Note

Phytochemical Studies and Quantitative HPLC Analysis of Rosmarinic Acid and Luteolin 5-*O*-*β*-D-Glucopyranoside on *Thymus praecox* subsp. *grossheimii* var. *grossheimii*

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***Thymus praecox* ssp. *grossheimii* (Ronniger) Jalas var. *grossheimii* (Lamiaceae) is used as an herbal tea for cold, stomachache, cough, and infections in Turkey. There are no phytochemical studies on this species. We performed phytochemical studies and quantitative analysis of rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside in the methanol extract of the plant. Several chromatographic methods were used for the isolation of major compounds. HPLC methods were applied for quantitative analysis of rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside in the methanol extract. In this study, ursolic acid (1), oleanolic acid (2), methyl rosmarinate (3), ethyl rosmarinate (4), rosmarinic acid (5), luteolin 5-*O*-*β*-D-glucopyranoside (6), and thymoquinol 2,5-*O*-*β*-diglucopyranoside (7) were isolated from the aerial parts of the plant. The relative contents of rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside in the extract were 15.2 and 57.8 mg/g of dry weight, respectively. Compounds isolated from this plant and the contents of rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside provided reasonable evidence for the traditional usages of this plant.**

Key words *Thymus praecox*; Lamiaceae; triterpene; phenolic compound; flavone glycoside; monoterpene glycoside

Thymus genus, belonging to the Nepetoideae subfamily of Lamiaceae family, is represented by 64 taxon and 27 of them are endemic for Turkish flora.[1–3] *Thymus* species are known as "Kekik, nemamulotu, sater" by the public, and the medicinal properties of this genus have made it one of the most popular plant in Turkey. In folk medicine, they are used as stomachic, sedative, antiseptic, antihelminthic and used against to common cold.[4,5] Also they are used for wound healing, mouth cavity diseases and providing oral hygiene. *Thymus* extracts have oral usage in dyspepsia and other gastrointestinal disorders, bronchitis and pertussis originated cough and they are used against laryngitis and tonsillitis as gargling.[4] Several studies showed that they have strong antibacterial and expectorant activity.[6,7] *Thymus* species growing in Turkey are rich in essential oils, triterpenes, phenolic acids, flavonoids and monoterpene glucosides.[6–18]

T. praecox ssp. *grossheimii* var. *grossheimii* is an aromatic plant which is often used as herbal tea and spice in the rural areas of Erzurum. The most important feature of this plant is its usage in folk medicine for cold, cough, stomachache, infection in the same area. There are no phytochemical studies on this species so we decided to perform several studies on the aerial parts the plant. The isolation of the compounds was carried out from chloroform ($CHCl_3$), ethyl acetate (EtOAc), and aqueous phases that were partitioned from methanol (MeOH) extract, using several and repeated chromatographic techniques. They were identified by using ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) data. HPLC methods were applied for quantitative analysis of rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside that were isolated from this plant, in the MeOH extract.

Results and Discussion

Extraction and Isolation At the end of the extraction and isolation processes of aerial parts of the plant 63.8 g MeOH extract was obtained. $CHCl_3$, EtOAc and remaining aqueous phase were 29.9 g, 4.5 g and 27.4 g, respectively after fractionation. Two triterpene compounds [ursolic acid (**1**) and oleanolic acid (**2**)] were isolated from $CHCl_3$ phase; three phenolic compounds [methyl rosmarinate (**3**), ethyl rosmarinate (**4**) and rosmarinic acid (**5**)] from EtOAc phase; one flavon glycoside [luteolin 5-*O*-*β*-D-glucopyranoside (**6**)] and one monoterpene glycoside [thymoquinol 2,5-*O*-*β*-diglucopyranoside (**7**)] from the aqueous phase (Fig. 1).

Subfamily Nepetoideae is characterized by the presence of caffeic acid esters such as rosmarinic acid, nepetoidin A, B for distinguishing from other subfamilies of Lamianeae.[19] Rosmarinic acid has many biological activities such as antibacterial, anti-inflammatory, and antiviral activities.[20] Rosmarinic acid, luteolin and luteolin glycosides have been isolated from various *Thymus* species before and *T. praecox* ssp. *grossheimii* var. *grossheimii* is also expected as one of the dietary and medicinal sources of those compounds.

