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Original article

PHENOLIC COMPOUNDS FROM THE ROOTS OF *Anchusa azurea* var. *azurea*

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Abstract

*In Turkish folk medicine, Anchusa L. species have been used as wound healing and diuretic agents. This study was performed on methanolic extract of the roots of A. azurea Miller var. azurea was resulted in the isolation and structure elucidation of four flavonol glycosides, kaempferol 3-O-β-glucopyranoside (=Astragalin, **1**), quercetin 3-O-β-glucopyranoside (=Isoquercitrin, **2**), quercetin 3-O-α-rhamnopyranosyl (1'''→6'')-β-glucopyranoside (=Rutin, **3**), kaempferol 3-O-α-rhamnopyranosyl (1'''→6'')-β-glucopyranoside (**4**) and a phenolic acid, rosmarinic acid (**5**). The structures of the isolated compounds were determined by spectroscopic methods [1D and 2D NMR spectra and MS]. This is the first report on the isolation of phenolic compounds from the roots of A. azurea.*

Key words: *Anchusa azurea, Boraginaceae, Phenolic compounds, Flavonol glycosides, Rosmarinic acid.*

Anchusa azurea var. *azurea*'nın Köklerinden Elde Edilen Fenolik Bileşikler

*Türk halk hekimliğinde Anchusa L. türleri yara iyi edici ve diüretik ajanlar olarak kullanılmaktadır. A. azurea Miller var. azurea'nın köklerinin metanol ektresi üzerinde gerçekleştirilen bu çalışma 4 flavonol glikozitin: kemferol 3-O-β-glukopiranozit (=Astragalin, **1**), kersetin 3-O-β-glukopiranozit (=Izokersitrin, **2**), kersetin 3-O-α-ramnopiranozil (1'''→6'')-β-glukopiranozit (=Rutin, **3**) ve kemferol 3-O-α-ramnopiranozil (1'''→6'')-β-glukopiranozit (**4**) ve bir fenolik asitin: rozmarinik asit (**5**) izolasyonu ve yapı aydınlatması ile sonuçlanmıştır. İzole edilen bileşiklerin yapıları spektroskopik yöntemlerle [1D ve 2D NMR spektrumu ve MS] saptanmıştır. A. azurea köklerinden fenolik bileşiklerin izolasyonu ilk defa kaydedilmiştir.*

Anahtar kelimeler: *Anchusa azurea, Boraginaceae, Fenolik bileşikler, Flavonol glikozitleri, Rozmarinik asit.*

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INTRODUCTION

The genus *Anchusa* L. (Boraginaceae) is represented by 15 species in the flora of Turkey (1). *Anchusa* L. species are used as a diuretic agent and an ointment prepared from a decoction of *Anchusa azurea* Miller roots mixed with egg yolk and beeswax is used externally for wound healing in Turkish folk medicine (2,3). In earlier investigations on *Anchusa* species, pyrrolizidine alkaloids, flavonoids, triterpene saponins and fatty acids were isolated (4-8). We previously reported the isolation and the structure determination of phenolic compounds and triterpene saponins from the aerial parts of *A. leptophylla* and *A. azurea* var. *azurea* (9,10). This paper describes the isolation and structure determination of four flavonoid glycosides, kaempferol 3-*O*-*β*-glucopyranoside (**1**), quercetin 3-*O*-*β*-glucopyranoside (**2**), quercetin 3-*O*-*α*-rhamnopyranosyl (1‴→6″)-*β*-glucopyranoside (**3**), kaempferol 3-*O*-*α*-rhamnopyranosyl (1‴→6″)-*β*-glucopyranoside (**4**), in addition to a phenolic acid, rosmarinic acid (**5**) from the methanolic extract of the roots of *A. azurea* Miller var. *azurea*.

