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Antioxidative Constituents from *Lycopus lucidus*

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Three phenolic compounds, rosmarinic acid (**1**), methyl rosmarinate (**2**), ethyl rosmarinate (**3**), and two flavonoids, luteolin (**4**), luteolin-7-*O*-β-D-glucuronide methyl ester (**5**) were isolated from the aerial part of *Lycopus lucidus* (Labiatae). Their structures were determined by chemical and spectral analysis. Compounds **1-5** exhibited potent antioxidative activity on the NBT superoxide scavenging assay. The IC_{50} values for compounds **1-5** were 2.59, 1.42, 0.78, 2.83, and 3.05 μg/mL respectively. In addition, five compounds were isolated from this plant for the first time.

Key words: *Lycopus lucidus*, Antioxidant, NBT superoxide scavenging assay, Ethyl rosmarinate

INTRODUCTION

Lycopus lucidus Turcz. is a perennial plant belonging to Labiatae family growing in Korea and has been used in traditional medicine for the treatment of inflammation, disorders of menstruation, and an edema. Previous phytochemical studies on *Lycopus* species have been very rare and led to the isolation of a few triterpenoid, and one flavonoid (Do *et al.*, 1991a, 1991b). Moreover, no biological studies of *L. lucidus* have been carried out up to date.

In an ongoing investigation into biologically active compounds from natural products, an ethyl acetate soluble fraction of *L. lucidus* was found to inhibit superoxide radical generation significantly *in vitro*. By means of a bioassay-directed chromatographic separation technique, rosmarinic acid (**1**), methyl rosmarinate (**2**), ethyl rosmarinate (**3**), luteolin (**4**), and luteolin-7-*O*-β-D-glucuronide methyl ester (**5**) were isolated. Five compounds have been isolated from this plant for the first time. The antioxidative activity of the isolated compounds were tested by a NBT superoxide scavenging assay according to the established method (Kirby and Schmidt 1997). This paper reports the isolation and identification of the constituents of *L. lucidus* and their antioxidative activities.

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MATERIALS AND METHODS

General experimental procedure

Optical rotation was measured on an Autopol-IV polarimeter. NMR spectra were recorded on a Varian Unity Inova 500 spectrometer. ^1H-^1H COSY, DEPT, HMQC, and HMBC NMR spectra were obtained with the usual pulse sequences. FABMS were determined on a JMS 700 (JEOL). TLC and column chromatography were carried out on precoated Si Gel F_{254} plates (Merck, art. 5715), RP-18 F_{254} plates (Merck, art. 15423), and Si gel 60 (Merck, 70-230 and 230-400 mesh).

Plant material

The whole plant of *L. lucidus* (Labiatae) was purchased from local Korean herb drug market in Gwangju, Korea, on Nov. 2001, and was authenticated by Department of Pharmacognosy, Chosun University. Voucher specimens were deposited in the Herbarium of College of Pharmacy, Chosun University (993-16).

Extraction and isolation

L. lucidus (1.2 kg) was extracted with MeOH at room temperature to afford 179.0 g of residue. The methanol extract was suspended in water and then partitioned by dichloromethane (68.6 g), ethyl acetate (16.3 g), and *n*-butanol (10.9 g) in turn. Six gram of the EtOAc fraction were subjected to column chromatography over a silica gel eluting with a $CHCl_3$-MeOH-H_2O (40:1:0 → 20:1:0 → 10:1:0.1 → 8:1:0.1 → 5:1:0.1 → 3:1:0.1 → 1:1:0.1 → MeOH

only) gradient system. Fractions were combined based on their TLC pattern to yield subfraction designated as E1-E15. Subfraction E14 (1.62 g) was further purified by column chromatography over a silica gel eluting with a $CHCl_3$-MeOH-H_2O (3:1:0.1) to give five subfractions (E141-E145). Subfraction E143 (506.98 mg) was finally purified by repeated column chromatography over a silica gel, RP-18, sephadex LH 20 to afford compound **1** (32.75 mg). Subfraction E6 (135.33 mg) was further purified by column chromatography over a silica gel eluting with a $CHCl_3$-MeOH-H_2O (10:1:0.1) to afford three subfractions (E61-E63). Subfraction E62 (110 mg) was purified by column chromatography over a silica gel eluting with $CHCl_3$-MeOH (15:1) to afford three subfractions (E621-E623). Subfraction E623 (80.82 mg) was purified by repeated column chromatography over a silica gel, RP-18, and sephadex LH 20 to give compound **2** (21.09 mg), and **3** (2.59 mg), respectively. Subfraction E622 (6.5 mg) was purified by column chromatography over a sephadex LH 20 to afford compound **4** (2.25 mg). In addition, subfraction E13 (517.04 mg) was purified by repeated column chromatography over a silica gel, sephadex LH 20, RP-18 to afford compound **5** (3.12 mg).

