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Secondary Metabolites from Bioactive Methanolic Extract of *Verbascum pycnostachyum* Boiss. & Helder Flowers

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Introduction

The genus *Verbascum*, which is known as 'mullein', is represented by 228 species, 185 of which are endemic to Turkey[1]. The leaves, flowers and whole aerial parts of *Verbascum* L. species have been used to treat respiratory problems, eczema and other types of inflammatory skin conditions in traditional Turkish medicine. They have also been widely utilized as a folk medicine to have a soothing and anti-inflammatory effect on the urinary tract. Additionally, various species are commonly used to treat hemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhea. They are traditionally consumed as a tea to relieve abdominal pains[2-4].

The iridoid and phenylethanoid glycosides are widely distributed in the genus *Verbascum*[5]. Although the taxonomic and morphological aspects of the genus *Verbascum* appear more or less complex, the frequent occurrence of the iridoid and phenylethanoid glycosides in the Scrophulariaceae has been used in chemotaxonomic studies[6, 7]. Iridoids display an interesting spectrum of biological activity such as anti-inflammatory[8]. Likewise, phenylethanoid glycosides are known to possess antioxidant activity[9].

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In order to evaluate folkloric utilization, both antinociceptive and anti-inflammatory activities of endemic *Verbascum* species, *V. pycnostachyum* Boiss. & Heldr. was investigated in our previous studies. Antinociceptive activity was investigated via *p*-benzoquinone-induced writhing test, while the anti-inflammatory activity was studied using carrageenan-induced hind paw edema, PGE_1- induced hind paw edema and 12-*O*-tetradecanoyl-13-acetate (TPA)-induced mouse ear edema models in mice. The methanolic extract of the flowers of *V. pycnostachyum* displayed significant antinociceptive and anti-inflammatory activity at 200 mg/kg dose, per os, without inducing any apparent acute toxicity as well as gastric damage[10].

As a part of our continuing search for bioactive agents from *Verbascum* species, we here have report the results of the isolation and structure elucidation of iridoid glycosides, aucubin[1], ajugol[2], ajugoside[3], harpagoside[4] and a phenylethanoid glycoside, verbascoside[5] from the bioactive methanolic extract of *Verbascum pycnostachyum* Boiss. & Helder flowers, which is an endemic species distributed in South Anatolia[1].

Materials and Methods

2.1. General Experimental Procedures

The UV spectra (λ_{max}) were recorded on a Hitachi HP 8452 A spectrophotometer. The IR spectra (ν_{max}) were determined on ATI Mattson Genesis Series FTIR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were obtained on Bruker Avance DRX 500 and 300 spectrometer operating at 500 and 300 MHz for ^{1}H NMR and at 125 and 75 MHz for ^{13}C NMR spectra. The chemical shift values are reported as parts per million (ppm) relative to tetramethylsilane (TMS), and the coupling constants are in hertz (Hz, in parentheses). LC-ESIMS FT data were obtained using a Bruker BioApex FT-MS instrument in the ESI mode. Reverse-phase material (C-18, Sepralyte 40 µm) was used for vacuum liquid chromatography (VLC). Medium pressure liquid chromatography (MPLC) separations were performed on a Labomatic glass column packed with LiChroprep RP-18 (Merck), using a Lewa M5 peristaltic pump. Si gel (230-400 mesh) (Merck) and Sephadex LH-20 were used for column chromatography (CC). Pre-coated silica gel 60 F_{254} aluminum sheets (Merck) were used for thin-layer chromatography (TLC) with developing solvent-system, $CHCl_3$-MeOH-H_2O (61:32:7).

Plates were examined by UV fluorescence and sprayed with 1% vanillin in concentrated H_2SO_4, followed by heating at 105°C for 1-2 mins.

2.2. Plant Material

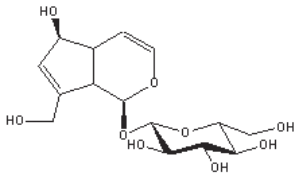
Verbascum pycnostachyum Boiss.& Heldr. (Scrophulariaceae) was collected from Mut to Karaman, 1300 m, in June 2000. A voucher specimen was deposited in the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 00182).

2.3. Extraction and Isolation

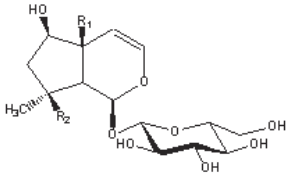
The air-dried and powdered flowers of *Verbascum pycnostachyum* (320.46 g) were extracted twice with MeOH (2x2000 ml) at 40°C. After evaporation of the combined extract in vacuo, 48.71 g MeOH extract was obtained. The isolation of compounds was guided on TLC autographic assay using 0.2 % DPPH solution in MeOH to search for potential antioxidant molecules. The crude extract (48.71 g) was fractionated by vacuum-liquid chromatography over reverse-phase material (VLC, 350 g), eluting with H_2O and gradient MeOH-H_2O mixtures (5-30 %) to yield compounds 1 (326.9 mg), 2 (317.1 mg) and fraction A. Fraction A (1.3 g) was subjected to vacuum liquid chromatography (VLC) using reversed-phase material (Sepralyte 40 µm, 175 g), employing MeOH/H2O mixtures (0-50 %) to give compound 5 (152.4 mg) and fraction A1. Fraction A1 (278.5 mg) was carried out on C_{18}-MPLC using gradient H2O-MeOH mixtures (10-100%) to give fractions A1a-d. Fraction A1a (53.6 mg) was chromatographed on a Si gel column (8 g) eluted with $CHCl_3$-MeOH mixtures (90:10, 85:15, 80:20, 70:30) and $CHCl_3$-MeOH-H_2O mixtures (80:20:2) to yield compound 3 (8.5 mg) and fraction A1aI. Fraction A1aI (6.7 mg) was further purified on a Sephadex LH-20 (10 g) column using MeOH to give compound 4 (3.3 mg).

