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Phytochemical Constituents from the Seeds of *Lithospermum erythrorhizon*

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Abstract – Phytochemical constituents were isolated from the seeds of *Lithospermum erythrorhizon* through open column chromatography and prep-HPLC. Their structures were identified as β-sitosterol (**1**), daucosterol (**2**), luteolin (**3**), and allantoin (**4**) on the basis of spectroscopic analysis. Among them, luteolin (**3**) was isolated for the first time from the plant.
Keywords – *Lithospermum erythrorhizon*, Boraginaceae, Column chromatography, Constituent, Luteolin

Introduction

Lithospermum erythrorhizon (Boraginaceae) is a perennial herbaceous species which is found in mountains and fields in Korea, Japan and China. Red pigments of this plant accumulate in the cork layers of the roots. The plant grows up to 0.7 m. Its floral season is June to September (Tabata, 1996; Cho *et al.*, 1999a; Lee, 2006). It has been used medicinally for wounds (Touno *et al.*, 2005), burns, and haemorrhoids (Tabata *et al.*, 1974) and as a dye for staining fabrics and food colorants (Lee *et al.*, 2000). It possesses a wide spectrum of biological properties, including anti-tumor, anti-fungus, anti-HIV (Han *et al.*, 2008), anti-inflammatory (Kang *et al.*, 1998), anti-microbial, immunostimulating and contraceptive (Shen *et al.*, 2002).

The color components of *L. erythrorhizon* roots are composed of shikonin and its derivatives, such as deoxyshikonin, acetylshikonin, isobutylshikonin, β,β-dimethylacrylshikonin, isovalerylshikonin, tetracrylshikonin, β-hydroxyisovalerylshikonin, α-methyl-*n*-butylshikonin, and propionylshikonin (Cho *et al.*, 1999b; Morimoto *et al.*, 1965; Zhang *et al.*, 2002). Also, lithospermic and rosmarinic acids (Fukui *et al.*, 1984), and lithosperman B (Yamamoto *et al.*, 2000) have been isolated from *L. erythrorhizon* roots.

Up to now, there have been many reports on the isolation and identification of compounds from *L. erythrorhizon* roots and on their medicinal effects, but no similar reports on compounds from *L. erythrorhizon* seeds. During the course of our continued studies on the compounds from *L. erythrorhizon* seeds, four compounds are isolated. This paper deal with the isolation and structure elucidation of compounds **1 - 4**.

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Experimental

Plant material – The seeds of *Lithospermum erythrorhizon* were collected from Jeongseon (Oct. 2007), Kangwon Province, Korea, and verified by Prof. Young-Hee Ahn, Chung-Ang University, Korea.

General procedures – The electron ionization mass spectrometry (EI-MS) was measured with a JEOL JMS-600W (Japan) mass spectrometer. ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AVANCE 300 NMR (Germany) spectrometer in $CDCl_3$, pyridine or DMSO using TMS as an internal standard. Chemical shifts were reported in parts per million (δ), and coupling constants (*J*) were expressed in hertz. TLC analysis was conducted with Kiesel gel 60 F254 (Art. 5715, Merck Co., Germany) plates (silica gel, 0.25 mm layer thickness), with compounds visualized by spraying with 10% H_2SO_4 followed by charring. Open column chromatography was conducted with silica gel (200 - 400 mesh ASTM; Merck Co., Germany). All other chemicals and reagents were analytical grade. Recycling preparative HPLC was conducted by a JAI LC-9104 (Japan) system and determination was performed by an L-6050 system pump with UV-3702 system UV/VIS detector.

