Studies on the Constituents of *Leonotis nepetaefolia*

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Three antioxidative phenylethanoid glycosides and two new iridoid glycosides, along with three known iridoids were isolated from the African medicinal plant, *Leonotis nepetaefolia* R. BR. (Labiatae). The new iridoid glycosides were established as 10-*O*-(*trans*-3,4-dimethoxycinnamoyl)geniposidic acid and 10-*O*-(*p*-hydroxybenzoyl)geniposidic acid. Antioxidative activities of the compounds were measured by α,α-diphenyl-β-picrylhydrazyl (DPPH) method, and the three known phenylethanoid glycosides, acteoside, martynoside and lavandulifolioside showed strong antioxidative activity.

Key words *Leonotis nepetaefolia*; Labiatae; acylated geniposidic acid; antioxidative activity; phenylethanoid glycoside

The plant *Leonotis nepetaefolia* R. BR. (Labiatae) is widespread throughout the West Indies, South America, and the African continent, and has been attributed to have a variety of salutary physiological effects. Chemical and biological studies of this plant have led to the isolation of labdanoid diterpenoids[1] and coumarins.[2] This paper deals with the isolation of two minor iridoids, 10-*O*-(*trans*-3,4-dimethoxycinnamoyl)geniposidic acid (**1**) and 10-*O*-(*p*-hydroxybenzoyl)-geniposidic acid (**2**), along with geniposidic acid (**3**),[3] mussaenoside (**4**)[4] and ixoside (**5**)[5] and three known phenylethanoid derivatives (**6**—**8**)[6] having antioxidant activity from the dried stem of *L. nepetaefolia*.

Dried stems (560 g) of this plant were extracted with MeOH, and the extract was partitioned between $CHCl_3$: MeOH : H_2O (4 : 4 : 3). The upper layer was concentrated (25.2 g) and subjected to porous polymer gel Mitsubishi Diaion HP-20 column and the adsorbed materials were eluted with H_2O, 25% MeOH, 75% MeOH and MeOH, successively.

The 75% MeOH eluate (3.56 g) was subjected to column chromatography on Sephadex LH-20, and silica gel followed by Lober RP-8 column chromatography to give compounds **1** (0.001% yield) and **2** (0.002% yield), together with compounds **6** (0.008% yield), **7** (0.001% yield) and **8** (0.01% yield). Compounds **6**, **7** and **8** were identified as acteoside, martinoside and lavandulifolioside by comparison of the ^{1}H- and ^{13}C-NMR data with published values.[6]

The 25% MeOH eluate (2.20 g) was subjected to column chromatography on Sephadex LH-20, silica gel followed by Lober RP-8 column chromatography to give compounds **3** (0.007% yield), **4** (0.014% yield) and **5** (0.004% yield). These compounds were identified as geniposidic acid, mussaenoside and ixoside by comparison of their ^{1}H- and ^{13}C-NMR data with reported values.

Compound **1** was isolated as a colorless powder. The molecular formula of **1** was determined as $C_{27}H_{33}O_{13}$ ($[M+H]^+$; *m/z* 565.1917) by HR-FAB-MS. In the ^{1}H-NMR spectrum of **1** were two trisubstituted olefinic proton signals at δ 7.46 (s) and 5.88 (br s), which were assignable to H-3 and H-7 of an iridoid skeleton, respectively. The signals at δ 3.86 and 3.87 in the ^{1}H-NMR spectrum (each 3H, s) and those at δ 56.4 and 56.5 in the ^{13}C-NMR spectrum, showed the presence of two aromatic methoxyl groups. It showed two *trans* olefinic proton resonances at δ 6.46 and 7.66 (each 1H, d, J=15.9 Hz). Furthermore, the appearance of an ABX system at δ 6.98—7.24 ppm in the ^{1}H-NMR spectrum (J_{AB}=7.9 and J_{BX}=1.8 Hz) indicated the presence of a 3,4-dimethoxycinnamoyl group. The other ^{1}H-NMR spectral data were very similar to those of geniposidic acid (**3**) except for the signals of 10-CH_2 group. A downfield shift (0.64 ppm) of the signal due to H-10 suggested that a 3,4-dimethoxycinnamoyl group was attached to C-10 of **3**. Comparison of the ^{13}C-NMR spectrum of **1** with that of **3** showed that C-10 and C-8 were shifted downfield (+1.5 ppm) and upfield (−3.1 ppm), respectively. The 3,4-dimethoxycinnamoyl group was determined to be attached at the C-10 position due to the correlation between the proton signal of C-10 methylene protons (4.90 ppm) and carbonyl signal at δ 168.9 ppm observed in the heteronuclear multiple bond correlation spectroscopy