Quantitative HPLC Analysis of Rosmarinic Acid and Luteolin 5-*O*-*β*-D-glucopyranoside Chromatograms of rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside in MeOH extract was shown Figs. 2 and 3, respectively. The limits of detection (LOD) and limits of quantification (LOQ) were es-

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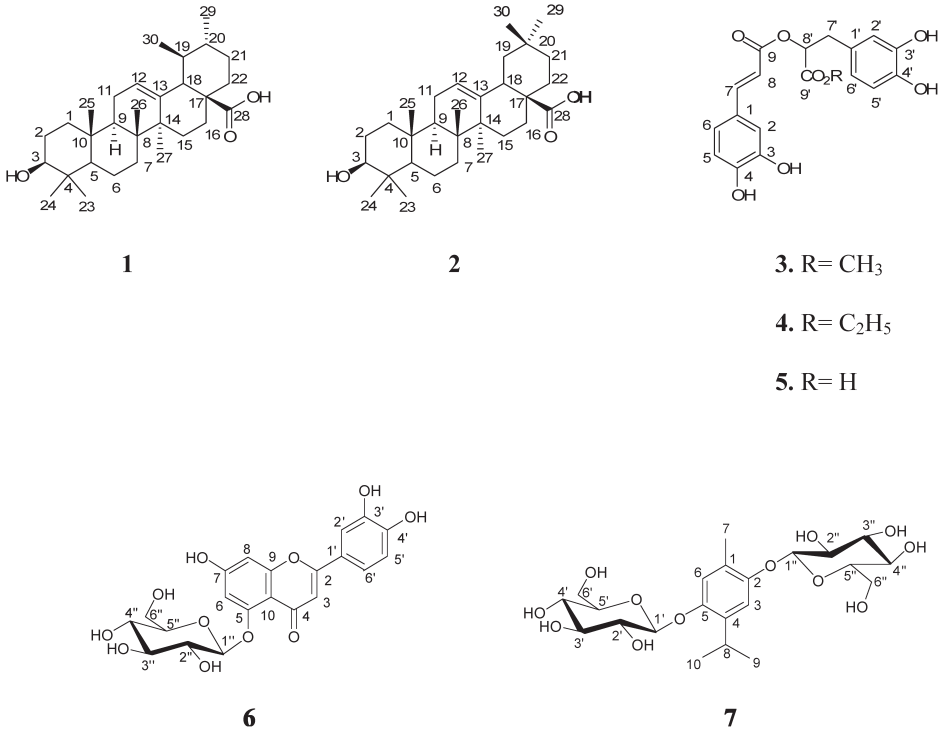

Fig. 1. Compounds Isolated from *T. praecox* subsp. *grossheimii* var. *grossheimii*