Extraction and isolation – The air-dried whole seeds of *L. erythrorhizon* (4,695 g) were ground into powder and extracted with MeOH (10 liters × 3) under reflux. The resultant extracts were combined and concentrated under reduced pressure to afford 325 g of the residue. The MeOH extract was suspended in water and then fractionated successively with equal volumes of *n*-hexane (230 g), $CHCl_3$ (9 g), EtOAc (4 g) and *n*-BuOH (23 g). A portion of the $CHCl_3$ fraction (8.0 g) was chromatographed on a silica gel column eluting with a gradient of *n*-hexane-EtOAc (100% *n*-hexane up to 80% EtOAc) to afford ten subfractions (subfrs. C1-C10). Among them, the subfrs. C3 and C10 were chromatographed on a silica gel column eluting with *n*-hexane : EtOAc to give compounds **1** (*n*-hexane : EtOAc, 97 : 3) and **2** (*n*-hexane-EtOAc, 4 : 6), respectively. A portion of the EtOAc fraction (3.0 g) was chromatographed on a silica gel column eluting with a gradient of *n*-hexane : EtOAc (100% *n*-hexane up to 100% EtOAc) and EtOAc : MeOH (EtOAc : MeOH mixture of increasing polarity) to afford 12 subfractions (subfrs. E1- E12). The subfr. E12 were eluted within 42 min by prep-HPLC using a JAIGEL-GS column with UV/VIS detection set at 245 nm. The mobile phase was $CHCl_3$-MeOH (80 : 20, v/v) at ambient temperature. A prep-HPLC separation of the subfrs. C9 and E12 led to the isolation of compound **3**. A portion of the *n*-BuOH fraction (10.0 g) was chromatographed on a silica gel column eluting with a gradient of $CHCl_3$: MeOH (100% $CHCl_3$ up to 80% MeOH) to afford 11 subfractions (subfrs. B1 - B11). The subfr. B7 was chromatographed on a silica gel column eluting with $CHCl_3$: MeOH (6 : 4) to give compound **4**.

Compound **1**: white powder; R_f = 0.24 (*n*-hexane : EtOAc = 8 : 2); EI-MS *m/z*: 414 $[M]^+$ (100.0), 396 (53.1), 381 (25.8), 329 (28.3), 303 (31.0), 289 (11.8), 273 (20.8), 255 (26.3), 231 (15.8), 213 (23.2), 159 (17.0), 145 (21.4); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 3.52 (1H, m, H-3), 2.27 (2H, m, H-4), 5.35 (1H, m, H-6), 1.99 (2H, m, H-11), 0.68 (3H, s, H-18), 1.01 (3H, s, H-19), 0.96 (3H, d, *J* = 6.3 Hz, H-21), 0.83 (3H, d, *J* = 6.3 Hz, H-26), 0.80 (3H, d, *J* = 3.3 Hz, H-27), 0.91(3H, t, *J* = 6.3 Hz, H-29); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 37.4 (C-1), 29.8 (C-2), 72.0 (C-3), 39.9 (C-4), 141.1 (C-5), 122.2 (C-6), 32.0 (C-7), 31.8 (C-8), 50.3 (C-9), 36.6 (C-10), 21.2 (C-11), 40.7 (C-12), 42.4 (C-13), 56.9 (C-14), 24.4 (C-15), 28.4 (C-16), 56.2 (C-17), 11.9 (C-18), 19.1 (C-19), 36.3 (C-20), 18,9 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.3 (C-25), 19.9 (C-26), 19.5 (C-27), 23.2 (C-28), 12.1 (C-29).