EXPERIMENTAL

General experimental procedures

The UV (MeOH) spectra were recorded on a Biotek MQX200 μQuant Microplate spectrophotometer. ^{1}H and ^{13}C NMR measurements in CD_3OD were performed on Varian Mercury plus 400 MHz for proton and 100 MHz for carbon by using TMS as internal standard. EIMS was performed on a Finnigan MAT 95 spectrometer. For open column chromatographic separations, Silica gel 60 (0.063-0.200 mm, Merck) and Sephadex LH-20 (Fluka) were used. TLC analyses were carried out on pre-coated Kieselgel 60 F_{254} aluminum sheets (Merck) and compounds were detected under UV fluorescence and spraying by 1% vanillin-H_2SO_4 reagent, followed by heating at 105°C for 1-2 min.

Plant material

Anchusa azurea Miller var. *azurea* was collected in July 2003 from Ankara-Beytepe, Turkey and identified by Prof. Dr. Hayri Duman, of the Department of Biology, Faculty of Sciences, Gazi University. A voucher specimen has been deposited at the Herbarium of Hacettepe University, Faculty of Pharmacy Ankara, Turkey under the number of HUEF 03012.

Extraction and isolation

Air dried and powdered roots of *Anchusa azurea* var. *azurea* (480 g) were extracted with 3 x 1.5 l methanol at 45 °C for 4 hours and combined MeOH extracts were concentrated under reduced pressure. MeOH extract (20 g) was chromatographed on a polyamide column eluting with methanol:water to give four main fractions (Fr. A-D). Fr. C (214 mg) was subjected to repeatedly normal phase column chromatography systems using EtOAc:CH_3OH: H_2O (100:5:1→100:10:5) and Sephadex LH-20 columns eluted with methanol to yield compound **1** (25 mg), compound **2** (5 mg), compound **3** (10 mg), compound **4** (15 mg) and compound **5** (37 mg).

Astragalin (=kaempferol 3-O-β-glucopyranoside, (**1** in Fig 1) Yellow amorphous powder; UV λ_{max} (MeOH) nm: 267, 350 ; IR υ_{max} (KBr) cm^{-1}: 1078, 1607, 1732, 3400; EIMS *m/z* 448 $[M]^+$ (calc. for $C_{21}H_{20}O_{11}$). ^{1}H (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz). NMR data of compound **1** (Table 1) were superimposable with those reported in the literature (11-14).

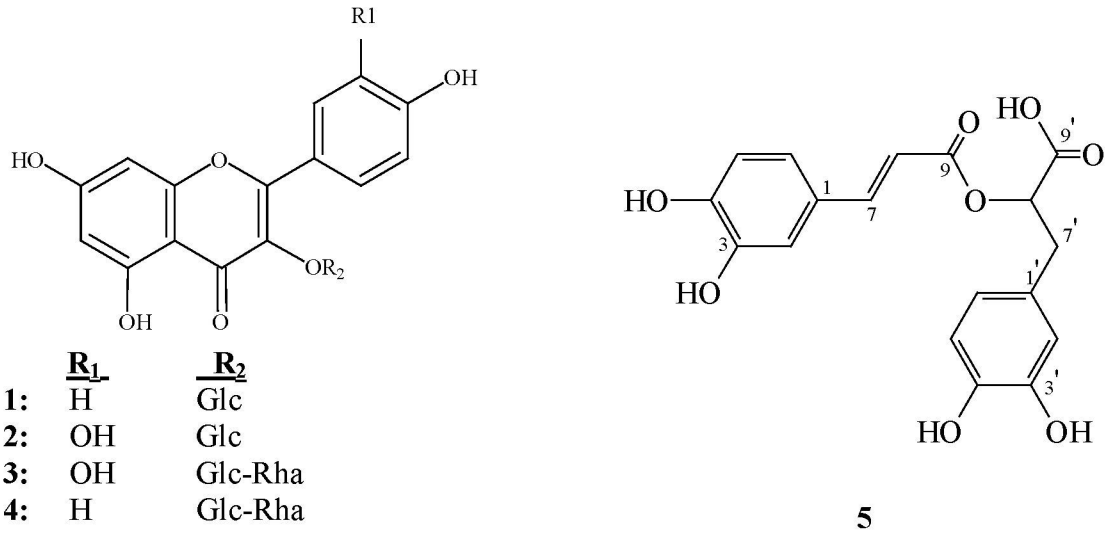
Isoquercitrin (=quercetin 3-O-β-glucopyranoside, (**2** in Fig 1) Yellow amorphous powder; UV (MeOH) λ_{max} 255, 365 nm; IR υ_{max} (KBr) cm^{-1}: 1603, 1654, 3421; EIMS *m/z* 464 $[M]^+$ (calc. for