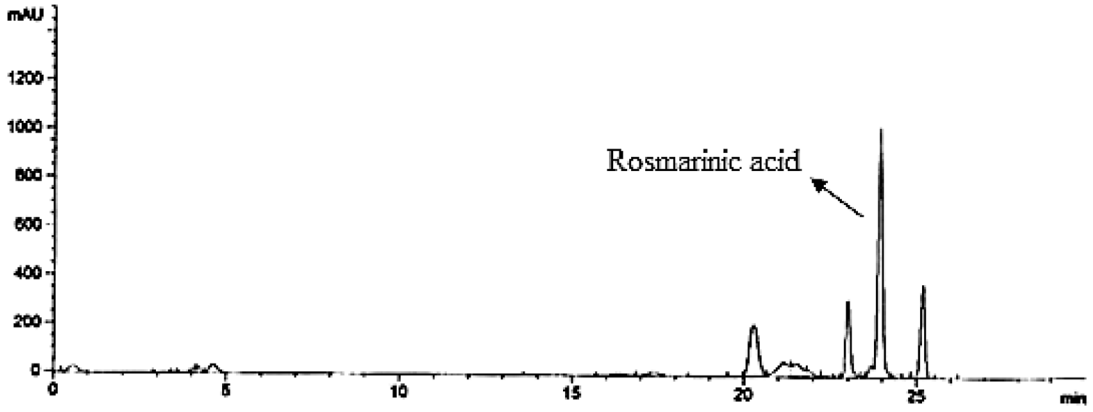

Fig. 2. HPLC Chromatogram of Rosmarinic Acid in Methanol Extract

tablished by decreasing analyte amounts to *S*/*N* of 3 : 1 and 10 : 1, respectively. The linearity of the detector response was determined at level ranges for rosmarinic acid (10–100 μg/mL) and luteolin 5-*O*-β-D-glucopyranoside (50–200 μg/mL). Evaluations were conducted by analysis of three working standard solutions of rosmarinic acid and luteolin 5-*O*-β-D-glucopyranoside. Calibration curves were plotted using rosmarinic acid and luteolin 5-*O*-β-D-glucopyranoside peak area ratios for HPLC. The calibration curve constructed was evaluated by its correlation coefficient with an excellent linearity. Results related calibration curves are presented in Table 1. The precision of the HPLC method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analyzing quality control samples six times per day, at three different concentrations which were quality control samples. The intermediate precision was evaluated by analyzing the same samples once daily for 3 d. The relative standard deviation (R.S.D.) of the predicted concentrations from the regression equation was taken as precision. The accuracy of this analytic method was assessed as the percentage relative error. For all the concentrations studied, intra- and inter-day R.S.D. of rosmarinic acid and luteolin 5-*O*-β-D-glucopyranoside values were 6.44% and for all concentrations of rosmarinic acid and luteolin 5-*O*-β-D-glucopyranoside the relative errors were 3.79%. Relative contents of the rosmarinic acid and luteolin 5-*O*-β-D-glucopyranoside in the extract are 15.2 and 57.8 mg/g of dry weight of the plant, respectively. There are some studies on several *Thymus* species (*T. nummularius, T. lotocephalus, T. mastichina* subsp. *mastichina*) about the quantitative HPLC analysis of rosmarinic acid and in these studies we can see there are differences in the amounts of the compound, changes from 131.9 μg/g to 43 mg/g.[21–23]

This is the first phytochemical study on *T. praecox* ssp. *grossheimii* var. *grossheimii* and 7 major compounds were isolated. Thymoquinol 2,5-*O*-β-diglucopyranoside was isolated from a *Thymus* species for the first time. The quantification of

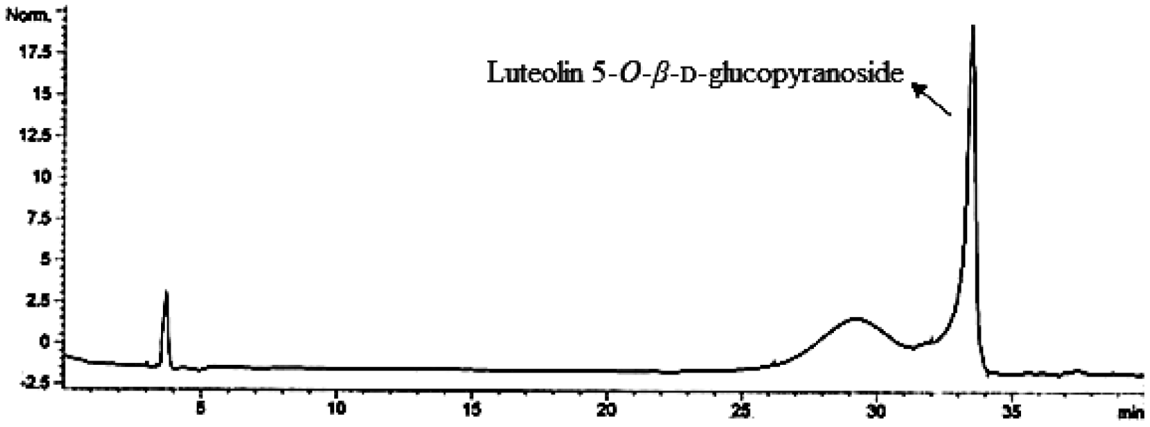

Fig. 3. HPLC Chromatogram of Luteolin 5-*O*-*β*-D-Glucopyranoside in Methanol Extract

Table 1. Linearity of Rosmarinic Acid and Luteolin 5-*O*-*β*-D-Glucopyranoside ($n=6$)[a)]

