SUPPLEMENTARY MATERIALS

Isolation, identification of secondary metabolites from *Salvia absconditiflora* and evaluation of their antioxidative properties

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Abstract: *Salvia absconditiflora* Greuter & Burdet (Synonym *Salvia cryptantha* Montbret & Aucher ex Benth) has been used extensively for traditional medicine. The aerial of plant material was boiled in water then filtrated. The filtrate was partitioned with ethyl acetate and *n*-butanol sequentially to yield the ethyl acetate and *n*-butanol extract. A sample of water was lyophilized to yield the water extract. Ethyl acetate extract revealed the highest antioxidant activity and included the most phenolic compounds among the extracts. Hence, ethyl acetate extract was subjected to chromatographic techniques. Ursolic acid (**1**), crismaritin **(2),** luteolin (**3**), rosmarinic acid methyl ester (**4**), 3,4-dihydroxyl benzaldehyde (protocatechuic aldehyde) (**5**), caffeic acid (**6**), apigenin-7-*O*-*β*-

glucoside (**7**), rosmarinic acid (**8**) and luteolin-7-*O*-*β*-glucoside (**9**) were isolated and the structures were elucidated by spectroscopic methods including 1D, 2D NMR, and LC-TOF/MS. Cirsimaritin (**2**), luteolin (**3**), rosmarinic acid methyl ester (**4**), rosmarinic acid (**8**), luteolin-7-*O*-*β*-glucoside (**9**) displayed the considerable antioxidant activity.

Keywords: *Salvia absconditiflora,chromatography, spectroscopy, natural products.*

Experimental

General experimental procedures

NMR spectra were recorded on a spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C NMR. Chemical shifts were given in ppm (δ scale), coupling constant *(J)* in Hz. DMSO-*d6* was used as deuterated solvent in the preparation of samples for NMR analysis. Column chromatography was performed on silica (60-230 mesh, Merck). TLC was carried out on analytical alumina plates (60 F_{254}). Hitachi U-290 UV-Vis spectrometer was used for UV measurements. HRMS analyses were recorded on Agilent 6210 LC-TOF/MS spectrometer. HPLC Perkin Elmer Series 200 was used for isolation of secondary metabolites. Ammonium thiocyanate and BHT were purchased from E. Merck (Darmstadt, Germany). Ferrous chloride, α-tocopherol, polyoxyethylene sorbitan monolaurate (Tween-20), radical 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (Ferrozine), nicotinamide adenine dinucleotide (NADH), BHA, and trichloracetic acid (TCA), formic acid purchased from Sigma-Aldrich (Darmstadt, Germany).

Plant Material

The aerial parts of *S. absconditiflora* (Synonym, *Salvia cryptantha* Montbret & Aucher ex Benth). were collected from Tokat, Artova at July 2015, altitude of 1279 m, coordinate of N

40° 06' 37.68" E 36° 20' 05.43". The plant material was identified by Asst. Prof. Dr. Bedrettin Selvi, Department of Biology, Faculty of Arts and Sciences, Gaziosmanpasa University where a voucher specimen was deposited (GOPU 6344).

Extraction and Isolation

The plant material was dried at shade for two weeks. Dried plant material (900 g ± 2 g) was boiled in water (1.5 L) for 2 h. After filtration, the filtrate was extracted with ethyl acetate (3 × 170 mL). Organic layer was seperated then the solvent was removed to yield the ethyl acetate extract (17.3 g). The water phase was extracted with *n*-butanol (3 × 170 mL), *n*-butanol phase was seperated and the solvent was removed to yield the *n*-butanol extract (24.8 g). Lyophilisation was applied for the water phase to remove the water to obtain the water extract (18.4 g).

