3.45 μg/ml and 0.85 μg/ml, respectively. Accordingly, the hexane fraction (113 g) was further subjected to column chromatography on a silica gel (9×40 cm, 70-230 mesh) eluted with gradient hexane-EtOAc (30:1→0:1). Eight fractions were obtained based on the monitoring of their TLC (silica gel) pattern. Silica gel column chromatography (5×30 cm, 230-400 mesh) of the Fr. 7 (Fr. 7, 13 g) was then carried out using a mobile phase of hexane-acetone (2:1) to yield three fractions (Fr. 7-1~Fr. 7-3). The Fr. 7-2 (2.1 g) was purified by preparative MPLC on an ULTRA PACK ODS-18 column (YAMAZEN, 26×300 mm; mobile phase: MeOH-H_2O (2:1); flow rate: 12 ml/min; detection:

254 nm) to give crude crystal, and recrystallized in MeOH to give compound **1** (10 mg, t_R 8 min). Compound **2** (9 mg, t_R 15 min) was isolated from Fr. 7-3 (1.6 g) by preparative MPLC on an ULTRA PACK ODS-18 column (YAMAZEN, 26×300 mm; mobile phase: MeOH-H_2O (2:1); flow rate: 12 ml/min; detection: 254 nm), and recrystallized in MeOH.

The EtOAc fraction (87 g) was subjected to column chromatography on a silica gel (9×40 cm, 70-230 mesh) eluted with gradient hexane-acetone (10:1→0:1). Eight fractions were obtained based on the monitoring of their TLC (silica gel) pattern. Silica gel column chromatography (5×30 cm, 230-400 mesh) of the Fr.6 (40 g) was then carried out using a mobile phase of $CHCl_3$ - MeOH (20:1) to yield four fractions (Fr. 6-1~Fr. 6-4). The Fr. 6-3 (18 g) was purified by preparative MPLC with MeOH - H_2O (1:1) to give compound **3** (30 mg, t_R 12 min) and compound **4** (200 mg, t_R 15 min). Compound **5** was isolated from seventh Fr. of EtOAc by revese phase (RP-18) column chromatography with MeOH-H_2O (1:1). Compound **3-5** were recrstallized in MeOH.

Compound 1 – Pale yellow prisms, mp : 205 - 207°C, IR (KBr) ν_{max} cm^{-1}: 3350 (-OH), 1650 (C=C), UV (MeOH) λ_{max}: 212, 278, 336 nm, FAB-MS m/z: 407 $[M+1]^+$, ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.83 (2H, *d*, *J* = 8.9 Hz, H-2′, H-6′), 6.93 (2H, *d*, *J* = 8.9 Hz, H-3′, H-5′), 6.57 (1H, *s*, H-3), 6.49 (1H, *s* , H-8), 5.25 (1H, *t*, *J* = 7.2 Hz, H-2″), 5.09 (1H, *t*, *J* = 7.0 Hz, H-7″), 3.35 (2H, *d*, *J* = 7.0 Hz, H-1″), 1.90 - 2.10(4H, *m*, H-5″, H-6″), 1.80 (3H, br. *d*, *J* = 7.0 Hz, H-4″), 1.60 (3H, br. *d*, *J* = 1.0 Hz, H-9″), 1.55 (3H, br. *d*, *J* = 0.5 Hz, H-10″), ^{13}C-NMR (75 MHz, CD_3OD) δ: 164.9 (s, C-2), 102.8 (d, C-3), 182.9 (s, C-4), 104.1 (s, C-4a), 161.6 (s, C-5), 112.2 (d, C-6), 158.9 (s, C-7), 93.0 (d, C-8),156.2 (s, C-8a), 122.5 (s, C-1′), 128.3 (d, C-2′), 115.9 (d, C-3′), 162.6 (s, C-4′), 115.9 (d, C-5′), 128.3 (d, C-6′), 21.2 (t, C-1″), 122.5 (d, C-2″), 134.7 (s, C-3″), 15.2 (q, C-4″), 39.9 (t, C-5″), 26.7 (t, C-6″), 124.4 (d, C-7″), 131.0 (s, C-8″), 16.6 (q, C-9″), 24.8 (q, C-10″).