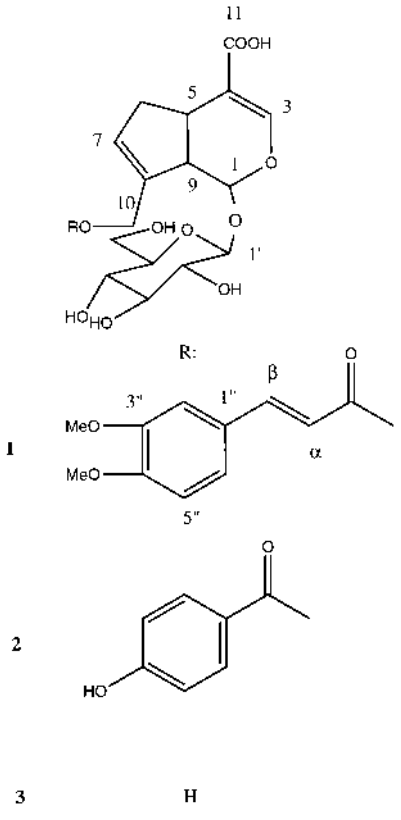

Chart 1

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Table 1. Radical Scavenging Effect of *Leonotis nepetaefolia* Extracts and Related Compounds on DPPH Radical

Sample	50% reduction (mg)	Sample	50% reduction (mg)
MeOH ext	0.085	Martinoside	0.008
Upper layer	0.065	Lavandulifolioside	0.006
Lower layer	0.091	Acteoside	0.004
100% MeOH eluate	0.027	BHT	0.017
75% MeOH eluate	0.010	α-Tocopherol	0.009
25% MeOH eluate	0.030		
H_2O eluate	2.480		

(HMBC) spectrum. To confirm the structure of **1** as 3,4-dimethoxycinnamoate, **1** was deacylated with methanolic potassium hydroxide. The products were identified as geniposidic acid and 3,4-dimethoxycinnamic acid by TLC and HPLC. The structure of **1** was thus determined to be 10-*O*-(*trans*-3,4-dimethoxycinnamoyl)geniposidic acid (**1**).

Compound **2** showed the same polarity and staining reaction (vanillin–H_2SO_4) as **1** on TLC. The molecular formula of **2** was determined from HR-FAB-MS evidence ($[M+H]^+$, *m*/*z* 495.1449). In the ^{1}H-NMR spectrum of **2**, two trisubstituted olefinic proton signals at δ 7.29 (s) and 5.88 (br s) were assignable, respectively, to H-3 and H-7 of an iridoid skeleton. Two doublets centered at δ 6.83 and 7.90 (each 2H, J=9.2 Hz) were assigned to the A_2B_2 system of the aromatic protons of a hydroxybenzoyl group. The other ^{1}H-NMR and ^{13}C-NMR spectral data were identical to those of **1**. A comparison with the ^{1}H-NMR spectrum of geniposidic acid (**3**) showed great similarity, except that the signals of the 10-CH_2 group were shifted downfield to δ 4.98 ppm in compound **2** as compared with δ 4.26 ppm in compound **3**. The ^{13}C-NMR spectrum of compound **2** showed 21 visible peaks. Of course, six could be assigned to a β-glucopyranosyl moiety. In the HMBC spectrum, long-range coupling ($^3J_{HCOC}$) was observed between the C-10 methylene proton signal at δ 4.98 and the carbon signal at δ 168.1 due to the *p*-hydroxylbenzoyl group. Together with the presence of the *p*-hydroxybenzoyl group, this suggested that C-10 was acylated. This structure was verified by alkaline hydrolysis of **2** to give *p*-hydroxybenzoic acid and geniposidic acid. Thus compound **2** is 10-*O*-(*p*-hydroxybenzoyl) geniposidic acid.

Active oxygen and free radical attacks biological molecules, leading to cancer, inflammation, atherosclerosis and aging.[7] For these reasons, antioxidants such as butylated hydroxytoluene (BHT), α-tocopherol, and ascorbic acid are widely used to protect food, and some of them are also used for the defense of biological molecules against oxidative damage.