Compound	Range (μg/mL)	LR	R	LOD (μg/mL)	LOQ (μg/mL)
Rosmarinic acid	10–100	$y=100.24x+16.048$	0.9999	0.024	0.081
Luteolin 5-*O*-*β*-D-glucopyranoside	50–200	$y=4.9039x-46.08$	0.9992	0.5	1.65

a) Based on six calibration curves, LR: Linear regression, *R*: Coefficient of correlation, LOQ: Limit of quantification, LOD: Limit of detection, *x*: Concentration of compounds, *y*: Peak area

rosmarinic acid and luteolin 5-*O*-*β*-D-glucopyranoside is done. Rosmarinic acid detected from *T. praecox* ssp. *grossheimii* var. *grossheimii* was in the same amount range as from other *Thymus* species reported before. This is the first quantification of luteolin 5-*O*-*β*-D-glucopyranoside in a *Thymus* species. Compounds isolated in this study have agreed with the traditional usages of this plant.

Experimental

General NMR spectra were recorded on a Varian-400 spectrometer at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C-NMR. Kieselgel 60 (0.063–0.200 mm Merck), Reversed Phase Kieselgel (LiChroprep RP-18, 25–40 μm, Merck 9303) and Sephadex LH-20 (Sigma) were used for column chromatography. Precoated Kieselgel 60 F_{254} (Merck 5554) aluminum sheets were used for preparative thin layer chromatography (TLC). All HPLC analyses were carried out on an Agilent 1200 series HPLC system, equipped with a vacuum degasser, a quaternary pump, an autosampler, a column compartment, and a diode-array detector which is controlled by Agilent 1200 LC Software. The reversed-phase ACE® C_{18} analytical column (250 mm×4.6 mm i.d., 5 μm) was used in chromatographic separations.

Plant Material The aerial parts of *T. praecox* ssp. *grossheimii* var. *grossheimii* were collected from Yarımca Village (1750 m), Ilıca District, Erzurum Province, in June 2007. It was identified by Prof. Dr. Hayri Duman. Voucher specimen is deposited in Ankara University Faculty of Pharmacy Herbarium (AEF 23861).

Extraction and Isolation of the Major Compounds Air-dried and powdered aerial parts of the plant (350 g) were extracted with MeOH (2000 mL×3) under reflux at 40°C for 3 h and combined MeOH extracts were concentrated under reduced pressure. MeOH extract was suspended with H_2O : MeOH (9 : 1, 300 mL). This mixture was partitioned with $CHCl_3$ (300 mL×3), EtOAc (300 mL×3), respectively. $CHCl_3$, EtOAc and aqueous phases were evaporated at reduced pressure at 40°C.

$CHCl_3$ extract (29 g) was subjected to silica gel column by eluting with *n*-hexane : EtOAc (9 : 1→8 : 2→7 : 3→6 : 4). Fractions 63–65 (1.1 g, **A**) were subjected to gel column by eluting with *n*-hexane : EtOAc (6 : 4). Fraction 13–29 (33 mg, **A1**) were subjected to Sephadex LH-20 column, eluting with MeOH. Fraction 5 and 6 (25 mg, **A2**) were subjected to preparative TLC using by $CHCl_3$: MeOH : H_2O (90 : 10 : 1) solvent system. Ursolic acid (**1**) and oleanolic acid (**2**) (17.6 mg) were obtained as a mixture. ^{1}H-NMR data and ^{13}C-NMR data of 1–7 agree with the literature.[24–34)]

Ursolic Acid (**1**)[24–27)]

White amorphous powder; ^{1}H-NMR (400 MHz, CD_3OD) δ_H: 5.22 (1H, quasi t, J=3.7 Hz, H-12,), 3.15 (1H, dd, J=10.9 Hz, J=5.1 Hz, H-3), 2.20 (1H, d, J=11.3 Hz, H-18), 1. 25 (3H, s, CH_3), 0.97 (3H, s, CH_3), 0.96 (3H, d, J=6.6 Hz, CH_3), 0.95 (3H, s, CH_3), 0.88 (3H, d, J=6.2 Hz, CH_3), 0.84 (3H, s, CH_3), 0.77 (3H, s, CH_3), 2.03–0.73 (24H, m); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 38.8 (C-1), 26.7 (C-2), 78.5 (C-3), 38.6 (C-4), 55.5 (C-5), 18.3 (C-6), 33.1 (C-7), 39.6 (C-8), 47.1 (C-9), 36.9 (C-10), 23.2 (C-11), 125.7 (C-12), 138.4 (C-13), 42.0 (C-14), 28.0 (C-15), 24.1 (C-16), 47.1 (C-17), 53.2 (C-18), 39.2 (C-19), 39.2 (C-20), 30.6 (C-21), 36.9 (C-22), 27.6 (C-23), 14.8 (C-24), 15.2 (C-25), 16.5 (C-26), 22.9 (C-27), 180.5 (C-28), 16.6 (C-29), 20.4 (C-30).