Compound 2 – Pale yellow prisms, mp: 174-177°C, IR (KBr) ν_{max} cm^{-1}: 3350 (-OH), 1650 (C=C). UV (MeOH) λ_{max}: 212, 278, 336 nm, FAB-MS m/z: 423 $[M+1]^+$, ^{1}H-NMR (300 MHz, CD_3OD) δ: 7.75 (1H, *d*, *J* = 9.0 Hz, H-6′), 6.47 (1H, *d*, *J* = 2.0 Hz, H-3′), 6.45 (1H, *s*, H-8), 6.42 (1H, *dd*, *J* = 2, 9.0 Hz, H-5′), 5.25 (1H, *t*, *J* = 7.0 Hz, H-2″), 5.06 (1H, *t*, *J* = 7.0 Hz, H-7″), 3.32 (2H, *d*, *J* = 7.0 Hz, H-1″), 1.90-2.10 (4H, *m*, H-5″, H-6″), 7.12 (1H, *s*, H-3), 1.78 (3H, br. *d*, *J* = 1.0Hz, H-4″), 1.61 (3H, br. *d*, *J* = 1.0Hz, H-9″), 1.56 (3H, br. *s*, H-10″), ^{13}C-NMR (75 MHz, CD_3OD) δ: 162.8 (s, C-2), 107.3 (d, C-3), 183.4 (s, C-4), 104.0 (s, C-4a), 156.3 (s, C-5), 111.8 (s, C-6), 162.5 (s, C-7), 92.9 (d, C-8), 158.8 (s, C-8a), 109.9 (s, C-1′), 159.2 (s, C-2′), 103.1 (d, C-3′), 162.2 (s, C-4′), 108.0 (d, C-5′), 129.8 (d, C-6′), 21.1 (t, C-1″), 122.6 (d, C-2″), 134.6 (s, C-3″), 15.2 (q, C-4″), 39.8 (t, C-5″), 26.7 (t, C-6″), 124.4 (d, C7″), 131.0 (s, C-8″), 24.8 (q, C-9″), 16.6 (q, C-10″).

Compound 3 – White needles, mp: 258-260°C, IR (KBr) ν_{max} cm^{-1}: 3350 (-OH), 1580 (C=C). 1510 (C-H), UV (MeOH) λ_{max}: 208, 217, 309, 322 nm , FAB-MS m/z: 229 $[M+1]^+$, ^{1}H-NMR (600 MHz, CD_3OD) δ: 7.35 (2H, *d*, *J* = 8.6 Hz, H-2, H-6), 6.76 (2H, *d*, *J* = 8.6 Hz, H-3, H-5), 6.95 (1H, *d*, *J* = 16.2 Hz, H-α), 6.79 (1H, *d*, *J* = 16.2 Hz, H-β), 6.44 (2H, *d*, *J* = 2.1 Hz, H-2′, H-6′), 6.16 (1H, *m*, H-4′), ^{13}C-NMR (150 MHz, CD_3OD) δ: 130.4(s, C-1), 128.8 (d, C-2), 116.5 (d, C-3), 158.4 (s, C-4), 116.5 (d, C-5), 128.8 (d, C-6), 141.3 (s, C-1′), 105.8 (d, C-2′), 159.7 (s, C-3′), 102.7 (d, C-4′), 159.7 (s, C-5′), 105.8 (d, C-6′), 129.4 (d, C-α), 127.0 (d, C-β).

Compound 4 – White needles, mp: 203-205°C, IR (KBr) ν_{max} cm^{-1}: 3400 (-OH), 1680 (C=C), 1380 (C-H), UV λ_{max} (MeOH): 208, 330, 350 nm, FAB-MS m/z: 245 $[M+1]^+$, ^{1}H-NMR (300 MHz, CD_2OD) δ: 7.33 (1H, *d*, *J* = 8.5 Hz, H-6), 7.28 (1H, *d*, *J* = 16.4 Hz, H-α), 6.83 (1H, *d*, *J* = 16.4 Hz, H-β), 6.46 (1H, *d*, *J* = 2.1 Hz, H-2′), 6.34 (1H, *d*, *J* = 2.1 Hz, H-6′), 6.32 (1H, *d*, *J* = 2.1 Hz, H-3), 6.23 (1H, *dd*, *J* = 8.5 Hz, 2.1 Hz, H-5), 6.15 (1H, *t*, *J* = 2.1 Hz, H-4′), ^{13}C-NMR (75 MHz, CD_2OD) δ: 116.9 (s, C-1), 156.3 (s, C-2), 101.3 (d, C-3), 158.2 (s, C-4), 107.4 (d, C-5), 123.9 (d, C-6), 141.2 (s, C-1′), 104.7 (d, C-2′), 158.6 (s, C-3′), 102.6 (d, C-4′), 158.6 (s, C-5′), 104.7 (d, C-6′), 125.6 (d, C-α), 127.4 (d, C-β).