Rosmarinic acid (1)

A yellow amorphous powder, $[\alpha]_D^{22}$ +78° (c 0.40, MeOH); FAB-MS: *m/z* 361 $[M+H]^+$; ^{1}H-NMR (CD_3OD, 500 MHz) δ: 7.51 (1H, d, *J* = 15.5 Hz, H-7'), 7.03 (1H, d, *J* = 2.0 Hz, H-2'), 6.91 (H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.77 (1H, dd, *J* = 8.0, 2.0 Hz, H-5'), 6.77 (1H, d, J = 2.0 Hz, H-2), 6.68 (1H, d, *J* = 8.0 Hz, H-5), 6.63 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.27 (1H, d, *J* = 15.5 Hz, H-8'), 5.09 (1H, dd, *J* = 10.0, 3.5 Hz, H-8), 3.10 (1H, dd, *J* = 14.5, 3.5 Hz, H-7a), 2.94 (1H, dd, *J* = 14.5, 10.0 Hz, H-7b); ^{13}C-NMR (CD_3OD, 125 MHz) δ: 177.64 (C-9), 169.24 (C-9'), 149.50 (C-4'), 146.85 (C-3'), 146.79 (C-7'), 146.08 (C-3), 144.93 (C-4), 131.29 (C-1), 128.12 (C-1'), 123.04 (C-6'), 121.89 (C-6), 117.63 (C-2), 116.60 (C-5'), 116.34 (C-5), 115.77 (C-8'), 115.27 (C-2'), 77.79 (C-8), 38.93 (C-7).

Methyl rosmarinate (2)

A yellow amorphous powder, $[\alpha]_D^{22}$ +98° (c 0.50, MeOH); FAB-MS: *m/z* 375 $[M+H]^+$; ^{1}H-NMR (CD_3OD, 500 MHz) δ: 7.55 (1H, d, *J* = 15.5 Hz, H-7'), 7.04 (1H, d, *J* = 2.0 Hz, H-2'), 6.95 (H, dd, *J* = 8.5, 2.0 Hz, H-6'), 6.78 (1H, d, *J* = 8.5 Hz, H-5'), 6.70 (1H, d, J = 2.0 Hz, H-2), 6.69 (1H, d, *J* = 8.0 Hz, H-5), 6.57 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.26 (1H, d, *J* = 15.5 Hz, H-8'), 5.19 (1H, dd, *J* = 7.5, 5.0 Hz, H-8), 3.70 (3H, s, OCH_3), 3.06 (1H, dd, *J* = 14.5, 5.5 Hz, H-7a), 3.00 (1H, dd, *J* = 14.5, 5.5 Hz, H-7b); ^{13}C-NMR (CD_3OD, 125 MHz) δ: 172.34 (C-9), 168.50 (C-9'), 150.10 (C-4'), 148.14 (C-7'), 147.03 (C-3'), 146.37 (C-3), 145.55 (C-4), 128.89 (C-1), 127.67 (C-1'), 123.38 (C-6'), 121.92 (C-6), 117.67 (C-2), 116.66 (C-5'), 116.45 (C-5), 115.34 (C-2'), 114.23 (C-8'), 74.82 (C-8), 52.82 (OCH_3), C-38.05 (C-7).

Ethyl rosmarinate (3)