Compound **2**: white powder; R_f = 0.61 ($CHCl_3$: MeOH : H_2O = 8 : 2 : 0.2); FAB-MS *m/z*: 577 $[M+H]^+$; ^{1}H-NMR (300 MHz, pyridine): δ 5.37 (1H, m, H-6), 0.67 (3H, s, H-18), 0.95 (3H, s, H-19), 1.00 (3H, d, *J* = 6.3 Hz, H-21), 0.94 (3H, d, *J* = 5.7 Hz, H-26), 0.90 (3H, d, *J* = 6.9 Hz, H-27), 0.89 (3H, m, H-29), 5.00 (1H, d, *J* = 7.8 Hz, H-1'); ^{13}C-NMR (75 MHz, pyridine): δ 36.8 (C-1), 29.3 (C-2), 78.7 (C-3), 38.3 (C-4), 140.4 (C-5), 121.2 (C-6), 31.4 (C-7), 31.3 (C-8), 49.6 (C-9), 36.2 (C-10), 20.6 (C-11), 40.1 (C-12), 41.8 (C-13), 56.2 (C-14), 23.9 (C-15), 27.8 (C-16), 55.1 (C-17), 11.7 (C-18), 19.1 (C-19), 35.5 (C-20), 18,6 (C-21), 33.3 (C-22), 25.4 (C-23), 45.1 (C-24), 28.7 (C-25), 18.9 (C-26), 19.7 (C-27), 22.6 (C-28), 11.8 (C-29), 100.8 (C-1'), 73.5 (C-2'), 76.9 (C-3'), 70.1 (C-4'), 76.7 (C-5'), 61.1 (C-6').

Compound **3**: yellow powder; R_f = 0.56 ($CHCl_3$: MeOH = 8 : 2); EI-MS *m/z*: 286 $[M]^+$ (100.0), 258 (12.2), 153 (18.5), 134 (6.8), 129 (9.4); ^{1}H-NMR (300 MHz, DMSO): δ 6.67 (1H, s, H-3), 6.19 (1H, d, *J* = 2.0 Hz, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 7.33 (1H, d, *J* = 2.1 Hz, H-2'), 6.94 (1H, d, *J* = 8.1 Hz, H-5'), 7.42 (1H, dd, *J* = 2.1, 8.1 Hz, H-6'), 12.98 (1H, s, 5-OH). ^{13}C-NMR (75 MHz, DMSO): δ 163.9 (C-2), 103.7 (C-3), 181.6 (C-4), 161.5 (C-5), 98.8 (C-6), 164.1 (C-7), 93.8 (C-8), 157.3 (C-9), 103.7 (C-10), 119.0 (C-1'), 113.4 (C-2'), 145.7 (C-3'), 149.7 (C-4'), 116.0 (C-5'), 121.5 (C-6').

Compound **4**: white crystal; R_f = 0.53 ($CHCl_3$: MeOH = 45:55); EI-MS *m/z*: 158 $[M]^+$ (9.8), 141 (10.9), 130 (100.0), 115 (31.4), 87 (80.8), 70 (10.8), 60 (22.4); ^{1}H-NMR (300 MHz, DMSO): δ 10.56 (1H, s, 1-NH), 8.07 (1H, s, 3-NH), 5.23 (1H, d, *J* = 8.1 Hz, H-4), 6.90 (1H, d, *J* = 8.1 Hz, 6-NH), 5.80 (2H, s, 8-NH); ^{13}C-NMR (75 MHz, DMSO): δ 156.9 (C-2), 62.5 (C-4), 173.7 (C-5), 157.4 (C-7).

Results and Discussion

A chromatographic separation of the MeOH extracts from the seed of *L. erythrorhizon* led to the isolation of compounds **1 - 4** (Fig. 1).

Compound **1** was obtained as white powders from the $CHCl_3$ fraction and it showed a molecular ion peak at *m/z* 414 $[M]^+$ in the EI-MS. The ^{1}H-NMR spectrum of **1** showed existence of sterol skeleton. The two angular methyl singlets of 18- and 19-Me at δ 0.68 and 1.01, and the three doublets of 21-, 26-, and 27-Me at δ 0.96, 0.83 and 0.80, and the one triplet of 29-Me at δ 0.91 were observed, respectively. The olefinic proton broad doublet one signal at δ 5.35 was showed H-6. The ^{13}C-NMR spectrum of **1** showed 27 resonances, and C-5 and -6 signals were noticed at δ 141.1 and 122.2, respectively. Accordingly, the structure of **1** was elucidated as β-