Oleanolic Acid (**2**)[24–27)]

White amorphous powder; ^{1}H-NMR (400 MHz, CD_3OD) δ_H: 5.22 (1H, quasi t, J=3.7 Hz, H-12,), 3.15 (1H, dd, J=10.9 Hz, J=5.1 Hz, H-3), 2.82 (1H, dd, J=12.7, J=4.3 Hz, H-18), 1.12 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.93 (3H, s, CH_3), 0.87 (3H, s, CH_3), 0.84 (3H, s, CH_3), 0.78 (3H, s, CH_3), 0.76 (3H, s, CH_3), 2.03–0.73 (24H,m); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 38.6 (C-1), 26.7 (C-2), 78.5 (C-3), 38.8 (C-4), 55.5 (C-5), 18.3 (C-6), 32.4 (C-7), 39.4 (C-8), 48.2 (C-9), 37.0 (C-10), 22.7 (C-11), 122.4 (C-12), 144.0 (C-13), 41.7 (C-14), 27.5 (C-15), 22.8

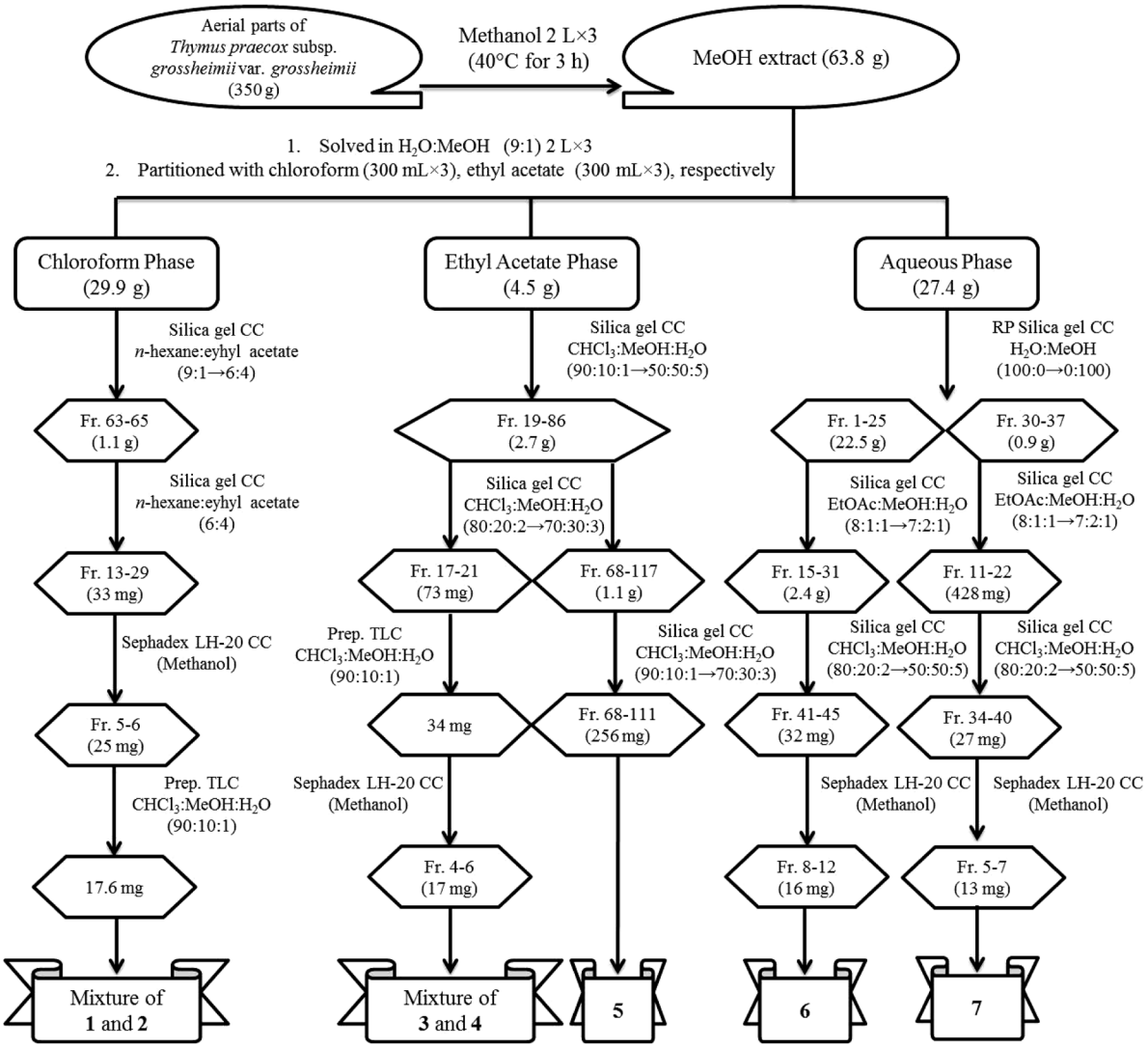

Fig. 4. Scheme of Fractionation and Isolation

(C-16), 46.4 (C-17), 41.5 (C-18), 46.1 (C-19), 30.4 (C-20), 33.7 (C-21), 32.3 (C-22), 27.7 (C-23), 15.1 (C-24), 14.7 (C-25), 16.5 (C-26), 25.2 (C-27), 180.5 (C-28), 32.8 (C-29), 23.3 (C-30).

EtOAc extract (4.5 g) was subjected to silica gel column by eluting with $CHCl_3:MeOH:H_2O$ (90:10:1→80:20:2→70:30:3→50:50:5). Fractions 19–86 (2.7 g, **A**) were subjected to gel column by eluting with $CHCl_3:MeOH:H_2O$ (80:20:2→70:30:3). Fraction 17–21 (73 