A yellow amorphous powder, $[\alpha]_D^{22}$ +110° (c 0.50, MeOH); FAB-MS: *m/z* 389 $[M+H]^+$; ^{1}H-NMR (CD_3OD, 500 MHz) δ: 7.56 (1H, d, *J* = 16.0 Hz, H-7'), 7.05 (1H, d, *J* = 2.0 Hz, H-2'), 6.96 (H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.78 (1H, d, *J* = 8.0 Hz, H-5'), 6.72 (1H, d, J = 2.0 Hz, H-2), 6.70 (1H, d, *J* = 8.0 Hz, H-5), 6.58 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.27 (1H, d, *J* = 16.0 Hz, H-8'), 5.15 (1H, dd, *J* = 7.5, 6.0 Hz, H-8), 4.15 (2H, q, *J* = 7.5 Hz, $O\underline{CH_2}CH_3$), 3.31 (1H, dd, *J* = 14.0, 4.8 Hz, H-7a), 3.03 (1H, dd, *J* = 14.0, 6.6 Hz, H-7b), 1.21 (3H, t, *J* = 7.5 Hz, $OCH_2\underline{CH_3}$); ^{13}C-NMR (CD_3OD, 125 MHz) δ: 171.91 (C-9), 168.54 (C-9'), 150.07 (C-4'), 148.09 (C-7'), 147.03 (C-3'), 146.37 (C-3), 145.56 (C-4), 128.87 (C-1), 127.70 (C-1'), 123.37 (C-6'), 121.99 (C-6), 117.75 (C-2), 116.66 (C-5'), 116.44 (C-5), 115.36 (C-2'), 114.29 (C-8'), 74.95 (C-8), 62.57 ($O\underline{CH_2}CH_3$), 38.07 (C-7), 14.52 ($OCH_2\underline{CH_3}$).

Luteolin (4)

A yellow amorphous powder, FAB-MS: *m/z* 287 $[M+H]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 7.40 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.37 (1H, d, *J* = 2.0 Hz, H-2'), 6.87 (1H, d, *J* = 8.0 Hz, H-5'), 6.64 (1H, s, H-3), 6.42 (1H, d, *J* = 2.0 Hz, H-8), 6.16 (1H, d, *J* = 2.0 Hz, H-6); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 164.58 (C-2), 103.44 (C-3), 182.28 (C-4), 162.14 (C-5), 99.58 (C-6), 165.07 (C-7), 94.58 (C-8), 158.00 (C-9), 104.26 (C-10), 121.99 (C-1'), 113.91 (C-2'), 146.48 (C-3'), 150.64 (C-4'), 116.69 (C-5'), 119.69 (C-6').

Luteolin-7-*O*-β-D-glucuronide methyl ester (5)

A yellow amorphous powder, FAB-MS: *m/z* 477 $[M+H]^+$; ^{1}H-NMR (DMSO-d_6, 500 MHz) δ: 13.0 (1H, s, OH-5), 7.51 (1H, dd, J = 8.5, 2.0 Hz, H-6'), 7.48 (1H, d, *J* = 2.0 Hz, H-2'), 6.96 (1H, d, *J* = 8.5 Hz, H-5"), 6.88 (1H, d, *J* = 2.0 Hz, H-8), 6.81 (1H, s, H-3), 5.39 (1H, d, J = 7.5 Hz, H-1"), 4.27 (1H, d, J = 9.5 Hz, H-5"), 3.73 (3H, s, OCH_3), 3.30-3.60 (3H, m, Glu H-2", 3", 4"); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ: 181.86 (C-4), 169.19 (Glu-6"), 164.54 (C-2), 162.40 (C-7), 161.19 (C-5), 156.95 (C-9), 150.14 (C-4'), 145.83 (C-3'), 121.19 (C-1'), 119.19 (C-6'), 115.97 (C-5'), 113.50 (C-2'), 105.47 (C-10), 103.12 (C-3), 99.31 (C-6), 99.05 (Glu-1"), 94.50 (C-8), 75.37 (Glu-3"), 75.14 (Glu-5"), 72.72 (Glu-2"), 71.30 (Glu-4"), 51.98 (OCH_3).

NBT superoxide scavenging assay

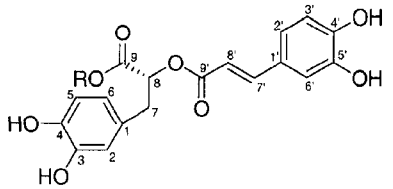
The NBT superoxide scavenging assay was carried out using a slight modification of the established method (Kirby and Schmidt 1997). The reaction mixture, which was equilibrated at 25°C, contained 20 μL of a 15 mM Na_2EDTA solution in a buffer (50 mM KH_2PO_4/KOH pH

7.4 in d.w.), 50 μL of 0.6 mM NBT in a buffer, 30 μL of a 3 mM hypoxanthine in 50 mM KOH solution, 50 μL of xanthine oxidase solution in a buffer (1 units in 10 mL buffer) and 100 μL of the sample. The plate reader (Molecular Devices Vmax) took readings every 20 *s* for 5 min at 570 nm. The control was 100 μL of 5% DMSO solution instead of the sample. Results were expressed as relative percentage inhibition to control, given by [(rate of control – rate of sample reaction)/rate of control] × 100. Allopurinol was used as a reference compound.