The radical scavenging activity was determined using the stable α,α-diphenyl-β-picrylhydrazyl (DPPH) radical. As shown in Table 1, 100%MeOH and 75% MeOH eluate scavenged the DPPH radical.[8] Acteoside (**6**), martinoside (**7**) and lavandulifolioside (**8**) scavenged the DPPH radical to the same degree as BHT and α-tocopherol.

Experimental

^{1}H- and ^{13}C-NMR spectra with tetramethylsilane(TMS) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard were recorded on a JEOL A500 FT-NMR spectrometer. FAB-MS and HR-FAB-MS were measured on a JEOL JMS-SX102. UV spectra were measured on a Shimadzu UV-160A. TLC was conducted on precoated plates (Merck 60 F_{254} and RP-18_{254s}), was detected at UV (254 nm) and spraying with vanillin-H_2SO_4 reagent followed by heating. Column chromatography was carried out on silica gel (Merck Kiesel gel 60), Diaion HP-20 (Mitsubishi Chemical Industries, Ltd.) and Sephadex LH-20 (Pharmacia). Low pressure liquid chromatography was carried out on a column of Lober LiChroprep RP-8 pre-packed column (240×10 mm i.d., Merck). HPLC was carried out on a column of RP-18 (250×4.6 mm i.d., Merck).

Plant Material Stems of *Leonotis nepetaefolia* were collected in 1995 in Saitama, in the botanical garden herbary of Kyoritsu College of Pharmacy, and voucher specimens have been deposited at the herbarium of this College.

Extraction and Isolation Air-dried stems of the plant (560 g) were powdered and extracted with methanol (2×5 l) under reflux. The methanol extract was concentrated under reduced pressure and the residue (35.6 g) was partitioned with $CHCl_3$–MeOH–H_2O (4 : 4 : 3) and the upper layer was concentrated *in vacuo* to give a residue (25.2 g). The residue was subjected to Diaion HP-20 column chromatography eluting with H_2O, 25% MeOH, 75% MeOH and MeOH.

The 25% MeOH eluate was chromatographed on Sephadex LH-20 (MeOH), silica gel ($CHCl_3$–MeOH–H_2O, 7 : 3 : 1—6 : 4 : 1) and Lober RP-8 (MeOH–H_2O, 4 : 6—6 : 4) to afford geniposidic acid (**3**) (36.5 mg), mussaenoside (**4**) (80.1 mg) and ixoside (**5**) (20.1 mg). 75% MeOH eluate was fractionated over Sephadex LH-20 (EtOH), silica gel ($CHCl_3$–MeOH–H_2O, 7 : 3 : 1) and Lober RP-8 (MeOH–H_2O, 2 : 8—1 : 0) column chromatography. The fractions were finally purified on Sephadex LH-20 (EtOH–H_2O, 1 : 1) and HPLC (MeOH–H_2O, 45 : 55) to give acteoside (**6**) (42.2 mg), martynoside (**7**) (6.7 mg), lavandulifolioside (**8**) (56.7 mg), 10-*O*-(*trans*-3,4-dimethoxycinnamoyl)geniposidic acid (**1**) (5.6 mg) and 10-*O*-(*p*-hydroxybenzoyl)geniposidic acid (**2**) (9.6 mg).

10-*O*-(*trans*-3,4-Dimethoxycinnamoyl)geniposidic Acid (1): A colorless powder, $[\alpha]_D^{25}$ −29.4° (c=0.19, MeOH). UV λ_{max}^{MeOH} nm (log ε)) 236 (4.08), 288 (2.45), 320 (2.61). FAB-MS *m*/*z*: 565$[M+H]^+$, HR-FAB-MS *m*/*z*: Calcd for $C_{27}H_{33}O_{13}$: 565.1920, Found: 565.1917. ^{1}H-NMR (CD_3OD) δ: 2.10 (1H, m, H-6eq), 2.78 (1H, m, H-9), 2.90 (1H, m, H-6ax), 3.20 (1H, m, H-5), 3.86, 3.87 (6H, s, OMe), 4.73 (1H, d, J=7.9 Hz, H-1′), 4.90 (2H, m, H-10), 5.17 (1H, d, J=7.3 Hz, H-1), 5.88 (1H, brs, H-7), 6.46 (1H, d, J=15.9 Hz, H-α), 6.98 (1H, d, J=7.9 Hz, H-5″), 7.18 (1H, dd, J=7.9, 1.8 Hz, H-6″), 7.24 (1H, d, J=1.8 Hz, H-2″), 7.46 (1H, s, H-3), 7.66 (1H, d, J=15.9 Hz, H-β). ^{13}C-NMR (CD_3OD) δ: aglycone moiety; 37.4 (C-5), 40.2 (C-6), 47.7 (C-9), 63.9 (C-10), 98.1 (C-1), 117.9 (C-4), 131.4 (C-7), 139.9 (C-8), 152.9 (C-3), 174.2 (C-11), cinnamoyl moiety; 111.6 (C-2″), 112.7 (C-5″), 116.5 (C-α), 124.1 (C-6″), 128.9 (C-1″), 146.6 (C-β), 150.3 (C-4″), 150.8 (C-3″), 168.9 (CO), 56.4, 56.5 (OMe), sugar moiety; 100.5 (C-1′), 75.0 (C-2′), 78.0 (C-3′), 71.5 (C-4′), 78.3 (C-5′), 62.8 (C-6′).