$C_{21}H_{20}O_{12}$), ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz). NMR data of compound **2** (Table 1) were superimposable with those reported in the literature (11-14).

Rutin (=quercetin 3-O-α-rhamnopyranosyl (1'''→6'')-β-glucopyranoside, (**3** in Fig 1) Yellow amorphous powder; UV (MeOH) λ_{max} 255, 365 nm; IR υmax. (KBr) cm^{-1}: 1603, 1654, 3421; EIMS *m/z* 610 $[M]^+$, 611$[M+H]^+$ (calc. for $C_{27}H_{30}O_{16}$), ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz). NMR data of compound **3** (Table 2) were superimposable with those reported in the literature (11-14).

Kaempferol 3-O-α-rhamnopyranosyl (1'''→6'')-β-glucopyranoside, (**4** in Fig 1) Yellow amorphous powder; UV λ_{max} (MeOH) nm: 255, 365 ; IR υmax. (KBr) cm^{-1}: 1600, 1654, 3400; EIMS *m/z* 593 $[M]^+$, 594 $[M+H]^+$ (calc. for $C_{27}H_{30}O_{15}$). ^{1}H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100 MHz). NMR data of compound **4** (Table 2) were superimposable with those reported in the literature (11-14).

Rosmarinic acid (=α-O-transcaffeeoyl-3',4'-dihydroxyphenyllactic acid, (**5** in Fig 1) Cream colour, amorphous powder, UV λ_{max} (MeOH) 215, 296, 321 nm. EI-MS: *m/z* 360 $[M]^+$ (calc. for $C_{18}H_{16}O_8$). IR υ_{max} (KBr) cm^{-1} 1070, 1600, 1693, 1740, 3300. ^{1}H NMR (400 MHz, CD_3OD): δH 7.02 (*d*, *J* = 1.8 Hz, H-2), 6.75 (*d*, *J* =8.0 Hz, H-5), 6.91 (*dd*, *J* = 8.0/1.8 Hz, H-6), 7.50 (*d*, *J* = 15.7 Hz, H-7), 6.26 (*d*, *J*= 15.7 Hz, H-8), 6.75 (*d*, *J* = 1.8 Hz, H-2'), 6.66 (*d*, *J* = 8 Hz, H-5'), 6.62 (*dd*, *J* = 8/1.8 Hz, H-6'), 2.92 (*dd*, *J* = 14.3/9.5 Hz, Ha-7'), 3.08 (*dd*, *J* = 14.3/3.3 Hz, Hb-7'), 5.07 (*dd*, *J* = 9.5/4 Hz, H-8'). ^{13}C NMR (CD_3OD, 100 MHz): δC 126,8 (C-1), 114.4 (C-2), 145.5 (C-3), 148.2 (C-4), 115.2 (C-5), 121.7 (C-6), 145.5 (C-7), 113.9 (C-8), 167,9 (C-9), 129.9 (C-1'), 116,3 (C-2'), 144.8 (C-3'), 143.6 (C-4'), 115 (C-5'), 120.5 (C-6'), 37.5 (C-7'), 76.4 (C-8'), 176.2 (C-9').



	R_1	R_2
1:	H	Glc
2:	OH	Glc
3:	OH	Glc-Rha
4:	H	Glc-Rha

5

Figure 1. The structures of compounds **1-5**.

RESULTS AND DISCUSSION

Compounds **1** – **4** (Figure 1) were isolated as yellow amorphous powders. Their UV and IR spectra were characteristic of a flavonol system. While the NMR spectra of compound **1** and **2** (Table 1) indicated one glucose moiety, the NMR spectra of compound **3** and **4** (Table 2) exhibited one glucose and one rhamnose units.