mg, **A1**) were subjected to preparative TLC $CHCl_3:MeOH:H_2O$ (90:10:1), and then, Sephadex LH-20 column eluting with MeOH. Fractions 4–6 (17 mg) gave methyl rosmarinate (**3**) and ethyl rosmarinate (**4**) as a mixture. Fractions 68–117 (1.1 g, **B**) were subjected to silica gel column by eluting with $CHCl_3:MeOH:H_2O$ (90:10:1→80:20:2→70:30:3). Fractions 68–111 (256 mg) gave rosmarinic acid (**5**).

Methyl Rosmarinate (**3**)[28]

Colorless amourphous solid; ^{1}H-NMR (400 MHz, CD_3OD)) δ_H: 7.55 (1H, d, J=15.8 Hz, H-7), 7.05 (1H, d, J=1.8 Hz, H-2), 6.95 (1H, dd, J=8.1 Hz, J=1.8 Hz, H-6), 6.78 (1H, d, J=8.1 Hz, H-5), 6.71 (1H, d, J=2.2, Hz, H-2′), 6.69 (1H, d, J=8.1 Hz, H-5′), 6.57 (1H, ddd, J=8.0 Hz, J=2.2 Hz, J=1.8 Hz, H-6′), 6.27 (1H, d, J=15.8 Hz, H-8), 5.19 (1H, m, H-8′), 3.70 (3H, s, $-OCH_3$), 3.03 (1H, m, H-7a′), 3.03 (1H, m, H-7b′); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 126.4 (C-1), 114.0 (C-2), 145.7 (C-3), 148.7 (C-4), 115.3 (C-5), 122.1 (C-6), 146.8 (C-7), 112.9 (C-8), 167.2 (C-9), 127.6 (C-1′), 116.4 (C-2′), 145.0 (C-3′), 144.2 (C-4′), 115.1 (C-5′), 120.6 (C-6′), 36.7 (C-7′), 73.5 (C-8′), 171.0 (C-9′), 51.5 ($-OCH_3$).