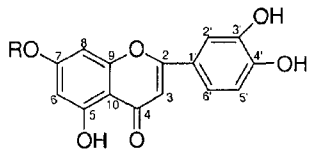
RESULTS AND DISCUSSION

Bioassay-guided chromatographic separation of an ethyl acetate soluble fraction of *L. lucidus* led to the isolation of compounds **1-5** (Fig. 1).

Compound **1** was obtained as a yellow amorphous powder. An $[M+H]^+$ peak at 361 in FAB-MS along with the analysis of ^{13}C-NMR, DEPT spectra showed its molecular formula to be $C_{18}H_{16}O_8$. The ^{1}H-NMR spectrum of **1** exhibited two groups of aromatic ABX type protons at δ 7.03 (1H, d, *J* = 2.0 Hz, H-2'), 6.91 (H, dd, *J* = 8.0, 2.0 Hz, H-6'), 6.77 (1H, dd, *J* = 8.0, 2.0 Hz, H-5'), 6.77 (1H, d, *J* = 2.0 Hz, H-2), 6.68 (1H, d, *J* = 8.0 Hz, H-5), 6.63 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), an olefinic protons at δ 7.51 (1H, d, *J* = 15.5 Hz, H-7'), 6.27 (1H, d, *J* = 15.5 Hz, H-8'), an oxygenated proton at δ 5.09 (1H, dd, *J* = 10.0, 3.5 Hz, H-8), and methylene protons at δ 3.10 (1H, dd, *J* = 14.5, 3.5 Hz, H-7a), δ 2.94 (1H, dd, *J* = 14.5, 10.0 Hz, H-7b). The ^{13}C-NMR spectrum showed four oxygenated phenyl carbons at δ 149.50 (C-4'), 146.85 (C-3'), 146.08 (C-3), and 144.93 (C-4), two esters carbons at δ 177.64 (C-9), and 169.24 (C-9'), a conjugated olefinic carbons at 146.79 (C-7'), and 115.77 (C-8'), a carbon bearing ester carbon at 77.79 (C-8). These results were further supported by HMQC spectrum. Based on the foregoing observations and a comparison of the data with the literature (Dapkevicius *et al.*, 2002; Hou *et al.*, 2002; Wang *et al.*, 1996; Okamura *et al.*, 1994), compound **1** was determined to be rosmarinic acid.



1 R = H (Rosmarinic acid)
2 R = CH_3 (Methyl rosmarinate)
3 R = CH_2CH_3 (Ethyl rosmarinate)



4 R = H (Luteolin)
5 R = glucuronide methyl ester
(Luteolin-7-*O*-β-D-glucuronide methyl ester)

Fig. 1. The chemical structures of compounds **1-5** isolated from *Lycopus lucidus*

Compound **2** was obtained as a yellow amorphous powder. An $[M+H]^+$ peak at 375 in FAB-MS along with the analysis of ^{13}C-NMR, DEPT spectra showed its molecular formula to be $C_{19}H_{18}O_8$. A comparison of the ^{1}H-, and ^{13}C-NMR spectral data with **1** and **2** revealed that both of compounds were much similar, except for the methoxyl proton at δ 3.70. Based on the analysis of the ^{1}H-, ^{13}C-NMR, HMQC, and HMBC spectrum, and a comparison of the data with the literature (Hou *et al.*, 2002; Kuo *et al.*, 2000; Wang *et al.*, 1996), compound **2** was determined to be methyl rosmarinate.

Compound **3** was obtained as a yellow amorphous powder. An $[M+H]^+$ peak at 389 in FAB-MS along with the analysis of ^{13}C-NMR, DEPT spectra showed its molecular formula to be $C_{20}H_{20}O_8$. In the ^{1}H-, and ^{13}C-NMR spectra, the signals were similar to those of compound **2**, except for the quartet ethyl proton at δ 4.15, and the triplet methyl proton at δ 1.21. Based on the NMR spectral evidences, and a comparison of the data with the literature (Hou *et. al.*, 2002), compound **3** was determined to be ethyl rosmarinate.

Compounds **4** and **5** were also identified as luteolin, and luteolin 7-*O*-β-D-glucuronide methyl ester, respectively, by comparing the MS, ^{1}H-NMR, and ^{13}C-NMR with those of reported in the literature (Schulz *et al.*, 1985; Matsuda *et al.*, 1995; Dapkevicius *et al.*, 2002; Lee *et al.*, 2002). Compounds **1-5** have been isolated from this plant for the first time.