10-*O*-(*p*-Hydroxybenzoyl)geniposidic Acid (2): Amorphous powder, $[\alpha]_D^{25}$ −18.7° (c=0.31, MeOH). UV λ_{max}^{MeOH} nm (log ε) 236 (3.64), 284 (1.82), 320 (1.89). FAB-MS *m*/*z*: 495 $[M+H]^+$, HR-FAB-MS *m*/*z*: Calcd for $C_{23}H_{27}O_{12}$: 495.1502, Found: 495.1449. ^{1}H-NMR (CD_3OD) δ: 2.14 (1H, m, H-6eq), 2.77 (1H, m, H-9), 2.89 (1H, m, H-6ax), 3.20 (1H, m, H-5), 4.72 (1H, d, J=7.9 Hz, H-1′), 4.98 (2H, m, H-10), 5.14 (1H, d, J=7.3 Hz, H-1), 5.88 (1H, br s, H-7), 6.83 (2H, d, J=9.2 Hz, H-3″, 5″), 7.29 (1H, s, H-3), 7.90 (2H, d, J=9.2 Hz, H-2″, 6″). ^{13}C-NMR (CD_3OD) δ: aglycone moiety; 37.3 (C-5), 40.2 (C-6), 47.9 (C-9), 64.1 (C-10), 98.1 (C-1), 116.9 (C-4), 131.3 (C-7), 140.0 (C-8), 150.3 (C-3), 174.0 (C-11), benzoyl moiety; 116.3 (C-3″, 5″), 122.3 (C-1″), 132.9 (C-2″, 6″), 163.7 (C-4″), 168.1 (CO), sugar moiety; 100.6 (C-1′), 75.0 (C-2′), 78.0 (C-3′), 71.5 (C-4′), 78.3 (C-5′), 62.8 (C-6′).

Alkaline Hydrolysis of 1 and 2 A solution of **1** or **2** (each 1 mg) in 2% KOH (5 ml) was kept at room temperature for 1 h. Each mixture was neutralized with 2 N HCl and partitioned with Et_2O. From the Et_2O layer, 3,4-dimethoxycinnamic acid or *p*-hydroxybenzoic acid was detected by TLC ($CHCl_3$–MeOH, 9 : 1). From the aqueous layer, geniposidic acid was detected by TLC ($CHCl_3$–MeOH–H_2O, 7 : 3 : 1, lower layer).

Geniposidic Acid (3): Colorless amorphous powder, ^{1}H-NMR (D_2O): δ 4.26 (2H, m, H-10), 4.81 (1H, d, J=7.9 Hz, H-1′), 5.30 (1H, d, J=7.0 Hz, H-1), 5.85 (1H, br s, H-7), 7.33 (1H, s, H-3). ^{13}C-NMR (D_2O) δ: aglycone moiety; 35.2 (C-5), 38.9 (C-6), 46.6 (C-9), 61.6 (C-10), 99.7 (C-1), 112.8 (C-4), 129.6 (C-7), 142.4 (C-8), 153.0 (C-3), 171.8 (C-11), sugar moiety; 97.9 (C-1′), 73.6 (C-2′), 76.5 (C-3′), 70.3 (C-4′), 76.9 (C-5′), 60.5 (C-6′).

Measurement of DPPH Radical Reducing Activity The sample dissolved in 0.1 M acetic acid buffer solution (pH 5.5, 2.0 ml) and EtOH solution (2.0 ml) was added to DPPH radical EtOH solution (1×10^{-4} M, 1.0 ml) in a 10 ml plastic dispocell 1938 PS (Kartell, Italy). After 30 minutes of the

incubation at 20 °C, the absorbance was measured at 517 nm using a Hitachi U-3000 spectrophotometer.

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