Antioxidant assays including total phenolic content, $DPPH^{\bullet}$ free radical, $ABTS^{+\bullet}$ scavenging and reducing power were used for ethyl acetate extract, *n*-butanol extract and water extract. Ethyl acetate extract consisted of the most phenolic contents and revealed the highest antioxidant activity in all assays among the extracts. Therefore, ethyl acetate extract (14.0 g) was used to isolate the secondary metabolites. EtOAc extract (14.0 g) was subjected to a silica gel column (2.5 cm × 80 cm, 520 g silica gel) eluting with *n*-hexane/EtOAc (from 100:0 to 0:100), EtOAc, EtOAc/MeOH (from 100:0 to 0:100) and MeOH. 400 fractions each 20 mL were collected. Ursolic acid (**1**) (12.9 mg) and crismaritin **(2)** (15.1 mg) were isolated from the fractions of 70-90 (*n*-hexane/EtOAc, 85/15 %) and 91-150 (*n*-hexane/EtOAc, 75/25 %) respectively. Luteolin (**3**), rosmarinic acid methyl ester (**4**), 3,4-dihydroxy-benzaldehyde (**5**) were isolated as a mixture in the fractions of 180-250, Afterward, luteolin (**3**) (6.9 mg), rosmarinic acid methyl ester (**4**) (5.2 mg), 3,4-dihydroxy-benzaldehyde (**5**) (6.3 mg) were purified by preperative HPLC. The fractions of 260-290 (*n*-hexane/EtOAc, 45/55 %) included the caffeic acid (**6**) (15.7 mg). Apigenin-7-*O*-*β*-glucoside (**7**) (13.8 mg) was generated from

the fractions of 295-320 (*n*-hexane/EtOAc, 30/70 %). Rosmarinic acid (**8**) (12.3 mg) was isolated from the fractions of 341-360 (EtOAc). Finally Luteolin-7-*O*-*β*-glucoside (**9**) (12.7 mg) was isolated from the fractions of 365-390 (EtOAc/MeOH, 90/10%).

Ursolic acid (**1**) was the first isolated compound during the chromatographic process. Its spectral data accorded with the literature (Venditti et al. 2015). The second isolated compound was cirsimaritin (**2**). It was isolated as a solid with yellow colour and its molecular formula was established as $C_{17}H_{13}O_6$ by LC-TOF/MS (*m/z* 313.0708 $[M-H]^-$) (calcd. 313.2815). In ^{1}H-NMR spectrum, the signal appeared at δ 12.9 belonged to the 5-OH proton. H-3 and H-8 resonated at δ 6.86 and 6.94 as a singlet respectively. The others peak at δ 7.98 (d, J = 8.8 Hz) and δ 6.93 (d, *J* = 8.8 Hz) belonged to H-2',6' and H-3',5' respectively. The ^{13}C NMR spectrum (APT, DEPT experiments) revealed two methoxy, one methyl, two methylene, sixteen methine, and nine quaternary carbon atoms, verifying the aglycone flavonoid structure named as cirsimaritin (**2**) (Venditti et al. 2017). The other isolated compound, luteolin (**3**) has been found in plant kingdom abundantly. Many vegetables and fruits such as tea, carrot, cabbage, artichoke, celery and apple include the rich luteolin. It revealed a large spectrum of biological activity (Luo et al. 2017). LC-TOF/MS (*m/z* 285.0387 $[M-H]^-$) (calcd. 285.0399) analysis confirmed the molecular formula as $C_{15}H_9O_6$. In ^{1}H NMR spectrum of luteolin, the signal appeared at δ 7.43 as a doublet with a coupling constant 2 Hz belonged to H-2'. H-6' resonated at δ 7.40 (dd, J = 8.1 Hz, J = 2.2 Hz). The resonance peak at δ 6.90 with doublet (J = 8.1 Hz) owned by H-5'. H-3 proton gave the signal at δ 6.67 as a singlet. The signal at δ 6.45 belonged to H-8 with meta coupling constant of 2.0 Hz. H-6 proton resonated at δ 6.20 (d, J= 2.0 Hz). Due to the neighbor hydroxyl groups with a free orbital, electon density increased at H-6 which led to a shielding of the corresponding hydrogen atom resulting in an upfield shift. The ^{13}C NMR spectrum displayed fifteen signals consisting of six methines, eight quaternary carbon atoms and one carbonyl confirmed the proposed stucture as luteolin (**3**) (Erenler et al. 2016). Fourth compound isolated from *S. absconditiflora* was rosmarinic acid methyl ester (**4**). In ^{1}H NMR spectrum, two ABX systems at δ 7.07 (d, *J* = 1.4 Hz), δ 7.02 (d, J = 8.1 Hz), δ 6.77 (d, J = 8.1 Hz) and δ 6.71 (d, J = 1.5 Hz), δ 6.64 (d, J = 8.2 Hz), δ 6.65 (d, J = 8.2 Hz) belonged to H-2, H-6, H-5 ve H-2', H-5', H-6' respectively. The resonance peaks at δ 7.48 (d, J = 15.8) and δ 6.26 (d, J = 15.9 Hz) matched with H-7 and H-8 respectively. Large coupling constant confirmed the trans orientation of olefinic protons. The signals at δ 5.08 (dd, J = 7.4 Hz, J = 5.3 Hz) was compatible with the H-8'. The diastereotopic methylene protons connected to C-7' gave the signals at δ 2.91 (dd, J = 10.3 Hz, J = 7.4 Hz)