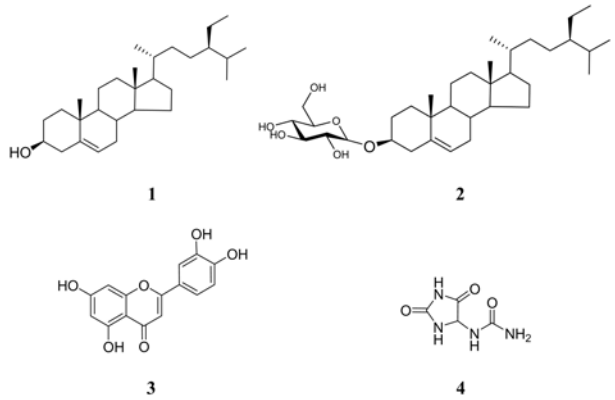

Fig. 1. Structures of compounds **1** - **4**.

sitosterol (stigmast-5-en-3-ol) by comparison of the spectral data in the literature (Rubinstein *et al*., 1976).

Compound **2** was obtained as white powders from the $CHCl_3$ fraction and it showed a molecular ion peak at *m/z* 577 $[M+H]^+$ in the FAB-MS. The ^{1}H-NMR spectrum of **2** showed the existence of sterol skeleton similar to compound **1**. The angular methyl two singlets of 18- and 19-Me at δ 0.67 and 0.95, and the three doublets of 21-, 26-, and 27-Me at δ 1.00, 0.94 and 0.90 were observed, respectively. The olefinic proton broad singlet one signal at δ 5.37 was showed H-6. The signals of δ 3.00-5.00 showed glycoside. And glucose position was at C-3 (β-linkage) of aglycone by the anomeric proton at δ 5.00 (d, J = 7.8 Hz). The ^{13}C-NMR spectrum of **2** showed 33 resonances, and C-5 and -6 signals were noticed at δ 140.4 and 121.2, respectively. Accordingly, the structure of **2** was elucidated as daucosterol (β-sitosterol-3-*O*-β-D-glucopyranoside) by comparison of the spectral data in the literature (Chang *et al*., 1981).

Compound **3** was obtained as yellow powder from the EtOAc fraction and it showed a molecular ion peak at *m/z* 286 $[M]^+$ in the EI-MS. The ^{1}H-NMR spectrum of **3** showed the typical flavonoid signals. It is showed the presence of two singlet signals at δ 6.67 (s, H-3) and 12.98 (s, 5-OH). The proton signals at δ 7.42 (dd, J = 2.1, 8.1 Hz, H-6'), 7.33 (d, J = 2.1 Hz, H-2') and 6.94 (d, J = 8.1 Hz, H-5') showed ABX splitting pattern of B ring, and *meta*-coupling of two doublets at δ 6.19 (d, J = 2.0 Hz, H-6) and 6.44 (d, J = 2.0 Hz, H-8) in A ring were observed. The ^{13}C-NMR spectrum of **3** showed 15 resonances, and C-2 signal (C=O) was noticed at δ 163.9. Accordingly, the structure of **3** was elucidated as luteolin (5,7,3',4'-tetrahydroxyflavone) by comparison of the spectral data in the literature (Ternai and Markham, 1976).

Compound **4** was obtained as white crystals from the *n*-BuOH fraction and it showed a molecular ion peak at *m/z* 158 $[M]^+$ in the EI-MS. The ^{1}H-NMR spectrum of **4** showed alkaloid compound. It curve was obtained relating the peak height of the down-field proton at δ 10.56. The broad signal at δ 5.80 was showed amino signal. The ^{13}C-NMR spectrum of **4** showed three carbonyl groups at δ 156.9, 157.4 and 173.7. Accordingly, the structure of **4** was elucidated as allantoin (2,5-dioxo-4-imidazolidinylurea) by comparison of the spectral data in the literature (Drewes and Staden, 1975).

To the best of our knowledge, this is the first report on the isolation of luteolin (**3**) from *L. erythrorhizon*.

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