The EIMS of **1** and **4** showed the ion peaks at *m/z* 448 $[M]^+$ and 593 $[M]^+$, respectively. Molecular formulae of compound **1** was determined as $C_{21}H_{20}O_{11}$ and compound **4** as $C_{27}H_{30}O_{15}$ by the NMR spectra and MS. The ^{1}H NMR spectrum of the compounds **1** and **4** indicated that B ring protons, H-6'and H-2' gave dublet (2H, *J*=8.8 Hz) at δ 8.05. H-3' and H-5' proton signals were observed at 6.88 ppm as a dublet (2H, *J*=8.8 Hz). H-6 and H-8 protons were at δ 6.20 (1H, d, *J*=2.0 Hz) and δ 6.40 (1H, d, *J*=2.0 Hz), respectively. Based on these findings and by comparison of NMR data, the aglycones of compounds **1** and **4** were identified as kaempferol. The proton signal appeared at δ 5.26 (1H, d, *J*=7.2 Hz) with the other resonances in the ^{1}H NMR spectrum of compound **1** was assigned to the anomeric proton of a β-glucose. In the ^{1}H NMR spectrum of compound **4,** which showed two anomeric proton signals at δ 5.12 (1H, d, *J*=7.6 Hz) and δ 4.51 d (1H, d, *J*=1.2 Hz) was revealed the presence of one β-glucose and one α-rhamnose. The ^{13}C NMR data confirmed the anomeric carbon resonances together with the other sugar resonances. The position of sugar units were determined by the HMBC experiment. On the basis of these data, the structure of the compound **1** was determined as kaempferol 3-*O*-*β*-glucopyranoside (=Astragalin) and the structure of the compound 4 was established as kaempferol 3-*O*-*α*-rhamnopyranosyl (1‴→6″)-*β*-glucopyranoside (11-14).

The molecular formula of compound **2** was determined as $C_{21}H_{20}O_{12}$ (MA:464) by the ^{1}H, ^{13}C NMR and EI Mass spectra. Based on the ^{1}H NMR spectrum of compound **2**, the data revealed H-6′-H-5′ *ortho*-coupling (8.4 Hz) at δ 7.58 ppm and δ 6.86 ppm respectively, and H-6′-H-2′ *meta*-coupling (*J*=2 Hz) at δ 7.58 and δ 7.70 ppm, respectively. The aromatic region exhibited another typical *meta*-coupled pattern for H-6 and H-8 protons (δ_H 6.18 and 6.37, *d*, *J* = 2.0 Hz). These results were identical with the quercetin. The ^{13}C NMR signals of **2** were assigned with the help of DEPT and HMQC experiments. The ^{13}C NMR spectrum of **2** showed the presence of 15 aromatic carbon signals. Additionally, one anomeric proton signal appeared at δ_H 5.22 (*d*, *J* = 7.6 Hz, H-1″) were assigned to the anomeric proton of β-glucose. The ^{13}C NMR data confirmed the anomeric carbon resonance together with the other sugar resonances. In the HMBC spectrum, a crosspeak between C-3 and H-1′ established the linkage point quercetin and sugar moiety. Based on the NMR data and comparison of the data given in the literature, the structure of compound **2** was identified as quercetin 3-*O*-*β*-glucopyranoside (=Isoquercitrin) (11-14).

The ^{1}H- and ^{13}C NMR spectra of **3** showed the presence of a quercetin moiety and sugar residue whose aglycone part was the same as that of compound **2**. However, other spectroscopic evidences indicated that compound **3** contained glucose and rhamnose, while compound **2** contained glucose as a sugar part.