and δ 2.96 (dd, J = 7.4 Hz and J = 5.3 Hz). The signal at δ 3.63 which was not observed in rosmarinic acid spectrum corresponded to the methoxyl group connected to C-9'. In ^{13}C NMR spectrum, observation of one methoxyl, one methylene, nine methine, six quaternery carbons and two carbonyl carbons fitted with the proposed structure. Beside these evidences, 2D NMR analyses also supported to the structure of rosmarinic acid methyl ester (**4**) (Abedini et al. 2013). The other isolated compounds were elucidated as 3,4-dihydroxy-benzaldehyde (protocatechuic aldehyde) (**5**) (Alvarenga et al. 2012), caffeic acid (**6**) (Dürüst et al. 2001), apigenin-7-*O*-*β*-glucoside (**7**) (Erenler et al. 2014), rosmarinic acid (**8**) (Aksit et al. 2014) and luteolin-7-*O*-*β*-glucoside (**9**) (Chiruvella et al. 2007).

Preperative HPLC analysis

Preparative separations were achieved using a Perkin Elmer Series 200 semi preparative HPLC equipped with Series 200 UV detector. A Chrometisil Kromaplus 100 C18 column was used (250 × 10 mm, 10 µm particle size) for elution of analyte. Deionize water (acidified by %1 formic Acid) (Solvent A) and acetonitrile (solvent B) were used as mobile phase at 5 mL/minute flow rate. Mobile phase program was as follows: linear gradient from 90:10 (A:B) to 55:45 (A:B) for 40 min. The detected peaks at 280 nm were collected using Foxy 200 fraction collector (%10 slope). The mixture was dissolved in DMSO (100 mg/1 mL) and manually injected over 200 µL loop for each run.

Antioxidant assays

Total phenolic content

Total phenolic content of the ethyl acetate, *n*-butanol and water extracts of *S. absconditiflora* was executed by Folin-Ciocalteu reagent with gallic acid as standard (Singleton and Slinkard 1977). An extract solution (1.0 mL, including 1000 mg sample), distilled water (46 mL) and Folin-Ciocalteu reagent (1.0 mL) were mixed in a volumetric flask. After adding Na_2CO_3 (3 mL, 2%), reaction mixture was shacked for 2 h. The absorbance was measured at 760 nm in a spectrophotometer (Hitachi U-2900). The concentration of total phenolic compounds in each extract was determined as mg gallic acid equivalent using an equation obtained from the standard gallic acid graph:

Absorbance = 0.0052 × Total phenols [Gallic acid eqv.(µg)] – 0.0057

$ABTS^{+\bullet}$ scavenging assay

At first, $ABTS^{\bullet+}$ was prepared by the reaction of ABTS (2 mM) with potassium persulfate (2.45 mM) then it was stored for 6 h in dark at room temperature. Subsequently, $ABTS^{\bullet+}$ solution (1.0 mL) reacted with each sample solution (3.0 mL) at various concentrations (2.5, 5.0, 10, 20, 40 µg/mL). The inhibition was calculated for each concentration comparative to a blank absorbance. The decolorization rate was calculated as absorbance (734 nm) of reduction percent. The capability of $ABTS^{\bullet+}$ was calculated by the equation:

$$ABTS^{\bullet+} \text{ scavenging effect (\%)} = [(A_c - A_s) / A_c] \times 100$$

in which, A_c is $ABTS^{\bullet+}$ initial concentration and A_s is $ABTS^{\bullet+}$ remaining concentration in the sample. The results were calculated as IC_{50} (Erenler et al. 2015).

$DPPH^{\bullet}$ free radical assay

$DPPH^{\bullet}$ scavenging activities of extracts and isolated compounds were measured according to the literature (Blois 1958). $DPPH^{\bullet}$ solution (0.26 mM, 1.0 mL) was added to the various concentrations of extracts and compounds (3 mL, 2.5-40 µg/mL). The reaction mixture was stirred at rt for 30 sec. The absorbance was measured at 517 nm on a spectrophotometer and lower absorbance of the reaction product showed the higher activity. The $DPPH^{\bullet}$ scavenging activity was calculated using the equation:

$$DPPH^{\bullet} \text{ scavenging effect (\%)} = [(Ac - As) / Ac] \times 100$$

Ac is the absorbance of the control and *As* is the absorbance of the sample. The results were calculated as IC_{50}.