Ethyl Rosmarinate (**4**)[28]

Colorless amourphous solid; ^{1}H-NMR (400 MHz, CD_3OD)) δ_H: 7.56 (1H, d, J=15.8 Hz, H-7), 7.05 (1H, d, J=1.8 Hz, H-2), 6.95 (1H, dd, J=8.1 Hz, J=1.8 Hz, H-6), 6.78 (1H, d, J=8.1 Hz, H-5), 6.71 (1H, d, J=2.2, Hz, H-2′), 6.69 (1H, d, J=8.1 Hz, H-5′), 6.57 (1H, ddd, J=8.0 Hz, J=2.2 Hz, J=1.8 Hz, H-6′), 6.27 (1H, d, J=15.8 Hz, H-8), 5.19 (1H, m, H-8′), 4.15 (2H, q, J=7.0 Hz -$O\underline{CH_2}CH_3$), 1.21 (3H, t, J=7.5 Hz, $-OCH_2\underline{CH_3}$), 3.03 (1H, m, H-7a′), 3.03 (1H, m, H-7b′); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 126.4 (C-1), 114.0 (C-2), 145.7 (C-3), 148.7 (C-4), 115.3 (C-5), 122.1 (C-6), 146.8 (C-7), 112.9 (C-8), 167.2 (C-9), 127.6 (C-1′), 116.4 (C-2′), 145.0 (C-3′), 144.2 (C-4′), 115.1 (C-5′), 120.6 (C-6′), 36.7 (C-7′), 73.5 (C-8′), 171.0 (C-9′), 61.3 ($-O\underline{CH_2}CH_3$),13.2 ($-OCH_2\underline{CH_3}$).

Rosmarinic Acid (**5**)[28–31]

Colorless amourphous solid; ^{1}H-NMR (400 MHz, CD_3OD)) δ_H: 7.50 (1H, d, J=16.1 Hz, H-7), 7.02 (1H, d, J=2.2 Hz, H-2,), 6.92 (1H, dd, J=8.3 Hz, J=2.0 Hz, H-6), 6.76 (1H, d, J=8.1 Hz,

Table 2. Gradient Programming for the Separation of Rosmarinic Acid and Luteolin 5-*O*-*β*-D-Glucopyranoside

Time (min)	Initial	Rosmarinic acid		Initial	Luteolin 5-*O*-*β*-D-glucopyranoside				
		20	35		25	40	50	55	60
A%	98	71.5	71.5	95	60	40	40	10	5
B%	2	28.5	28.5	5	40	60	60	90	95

H-5), 6.74 (1H, d, *J*=2.2 Hz, H-2′), 6.68 (1H, d, *J*=8.1 Hz, H-5′), 6.62 (1H, dd, *J*=8.1 Hz, *J*=1.8 Hz, H-6′), 6.26 (1H, d, *J*=16.1 Hz, H-8,), 5.10 (1H, dd, *J*=9.7 Hz, *J*=3.4 Hz, H-8′), 3.09 (1H, dd, H-7a′, *J*=14.3 Hz, *J*=3.5 Hz), 2.94 (1H, dd, *J*=14.3 Hz, *J*=9.5 Hz, H-7b′); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 126.7 (C-1), 114.3 (C-2), 145.6 (C-3), 148.2 (C-4), 115.3 (C-5), 121.8 (C-6), 145.5 (C-7), 113.9 (C-8), 167.9 (C-9), 129.8 (C-1′), 116.4 (C-2′), 144.7 (C-3′), 143.6 (C-4′), 115.1 (C-5′), 120.6 (C-6′), 37.5 (C-7′), 76.4 (C-8′), 176.5 (C-9′).

Remaining aqueous extract (27 g) was subjected to reversed phase silica gel column and eluted with H_2O : MeOH (100 : 0→0 : 100). Fractions 1–25 (22.5 g, **A**) were subjected to silica gel column eluting with EtOAc : MeOH : H_2O (8 : 1 : 1→7 : 2 : 1). Fractions 15–31 (2.4 g, **A1**) were subjected to silica gel column eluting with $CHCl_3$: MeOH : H_2O (80 : 20 : 2→70 : 30 : 3→50 : 50 : 5). Fractions 41–45 (32 mg, **A2**) were subjected to Sephadex LH-20 column eluting with MeOH. Fraction 8–12 (16 mg) gave luteolin 5-*O*-*β*-D-glucopyranoside (**6**).

