韓藥作誌(Korean J. Medicinal Crop Sci.) 18(5) : 311 – 314 (2010)

HPLC를 이용한 지치중의 부위별 Luteolin 정량

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Determination of Luteolin in the Different Parts of *Lithospermum erythrorhizon* by HPLC

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ABSTRACT : The content of luteolin in the different parts of *Lithospermum erythrorhizon* was determined by high performance liquid chromatography (HPLC). Luteolin was quantified by a reverse-phase column with gradient solvent program (water : ACN = 90 : 10 to 77 : 23 for 60 min). UV detection was conducted at 330 ㎚. The content of luteolin was measured in the seeds (0.28 ㎎/g), stems (0.30 ㎎/g), and roots (0.16 ㎎/g) of *L. erythrorhizon*. The presence of luteolin in *Lithospermum* species is important in agricultural crop production for increasing the amounts of clinically available medicine and health supplements.

Key Words **:** *Lithospermum erythrorhizon*, Boraginaceae, Quantitative Analysis, Luteolin

INTRODUCTION

The genus *Lithospermum*, which is perennial plant belonging to the Boraginaceae family, distributed in Korea, Japan, and China. Among the genus, three species such as *L. erythrorhizon*, *L. arvense*, and *L. zollingeri* are spontaneously grown in Korea. Traditionally, the red pigments in *L. erythrorhizon* roots have been used as a dye for staining fabrics and food colorants (Lee *et al*., 2000). Also, it has been used medicinally for wounds (Touno *et al*., 2005), burns, and haemorrhoids (Tabata *et al*., 1974).

Many studies have shown that *Lithospermum* species exhibits a variety of pharmacological activities such as anti-tumor, anti-fungal, anti-HIV (Han *et al*., 2008), anti-inflammatory (Kang *et al*., 1998), anti-microbial, immunos-timulating, and contraceptive (Shen *et al*., 2002). The extracts from *L. erythrorhizon* are reported to have antioxidant and antitumor effects (Yingming *et al*., 2004). The plant has been widely used as various materials in Korea. The color components of *L. erythrorhizon* roots such as deoxyshikonin, acetylshikonin, isobutylshikonin, isovalerylshikonin, tetracrylshi-konin, and propionylshikonin (Cho *et al*., 1999; Morimoto *et al*., 1965; Hwang *et al*., 2002; Zhang *et al*., 2002) are reported to various effects (Chen *et al*., 2003; Ishida and Sakaguchi, 2007; Kim *et al*., 2006; Kuroda *et al*., 1997; Yamasaki *et al*., 1993; You *et al*., 2000).

In previous papers, we reported the isolation of compounds such as two sterols, two phenolics, and an alkaloid from the plant (Park *et al*., 2009a; Park *et al*., 2009b). Among them, luteolin, a flavonoid, was isolated for the first time from *L. erythrorhizon*. It is clear that luteolin plays a role in suppressing the protein expression of iNOS and COX-2, two critical inducible enzymes responsible for the production of nitric oxide PGE_2 (Hu and Kitts, 2004). Therefore, the determination of the part-specific preponderance of luteolin in *L. erythrorhizon* was performed by HPLC.

MATERIALS AND METHODS

1. Plant materials

The seeds, stems, and roots of *L. erythrorhizon* were collected from Jeongseon (Oct. 2007), Gangwon Province,

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Received 2010 August 11 / 1st Revised 2010 October 8 / 2nd Revised 2010 October 11 / Accepted 2010 October 13

Korea. The plant is verified by Prof. Young-Hee Ahn, Chung-Ang University, Korea.

2. Instruments and reagents

The mass spectrometry (MS) was measured with a Jeol JMS-AX505WA mass spectrometer (Japan). ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded with a Bruker AVANCE 300 NMR spectrometer (Germany) in DMSO using TMS as an internal standard. HPLC chromatograms were recorded with a GILSON 305 system pump with a GILSON 188 system UV/VIS detector (France). Water and ACN used in this research were of HPLC grade, and all other reagents were of analytical grade.

3. Preparation of luteolin

The air-dried whole seeds of *L. erythrorhizon* (4,695 g) were ground into powder and extracted with MeOH (1,000 mℓ × 3) under reflux. The MeOH extract was suspended in water, and then fractionated successively with equal volumes of *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH (yield: 230, 9, 4, and 23 g, respectively). Among them, a portion of the *n*-BuOH fraction (10.0 g) was chromatographed on a silica gel column (No. 7734, 6 × 80 ㎝) using a stepwise gradient of $CHCl_3$ and MeOH solvent system to yielded 11 sub-fractions. Luteolin was isolated from the sub-fr. 7 (60% MeOH) after recrystallization with MeOH.