Reducing power

Sodium phosphate buffer (0.2 M, pH 6.7) was prepared. Potassium ferricyanide [$K_3Fe(CN)_6$] (1.25 mL, 1%) were treated with the each extract and compounds at different concentrations

(2.5-40 µg/mL) at 50 °C for 30 min and total volume was completed to 2.5 mL with buffer solution. The reaction mixture was stirred at for 20 min. Trichloroacetic acid (1.25 mL, 10%) was added to the reaction mixture and then $FeCl_3$ (0.25 mL, 0.1%) was added. The absorbance was measured at 700 nm in a spectrophotometer. High absorbance value of the reaction mixture indicated high reducing capability (Demirtas et al. 2013).

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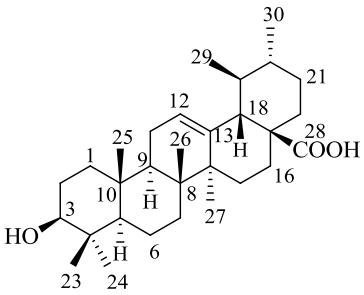
Table S1: Antioxidant activity of *S. absconditiflora* extracts

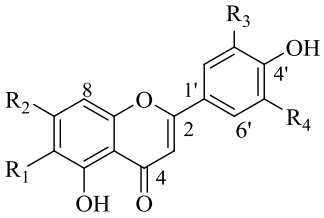
Extracts and standards	**Total phenolic contents (g GAE/Kg extract)**	**Total flavonoid (g quercetin /kg extract)**	**DPPH$^{\bullet}$ scavenging [IC_{50} (µg/mL)]**	**FRAP (mmol TE/g extract)**	**ABTS$^{\bullet+}$ Scavenging [IC_{50} (µg/mL)]**
EtOAc ext	228.55±16.80	2.66±0.15	6.00±0.74	4.63 ±0.05	2.30±0.18
BuOH ext	136.22±13.94	3.89±0.22	6.30 ± 1.35	3.42 ±0.17	3.22±0.12
Water ext	27.18 ± 3.16	1.44±0.02	48.12±2.07	0.66 ±0.05	6.78±0.41
BHT	-	-	8.51 ± 1.01	4.33±0.27	2.32±0.11
BHA	-	-	3.30 ± 0.28	7.27 ±0.27	2.48±0.25
Trolox	-	-	3.54± 0.26	3.96 ±0.26	4.84±0.08

Table S2: Antioxidant activity of compounds from *S. absconditiflora*

Compounds	**DPPH$^{\bullet}$ scavenging [IC_{50} (µg/mL)]**	**FRAP (mmol TE/g sample)**	**ABTS$^{\bullet+}$ Scavenging [IC_{50} (µg/mL)]**
Ursolic acid (1)	151.52 ± 4.48	0.28 ± 0.01	6.32 ± 0.58
Crismaritin (2)	11.82 ± 0.02	2.76 ± 0.06	1.51 ±0.01
Luteolin (3)	16.29 ± 0.78	3.56 ± 0.12	5.61± 0.04
Rosmarinic acid methyl ester (4)	4.84 ± 0.07	5.86 ± 0.50	2.05 ± 0.15
3,4-dihydroxy-benzaldehyde (5)	35.34 ± 0.77	1.51 ±0.06	53.39± 2.41
Caffeic acid (6)	10.83 ± 0.25	3.82 ± 0.33	8.07 ± 0.38
Apigenin-7-*O*-*β*-glucoside (7)	91.90 ± 5.18	1.30 ± 0.01	15.48 ± 0.14
Rosmarinic acid (8)	7.52 ± 0.06	4.23 ± 0,06	6.00± 0.26
Luteolin-7-*O*-*β*-glucoside (9)	19.36± 0.78	1.57± 0.05	3.9± 0.18
BHT	8.51 ± 1.01	4.33± 0.23	2.32 ±0.11
BHA	3.30 ± 0.28	7.26 ± 0.26	2.48± 0.25
Trolox	3.54 ± 0.26	3.96 ± 0.26	4.84± 0.08



Ursolic acid (**1**)

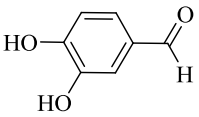


$R_1 = R_2 = OMe$, $R_3 = R_4 = H$: cirsimaritin (**2**)