The anomeric proton resonances of compound **3** were observed at δ_H 5.10 (*d*, *J*= 7.2 Hz, H-1″, δ_C 103.6) and δ_H 4.51 (1H, *d*, *J* = 1.2 Hz, H-1‴, δ_C 101.2). In the HMBC spectra of **3**, crosspeaks between H-1″→C-3 and H-1‴→C-6″ established the linkage point between quercetin and sugar moieties. EIMS displayed the molecular ion peak at *m/z* 610 $[M]^+$. Therefore, compound **3** were characterized as quercetin 3-*O*-*α*-rhamnopyranosyl (1‴→6″)-*β*-glucopyranoside (=Rutin) (11-14).

Table 1. ^{13}C ve ^{1}H NMR spectroscopic data of kaempferol 3-*O*-β-glucopyranoside (=Astragalin, **1**) and quercetin 3-*O*-β-glucopyranoside (=Isoquercitrin, **2**).

	1				**2**			
Atom No	DEPT	δ_C (ppm)	δ_H (ppm)	*J* (Hz)	DEPT	δ_C (ppm)	δ_H (ppm)	*J* (Hz)
2	C	157.4			C	157.3		
3	C	134.3			C	134.5		
4	C	178.4			C	178.3		
5	C	161.9			C	161.9		
6	CH	98.7	6.20 d	2.0	CH	98.9	6.18 d	2.0
7	C	164.9			C	165.3		
8	CH	93.6	6.40 d	2.0	CH	93.6	6.37 d	2.0
9	C	157.9			C	157.8		
10	C	104.6			C	104.4		
1′	C	121.6			C	121.9		
2′	CH	131.1	8.05 d	8.8	CH	114.8	7.70 d	2.0
3′	CH	114.9	6.88 d	8.8	C	144.7		
4′	C	160.4			C	148.7		
5′	CH	114.9	6.88 d	8.8	CH	116.4	6.86 d	8.4
6′	CH	131.1	8.05 d	8.8	CH	122.0	7.58 dd	8.4 / 2.0
Glucose								
1″	CH	102.9	5.26 d	7.2	CH	103.2	5.22 d	7.6
2″	CH	74.6	†		CH	74.6	†	
3″	CH	76.9	†		CH	77.0	†	
4″	CH	70.2	†		CH	70.1	†	
5″	CH	77.3	†		CH	77.2	†	
6″	CH_2	61.5	3.52 dd	12.0 / 5.4	CH_2	61.4	3.57 dd	12.0/5.4
			3.69 dd	12.0 / 2.0			3.71 dd	12.0/2.0

† Signal patterns are unclear due to overlapping.

Table 2. ^{13}C ve ^{1}H NMR spectroscopic data of Quercetin 3-*O*-α-rhamnopyranosyl (1‴→6″)-β-glucopyranoside (=Rutin, **3**), Kaempferol 3-*O*-α-rhamnopyranosyl (1‴→6″)-β-glucopyranoside (**4**)

	3				**4**			
Atom No	DEPT	δ_C (ppm)	δ_H (ppm)	*J* (Hz)	DEPT	δ_C (ppm)	δ_H (ppm)	*J* (Hz)
2	C	157.4			C	157.4		
3	C	134.5			C	134.3		
4	C	178.2			C	178.2		
5	C	161.8			C	161.8		
6	CH	98.9	6.20 d	2.0	CH	98.9	6.20 d	2.0
7	C	165.1			C	165.1		
8	CH	93.7	6.39 d	2.0	CH	93.8	6.40 d	2.0
9	C	158.1			C	158.2		
10	C	104.4			C	104.4		
1′	C	122.0			C	121.6		
2′	CH	114.9	7.66 d	2.0	CH	131.2	8.06 d	8.8
3′	C	144.7			CH	114.9	6.88 d	8.4
4′	C	148.7			C	160.3		
5′	CH	116.5	6.87 d	8.0	CH	114.9	6.88 d	8.4
6′	CH	122.4	7.63 dd	8.0/2.0	CH	131.2	8.06 d	8.8
Glucose								
1″	CH	103.6	5.10 d	7.2	CH	103.4	5.12 d	7.6
2″	CH	74.6	†		CH	74.6	†	
3″	CH	77.0	†		CH	77.0	†	
4″	CH	70.2	†		CH	70.3	†	
5″	CH	76.1	†		CH	76.1	†	
6″	CH_2	67.4	†		CH_2	67.4	†	
Rhamnose								
1‴	CH	101.2	4.51 d	1.2	CH	101.2	4.51 d	1.2
2‴	CH	70.9	†		CH	70.9	†	
3‴	CH	71.1	†		CH	71.1	†	
4‴	CH	72.8	†		CH	72.7	†	
5‴	CH	68.5	†		CH	68.6	†	
6‴	CH_3	16.7	1.11 d	5.7	CH_3	16.7	1.11 d	5.6