Luteolin 5-*O*-*β*-D-Glucopyranoside (**6**)[32,33]

Yellow amourphous powder; ^{1}H-NMR (400 MHz, CD_3OD)) δ_H: 7.36 (1H, dd, *J*=8.8 Hz, *J*=2.2 Hz, H-6′), 7.35 (1H, br s, H-2′), 6.85 (1H, d, *J*=8.8 Hz, H-5′), 6.77 (1H, d, *J*=2.2 Hz, H-8), 6.67 (1H, d, *J*=2.2 Hz, H-6), 6.54 (1H, s, H-3), 4.84 (1H, d, *J*=7.7 Hz, H-1″), 3.73 (1H, dd, *J*=12.1 Hz, *J*=1.8 Hz, H-6a″), 3.53 (1H, dd, *J*=12.1 Hz, *J*=4.9 Hz, H-6b″), 3.44–3.14 (4H, m, H-2″,-3″,-4″,-5″, sugar protons); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 164.2 (C-2), 105.3 (C-3), 179.2 (C-4), 159.4 (C-5), 103.7 (C-6), 163.1 (C-7), 98.2 (C-8), 158.9 (C-9), 108.0 (C-10), 122.3 (C-1′), 112.8 (C-2′), 145.8 (C-2′), 149.6 (C-2′), 115.6 (C-2′), 118.9 (C-2′), 103.8 (C-1″), 73.5 (C-2″), 76.1 (C-3″), 70.0 (C-4″), 77.4 (C-5″), 61.3 (C-6″).

Fractions 30–37 (900 mg, **B**) were subjected to silica gel column eluting with EtOAc : MeOH : H_2O (8 : 1 : 1→7 : 2 : 1). Fractions 11–22 (428 mg, **B1**) were subjected to silica gel column eluting with $CHCl_3$: MeOH : H_2O (80 : 20 : 2→70 : 30 : 3→50 : 50 : 5). Fractions 34–40 (27 mg, **B2**) were subjected to Sephadex LH-20 column eluting with MeOH. Fractions 5–7 (13 mg) gave thymoquinol 2,5-*O*-*β*-diglucopyranoside (**7**).

Thymoquinol 2,5-*O*-*β*-Diglucopyranoside (**7**)[34]

White crystals; ^{1}H-NMR (400 MHz, CD_3OD)) δ_H: 7.03 (1H, s, H-3), 6.96 (1H, s, H-6), 4.75 (1H, d, *J*=7.7 Hz, H-1′), 4.73 (1H, d, *J*=7.3 Hz, H-1″), 3.87 (1H, m, H-8), 3.72–3.36 (14H, m, sugar protons), 2.23 (s, 3H, Me-7), 1.19 and 1.17 (each 3H, d, *J*=7.0 Hz, Me-9 and Me-10); ^{13}C-NMR (100 MHz, CD_3OD) δ_C: 151.6 (C-5), 150.1 (C-2), 136.9 (C-1), 126.1 (C-4), 118.4 (C-3), 114.1 (C-6), 102.8 and 102.7 (C-1′ and C-1″ of glucose molecules), 77.2 and 77.1 (C-5′ and C-5″ of glucose molecules), 76.9 (C-3′ and C-3″ of glucose molecules), 73.9 (C-2′ and C-2″ of glucose molecules), 70.4 (C-4′ and C-4″ of glucose molecules), 61.5 (C-6′ and C-6″ of glucose molecules).

The fractionation and isolation scheme is given in Fig. 4.

Quantitative HPLC Analysis of Rosmarinic Acid and Luteolin 5-*O*-*β*-D-Glucopyranoside The stock standard solution of rosmarinic acid and luteolin 5-*O*-*β*-D-glucoside were prepared in methanol as a concentration of 1000 *μ*g/mL separately. Working standard solutions were prepared from the stock solution. The identification and quantification of rosmarinic acid at 330 nm with pump A (water containing 0.05% phosphoric acid), pump B (acetonitrile) and luteolin 5-*O*-*β*-D-glucopyranoside at 350 nm with pump A (water containing 0.1% acetic acid), pump B (MeOH) were achieved respectively with a gradient programme given in Table 2. The flow rates and injection volumes were 1 mL/min and 100 *μ*L during the gradient runs. Column temperatures were maintained at 30°C for both methods.

Conflict of Interest The authors declare no conflict of interest.

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