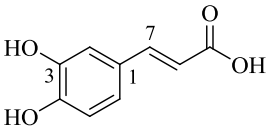
$R_1 = R_4 = H$, $R_2 = R_3 = OH$: luteolin (**3**)

$R_1 = R_3 = R_4 = H$, $R_2 = OGlc$: apigenin-7-O-β-glucoside (**7**)

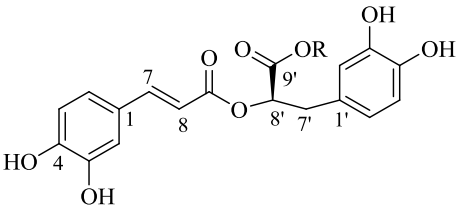
$R_1 = R_3 = H$, $R_2 = OGlc$, $R_4 = OH$: luteolin-7-O-β-glucoside (**9**)



3,4-dihydroxy benzaldehyde (**5**)



Caffeic acid (**6**)



R = H : rosmarinic acid (**8**)

R = CH_3 : rosmarinic acid methyl ester (**4**)

Figure S1: Isolated compounds from *S. absconditiflora.*

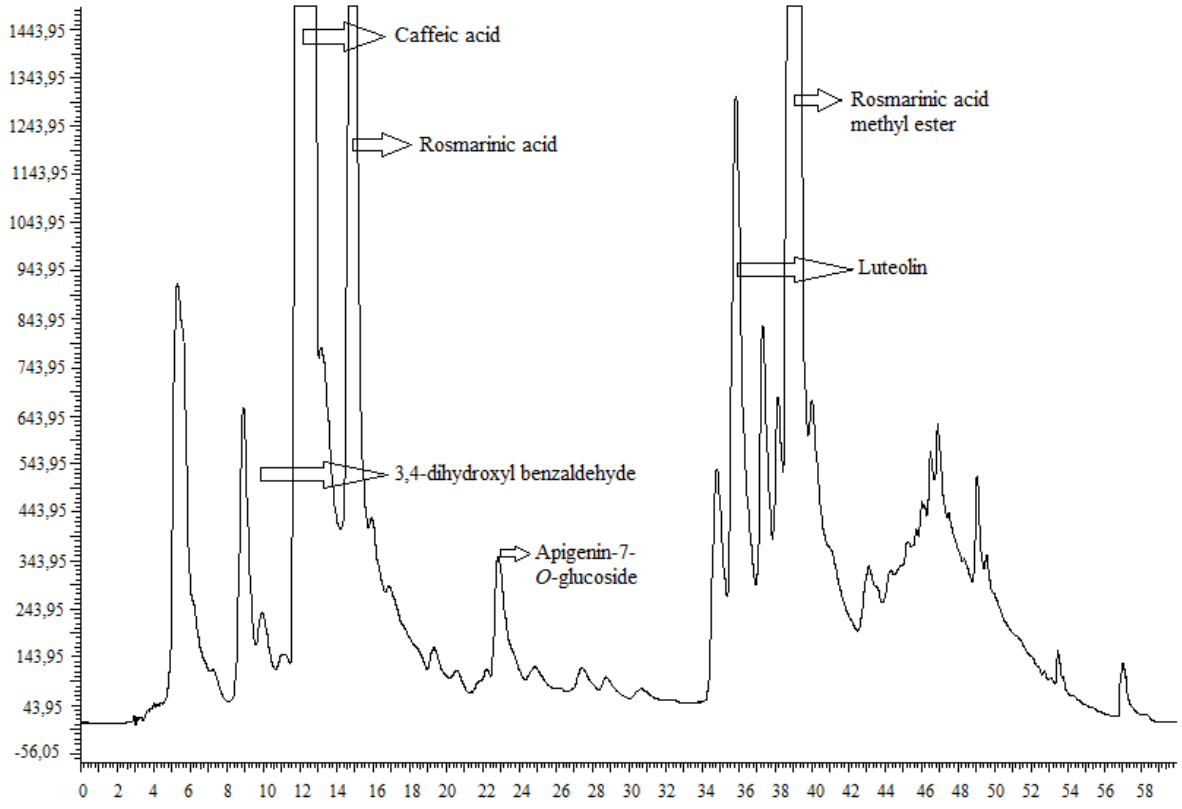


Figure S2: HPLC chromatogram of ethyl acetate extract.