† Signal patterns are unclear due to overlapping.

Grey amorphous compound **5** gave the pink-purplish spot with 1 % vanillin/ sulfuric acid on TLC plate. The IR spectrum exhibited absorbtion bands due to hydroxyl groups (3300 cm^{-1}) and carboxylic acid function (1693, 1740 cm^{-1}). The molecular formula of compound **5** was found as $C_{18}H_{16}O_8$ (MA:360) by the ^{1}H NMR, ^{13}C NMR and EIMS. The ^{1}H NMR spectrum exhibited two aromatic ABX type signals at δ_H 6.62 (*dd*, *J* = 8/1.8 Hz), 6.66 (*d*, *J* = 8 Hz) and 6.75 (*d*, *J* = 1.8 Hz) assignable to H-6', H-5' ve H-2', respectively and at δ_H 6,75 (*d*, *J* =8.0 Hz, H-5), 6,91 (*dd*, *J* = 8.0/1.8 Hz, H-6) and 7,02 (*d*, *J* = 1.8 Hz, H-2) assignable to H-5, H-6 ve H-2, respectively. The doublet–doublet signals at δ_H 2.92 (*dd*, *J* = 14.3/9.5 Hz, H_a-7') and δ_H 3,08

(*d*, *J* = 14.3/3.3 Hz, H_b -7') and the peaks at δ_H 5.07 (*dd*, *J* = 9.5/4 Hz, H-8') together with ^{13}C NMR data [37.5 (C-7'), 76.4 (C-8'), 176.2 (C-9')] showed the presence of 3,4-dihydroxyphenyllactic acid unit. Additionally, the signals of olefinic protons at δ_H 7,50 (*d*, *J* = 15.7 Hz, H-7) and 6,26 (*d*, *J*= 15.7 Hz, H-8) suggested the compound to be a transcaffeoyl ester of 3,4-dihydroxyphenyllactic acid. ^{13}C-NMR spectrum of **5** showed signals attributable to twelve aromatic carbons at δ_C 126,8 (C-1), 114.4 (C-2), 145.5 (C-3), 148.2 (C-4), 115.2 (C-5), 121.7 (C-6), 129.9 (C-1'), 116,3 (C-2'), 144.8 (C-3'), 143.6 (C-4'), 115 (C-5'), 120.5 (C-6'), one ester carbonyl at 167.9 ppm (C-9) and one carboxylic acid at δ_C 176.2 (C-9'). These chemical shifts corroborated by ^1H-^1H COSY and HMQC studies. The manner in which the caffeic acid and 3,4-dihydroxyphenyllactic acid were linked together in **5** was established by application of HMBC which showed correlations from H-8' (δ 5.07) to C-9 (δ 167.9), C-1' (δ 129.9) and C-9' (δ 176.2), hence, the ester linkage was proved to be between the α-OH of phenyl lactic acid and the COOH (9) of the caffeoyl unit. Therefore, compound **5** was assumed to be *α-O*-transcaffeoyl-3′,4′-dihydroxyphenyllactic acid (=Rosmarinic acid) (15, 16).

This is the first report on the isolation of phenolic compounds from the roots of *A. azurea* var. *azurea* growing in Turkey. All isolated phenolics are bioactive compounds (17, 18). Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's). It is known that phenolic compounds which provide protection against a lot of diseases are very important. The main compounds of *A. azurea* roots are phenolic subtances which possessed significant its biological properties.

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