Luteolin: Yellow powder; R_f= 0.56 ($CHCl_3$: MeOH = 8 : 2); EI-MS *m/z* 286 $[M]^+$ (100), 258 (12.2), 153 (18.5), 134 (6.8), 129 (9.4); ^{1}H-NMR (300 MHz, DMSO) δ_H 12.98 (1H, s, 5-OH), 10.44 (1H, s, 7-OH), 9.70 (1H, s, 3'-OH), 9.41 (1H, s, 4'-OH), 7.43 (1H, dd, *J* = 8.0, 2.0 Hz, H-6'), 7.39 (1H, d, *J* = 2.0 Hz, H-2'), 6.90 (1H, d, *J* = 8.0 Hz, H-5'), 6.67 (1H, s, H-3), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.19 (1H, d, *J* = 2.0 Hz, H-6); ^{13}C-NMR (75 MHz, DMSO) δ_C 181.6 (C-4), 164.1 (C-7), 163.9 (C-2), 161.4 (C-5), 157.3 (C-9), 149.7 (C-4'), 145.7 (C-3'), 121.5 (C-6'), 119.0 (C-1'), 116.0 (C-5'), 113.7 (C-2'), 104.3 (C-10), 102.9 (C-3), 98.8 (C-8), 93.8 (C-6).

4. Sample preparation

For the quantitative analysis of luteolin in different parts of *L. erythrorhizon*, each 60.0 g was minced. The seeds, stems, and roots of *L. erythrorhizon* were extracted with methanol under reflux and removed to yielded crude extracts. The resultant solution was filtered through Whatman 0.45 ㎛ PVDF syringe filter (Cat No. 6779, USA) prior to HPLC.

5. HPLC condition

Qualitative and quantitative analysis of luteolin was performed using a HPLC system. Determination was achieved on a reversed phase column (Watchers 100 ODS, 4.6 × 250 ㎜, 5 ㎛, Daiso, Japan) and gradient solvent system (water : ACN) was employed as the mobile phase. The elution program was 90 : 10 initially, increased in linear gradients to 77 : 23 for 60 min. The flow rate was kept constant at 1.0 mℓ/min and the peaks were identified using UV absorbance at 330 nm. Injection volume is 20 ㎕ of the prepared methanol solutions. All solvents for HPLC analysis were degassed before use. HPLC analyses were done in triplicates.

6. Calibration

The standard stock solution (1 ㎎/300 ㎕) of luteolin isolated the seeds of *L. erythrorhizon* was prepared in MeOH, and blended with same solvent. This solution was diminished of content to 50% to different concentration. The luteolin levels were ascertained by comparing the integrated peak areas of the individual compounds with that of a standard curve prepared from the corresponding standards. The calibration functions are calculated with peak area (Y), concentration (X, ㎎/㎕), and mean values (n = 4).

RESULTS AND DISCUSSION

A chromatographic separation of the MeOH extract from the seeds of *L. erythrorhizon* led to the isolation of luteolin (Fig. 1). In a previous paper, the presence of luteolin in the seeds of *L. erythrorhizon* was identified by NMR and MS spectroscopy (Park *et al*., 2009b). The typical flavonoid signals were identified in the ^{1}H-NMR spectrum. The singlet at δ 12.98 and 10.44 showed the aromatic 5-OH and 7-OH of the flavonoid A-ring. The proton signals at δ 6.90 (dd, *J* = 8.0 Hz, H-5'), 7.39 (d, *J* = 2.0 Hz, H-2') and 7.43 (d, *J* = 8.0, 2.0 Hz, H-6') showed the ABX splitting type of the flavonoid B-ring. The ^{13}C-NMR spectrum showed C = O at δ 181.6. It showed molecular ion peak at *m/z* 286 $[M]^+$ in the EI-MS, which expect to a molecular formula of $C_{15}H_{10}O_6$. Accordingly, the structure was

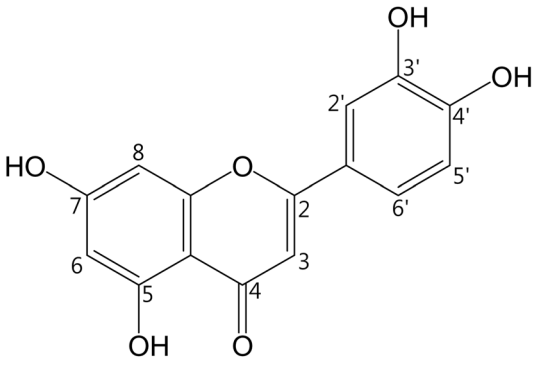

Fig. 1. Structure of luteolin from *L. erythrorhizon*.