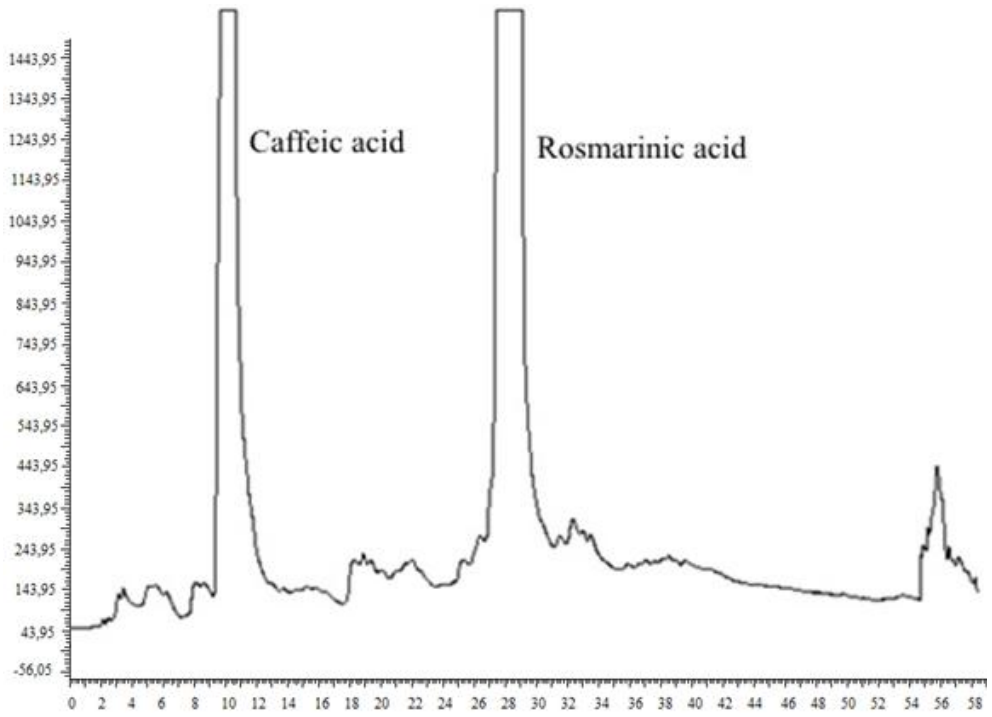


Figure S3: HPLC chromatogram of caffeic acid and rosmarinic acid isolated.

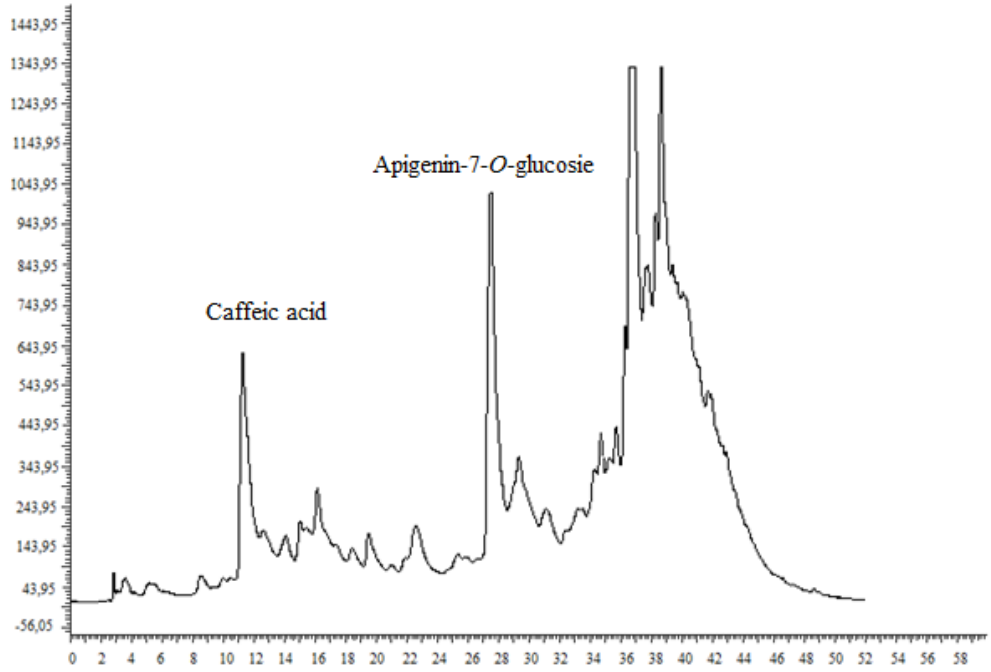


Figure S4: HPLC chromatogram of caffeic acid and apigenin-7-*O*-glucoside isolated.