Compound 5 – Yellow needles, mp: 313-314°C, IR (KBr) ν_{max} cm^{-1}: 3350 (-OH), 1680 (C=C), UV (MeOH) λ_{max}: 210, 259, 374 nm, FAB-MS m/z: 303 $[M+1]^+$, ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 7.67 (1H, *d*, *J* = 2.0 Hz, H-2′), 7.53 (1H, *dd*, *J* = 2.0, 8.5 Hz, H-6′), 6.88 (1H, *d*, *J* = 8.5 Hz, H-5′), 6.40 (1H, *d*, *J* = 2.0 Hz, H-8), 6.19 (1H, *d*, *J* = 2.0 Hz, H-6), ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 147.7 (s, C-2), 136.6 (s, C-3), 176.7 (s, C-4), 161.6 (s, C-5), 99.0 (d, C-6), 164.7 (s, C-7), 94.2 (d, C-8), 157.0 (s, C-9), 103.9 (s, C-10), 122.9 (s, C-1′), 115.9 (d, C-2′), 145.9 (s, C-3′), 148.6 (s, C-4′), 116.5 (d, C-5′), 120.8 (d, C-6′).

Preparation of rat brain homogenate – The rat brain homogenate was prepared as described previously (Na *et al.*, 2002). Sprague-Dawley rat brain was removed and washed with ice-cold saline. The brain was homogenized in 9 volume of ice-cold phosphate buffer (pH 7.4) using a glass homogenizer and then centrifuged at 1,000 rpm for 10 min. The supernatant was stored at –70°C until the lipid peroxidation experiment.

Lipid peroxidation inhibitory activity (LPIA) – The lipid peroxidation inhibitory activity (LPIA) in rat brain homogenate was evaluated by the thiobarbituric acid (TBA)

method (Na *et al.*, 2002). The reaction mixture was composed of 10 μl of sample (DMSO), 740 μl of 50 mM phosphate buffer (pH 7.4), 50 μl of rat brain homogenate (10 mg protein/ml) and 200 μl of the free radical generating system: 0.1 mM $FeSO_47H_2O$ + 1 mM ascorbic acid. The reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by adding 250 μl of 20% TCA and 250 μl of 1% TBA (in 50 mM NaOH). After boiling at 95 °C for 5 min, the mixture was centrifuged at 10000 rpm for 10 min. The absorbance of supernatant was measured at 532 nm. LPIA (%) was expressed as follows:

$$\text{LPIA (\%)} = (A_{control} - A_{sample})/(A_{control} - A_{blank}) \times 100$$

Where, $A_{control}$ is the absorbance of control group, A_{sample} is the absorbance of test group and A_{blank} is the absorbance of blank to which the sample and the free radical generating system (Fe^{2+} /ascorbate) were not added.

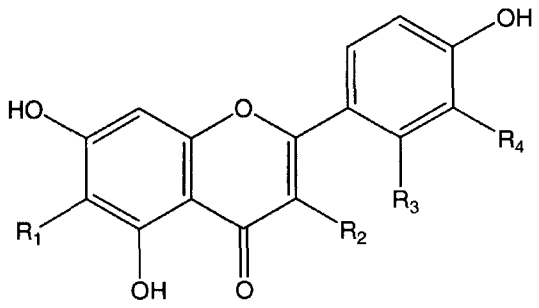
Results and Discussion

The MeOH extract of the twig of *M. alba* and its sequential solvent fractions were tested their LPIA. As a result, the hexane-soluble fraction and EtOAc-soluble fraction showed activity with IC_{50} value of 3.45 μM and 0.85 μM, respectively. Further investigation on these solvent fractions, three flavonoids and two stilbenes were isolated. Compound **1**, **2** and **5** showed greenish brown color in $FeCl_3$ test, and showed red color in HCl-Mg reaction. The structure of compound **1**, **2**, and **5** were identified as 6-geranylapigenin (Fuaki *et al.*, 1991), 6-geranylnorartocarpetin (Fuaki *et al.*, 1991) and quercetin (Kim *et al*, 1999), respectively, by comparison of their mp, UV, IR, Mass, and NMR spectral data with those reported in the literatures (Fig. 1). Compound **1** and **2** were isolated from the root bark of *M. alba*, but it is first isolated from the twig of the same plant.