The antioxidative activity of the isolated compounds were tested by a NBT superoxide scavenging assay and summarized in Table I. Compounds **1-5** showed similar potent antioxidative activity. The IC_{50} values for rosmarinic acid (**1**), methyl rosmarinate (**2**), ethyl rosmarinate (**3**), luteolin (**4**), and luteolin-7-*O*-β-D-glucuronide methyl ester (**5**) were 2.59, 1.42, 0.78, 2.83, and 3.05 μg/mL respectively. The antioxidative activities of these compounds were comparable to allopurinol, which was used as a positive control. Moreover, no clear difference in antioxidative activity was observed between rosmarinic acid and methyl, ethyl rosmarinate. These results suggest that the *ortho*-dihydroxy (catechol) structure play an important role in the antioxidative function as exhibited in flavonoids (Pietta 2000; Akdemir *et al.*, 2001), and the substitution of the free carboxylic acid in rosmarinic acid has no effect on the antioxidative activity.

These results also demonstrate that compounds **1-5**

Table I. Antioxidative activity of compounds **1-5** isolated from *L. lucidus*

Compounds	IC_{50} (μg/mL)
Rosmarinic acid (**1**)	2.59
Methyl rosmarinate (**2**)	1.42
Ethyl rosmarinate (**3**)	0.78
Luteolin (**4**)	2.83
Luteolin-7-*O*-β-D-glucuronide methyl ester (**5**)	3.05
Allopurinol*	1.70

*Used as a positive control

could be mainly responsible for the potent antioxidative effect of an ethyl acetate soluble fraction of *L. lucidus*, and might be suitable for further development as a leading natural antioxidant.

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REFERENCES

Akdemir, Z. Ş., Tati, İİ., Saraccoğlu, İ., İsmailoğlu, U. B., Şahin-Erdemli, İ., and Caliş, İ., Polyphenolic compounds from *Geranium pratense* and their free radical scavenging activities. *Phytochemistry*, 56, 189-193 (2001).

Dapkevicius, A., Beek, T. A., Lelyveld, G. P., Veldhuizen, A., Groot, A., Linssen, J. P. H., and Venskutonis, R., Isolation and structure elucidation of radical scavengers from *Thymus vulgaris* leaves. *J. Nat. Prod.*, 65, 892-896 (2002).

Do, J. C., Chai, J. Y., and Son, J. K., Studies on the constituents of *Lycopus lucidus* (I). *Kor. J. Pharmacogn.*, 22, 162-165 (1991a).

Do, J. C., Chai, J. Y., and Son, J. K., Studies on the constituents of *Lycopus lucidus* (II). *Kor. J. Pharmacogn.*, 22, 166-170 (1991b).

Hou, Z.-F., Tu, Y.-Q., and Li, Y., Three new phenolic compounds from *Nepeta prattii*. *J. Chin. Chem. Soc.*, 49, 255-258 (2002).

Kirby, A. J. and Schmidt, R. J., The antioxidant activity of Chinese herbs for eczema and of placebo herbs - I. *J. Ethnopharmacol.*, 56, 103-108 (1997).

Kuo, Y.-H., Lee, S.-M., and Lai, J.-S., Constituents of the whole herb of *Clinoponium laxiflorum*. *J. Chin. Chem. Soc.*, 47, 241-246 (2000).

Lee, M. H., Son, Y. K., and Han, Y. N., Tissue factor inhibitory flavonoids from the fruits of *Chaenomeles sinensis*. *Arch. Pharm. Res.*, 25, 842-850 (2002).

Matsuda, H., Cai, H., Kubo, M., Tosa, H., and Inuma, M., Study on anti-cataract drugs from natural sources. II. Effects on Buddlejae Flos on *in vitro* aldose reductase activity. *Biol. Pharm. Bull.*, 18, 462-466 (1995).

Okamura, N., Haraguchi, H., Hashimoto, K., and Yagi, A., Flavonoids in *Rosmarinus officinalis* leaves. *Phytochemistry*, 37, 1463-1466 (1994).

Pietta, P-G., Flavonoids as antioxidants. *J. Nat. Prod.*, 63, 1035-1042 (2000).

Schulz, M., Strack, D., Weissenböck, G., Markham, K. R., Dellamonica, G., and Chopin, J., Two luteolin *O*-glucuronides from primary leaves of *Secale cereale*. *Phytochemistry*, 24, 343-345 (1985).

Wang, Y., Ohtani, K., Kasai, R., and Yamasaki, K., Flavonol glycosides and phenolics from leaves of *Cordia dichotoma*. *Nat. Med.*, 50, 367-367 (1996).