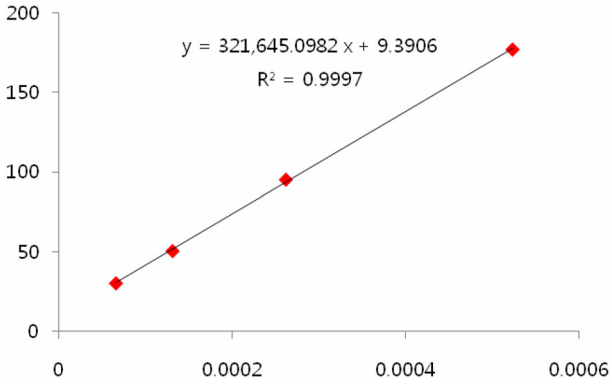

Fig. 2. Calibration curve for luteolin (X axis, μg/20 μℓ; Y axis, Area).

Table 1. Content of luteolin in seeds, stems, and roots of *L. erythrorhizon*.

Samples	Content (㎎/g)
Seeds	0.277 ± 0.078*
Stems	0.297 ± 0.042
Roots	0.162 ± 0.100

*Data are given as the mean ± S.D. (n = 3) in mg/g dried samples.

elucidated as luteolin by comparing its spectral data in the literature (Park *et al*., 2009b; Ternai and Markham, 1976).

Luteolin was a common constituent in *Taraxacum* and *Citrus* species (Jung *et al*., 2008; Elisa *et al*., 2007). However, there have been no reports on the determination of luteolin in *Lithospermum* species. The content of part-specific preponderance of luteolin in *L. erythrorhizon* was determined by HPLC. The standard curve for luteolin is Y = 321645.0982X – 9.3906 (r^2 = 0.9997) (Fig. 2). Luteolin was shown at the retention time 27.2 min. The retention time of the expected peak of luteolin in *L. erythrorhizon* was the same as that of the standard compound. Table 1 shows that

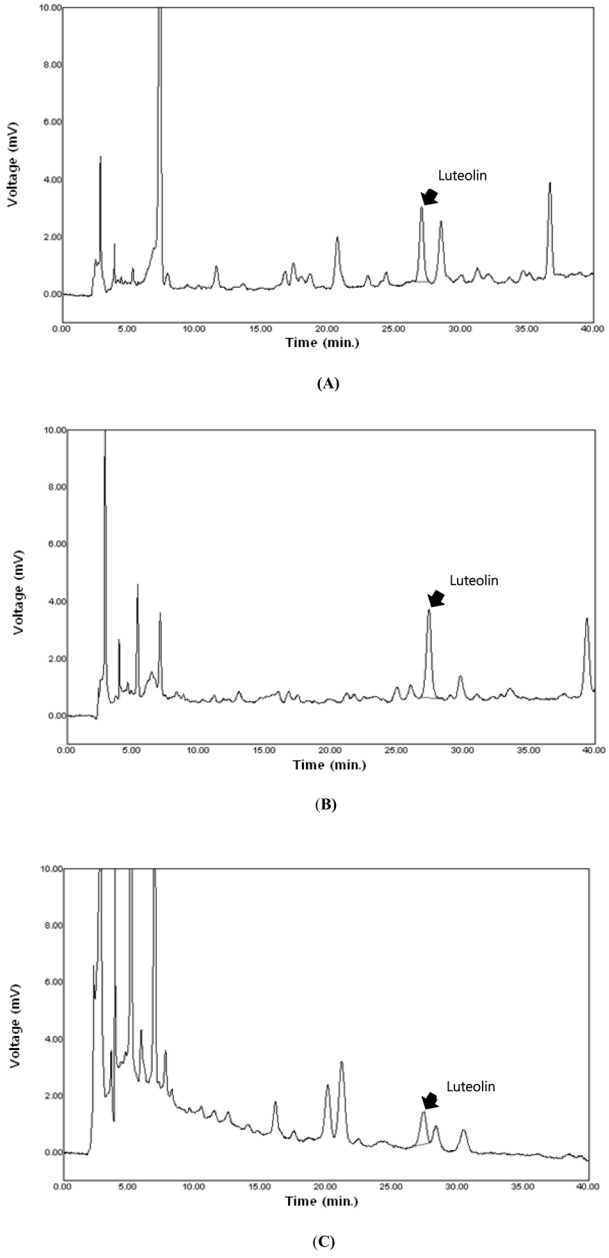

Fig. 3. HPLC chromatograms of the seeds (**A**), stems (**B**), and roots (**C**) of *L. erythrorhizon*.

luteolin was detected in the seeds (0.28 ㎎/g), stems (0.30 ㎎/g), and roots (0.16 ㎎/g) of *L. erythrorhizon*. In particular, the content of luteolin in the stems and seeds were two times more than that of the roots.

Luteolin plays a key inhibitor of *N*-acetyltransferase as a growth factor of *Helicobacter pylori* (Chung *et al*., 2001), and the inhibition of PAR-2 and -4 dependent inflammation (Lee *et al*., 2007). The presence of luteolin in *Lithospermum*

species is especially important in agricultural crop production for increasing the amounts of clinically available medicine and health supplements. Accordingly, the use of the plant *L. erythrorhizon* will indicate medicinal possibility as new additives to natural products for the development of wine, food products, and health supplements in Korea.

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