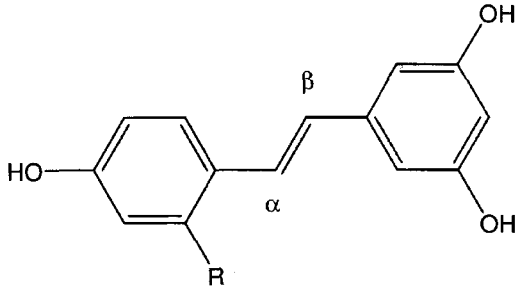
Compound **3** and **4**, isolated from the EtOAc fraction, showed different pattern in NMR spectrum. Compound **3** and **4** were determined as resveratrol (Ryu *et al.*,1994; Ko *et al.*,1998) and oxyresveratrol (Hirakura *et al.*,1985.), respectively, by comparison of their mp, UV, IR, Mass, and NMR spectral data with those reported in the literatures (Fig. 1).

In order to evaluate antioxidant effect of compounds **1-5**, *in vitro* lipid peroxidation tests on the rat brain homogenate had been performed. As shown in Table 1, compound **1**, **2**, exhibited moderate inhibitory activity with IC_{50} values of 3.37, 3.74 μM, respectively. **3**, **4** and **5** exhibited potent inhibitory activity with IC_{50} values of 0.23, 0.29 and 0.06 μM, respectively,. These compounds showed higher activity than α-tocopherol (IC_{50} 4.9 μM), which were used as positive control. Compounds **1** and **2** were isolated from hexane fraction and compounds **3**, **4**, and **5** were isolated from EtOAc fraction. The result is consistent with the corresponding to the preliminary screening of hexane (IC_{50} value, 3.45 μM) and EtOAc (IC_{50} value, 0.85 μM) fractions. The compound **3**, **4** and **5** exhibited potent activity in the lipid peroxidation inhibitory assay.

In this study, quercetin (**5**) exhibited the most potent activity. While, 6-geranylapigenin (**1**) and 6-geranylnorartocarpetin



1:R_1=geranyl, R_2=H, R_3=H, R_4=H
2: R_1=geranyl, R_2=H, R_3=OH, R_4=H
5: R_1=H, R_2=OH, R_3=H, R_4=OH



3: R=H
4: R=OH

Fig. 1. Structures of compound **1-5**.

Table 1. Antioxidant activities of compound 1-5 isolated from the twig of *Morus alba* L.

Compounds	Lipid peroxidation inhibitory activity IC_{50} [a] (M)
6-geranylapigenin (**1**)	3.37
6-geranylnorartocarpetin (**2**)	3.74
resveratrol (**3**)	0.23
oxyresveratrol (**4**)	0.29
quercetin (**5**)	0.06
α-tocopherol[b]	4.90

[a]IC_{50} values were calculated from regression lines using five different concentration in triplicate experiments.
[b]α-tocopherol is positive control.

(**2**) which has geranyl group at position C-6 showed negative effect on the lipid peroxidation inhibitory activity. This result indicated that flavone which has 5,7-dihydroxyl groups without geranyl group at C-6 is essential for the activity. In many antioxidant studies of flavonoids, a catchol or pyrogallol moiety in the B-ring and free hydroxyl group at position C-3 have been clarified to essential for a high antioxidant effect. In the case of stilbene derivatives, resveratrol (**3**) and oxyresverartol (**4**), both compounds also exhibited potent inhibitory activity with IC_{50} values of 0.23 and 0.29 μg/ml, but much less active than 3,3,4,5-tetrahydroxystilbene (data are not shown). Oxyresveratol (**4**) has one more hydroxyl group at *meta*-position, nevertheless, the activity was similar to that of compound **3**. Moreover, in the case of compound **2** substituted two hydroxyl group at *meta*-position in the B-ring of flavone, the activity was also similar to that of compound **1**. It is concluded that *meta*-dihydroxy groups of the B-ring do not influence to the antioxidant activity. Consequently, 5,7-dihydroxyphenolic groups in the A-ring and *ortho*-dihydroxy ones in the B-ring may be essential for the antioxidant activity.

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