Results and Discussion

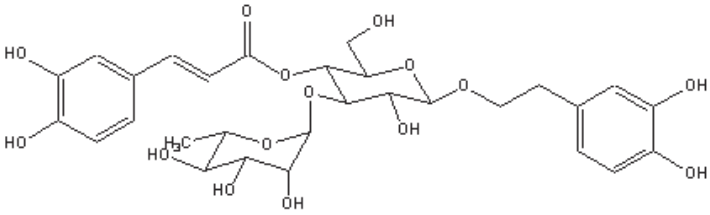
Compounds 1-5 were isolated from the methanolic extract of the flowers of *Verbascum pycnostachyum* by a combination of vacuum liquid chromatography (VLC) and open column chromatographic methods, with the following results (Fig.).



Aucubin (**1**)



	R_1	R_2
Ajugol (2)	H	OH
Ajugoside (3)	H	$OCOCH_3$
Harpagoside (4)	OH	trans-cinnamoyl



Verbascoside (**5**)

Figure 1

Isolated compounds from Verbascum pycnostachyum

Aucubin (1): UV (MeOH, λ_{max}, nm): 202. IR (KBr, ν_{max}, cm^{-1}): 3630 (OH), 1665 (C=C), 1545, 1360 (aromatic ring). Positive ion LC-ESIMS *m/z* 368 ($(M+Na)^+$, calc. for $C_{21}H_{32}O_{13}$). ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data were superimposable with those reported in the literature[11].

Ajugol (2): UV (MeOH, λ_{max}, nm): 220. IR (KBr, ν_{max}, cm^{-1}): 3470 (OH), 1655 (C=C). Positive ion- LC-ESIMS *m/z* 370 ($(M+Na)^+$, calc. for $C_{15}H_{24}O_9$). ^1H (500 MHz, DMSO-d_6) and ^{13}C (125 MHz, DMSO-d_6) NMR data (Table) were superimposable with those reported in the literature[12].

TABLE I
^1H- and ^{13}C-NMR (500 and 125 MHz, DMSO-d_6) data of compounds **2, 3.**

	2			3		
	δ_C	δ_H	J (Hz)	δ_C	δ_H	J (Hz)
Aglycone						
1	92.7	5.45 s	-	92.1	5.63 s	-
3	139.4	6.15 d	5.1	139.7	6.20 d	6.4
4	104.9	4.85 †	-	103.4	4.70 d	6.4
5	40.3	2.72 m	-	39.7	2.62 m	-
6	77.2	3.90 †	-	74.5	3.86 d	7.6
7a	49.0	1.79 dd	4.5/13.4	47.3	2.00 dd	5.5/15.0
7b		2.04 dd	5.6/13.4		2.10 d	15.0
8	78.5	-	-	87.8	-	-
9	50.8	2.54 d	9.4	48.0	2.66 m	-
10	24.2	1.31 s	-	22.3	1.45 s	-
$O\underline{C}OCH_3$	-			170.0	-	-
$OCO\underline{C}H_3$	-			22.0	1.94 s	-
β-Glucose						
1′	98.4	4.64 d	7.9	97.8	4.46 d	7.8
2′	73.8	3.15-3.40 †	-	73.0	2.96 t	8.9
3′	76.8	3.15-3.40 †	-	76.6	3.14 t	8.9
4′	70.7	3.19 t	8.7	70.0	3.05 d	9.0
5′	77.0	3.15-3.40 †	-	76.8	3.13 m	-
6′a	61.9	3.66 dd	4.8/11.6	61.1	3.46 dd	6.0/12.0
6′b		3.89 †	-		3.69 d	12.0

† Signal patterns are unclear due to overlapping

Ajugoside (3): UV (MeOH, λ_{max}, nm): 224. IR (KBr, ν_{max}, cm^{-1}): 3450 (OH), 1705 (C=O), 1650 (C=C). Positive ion- LC-ESIMS *m/z* 412 ($(M+Na)^+$, calc. for $C_{17}H_{26}O_{10}$). ^1H (500 MHz, DMSO-d_6) and ^{13}C (125 MHz, DMSO-d_6) NMR data (Table) were superimposable with those reported in the literature[13, 14].

Harpagoside (4): UV (MeOH, λ_{max}, nm): 228. IR (KBr, ν_{max}, cm^{-1}): 3600 (OH), 1705 (C=O), 1637 (C=C), 1604, 1363 (aromatic ring). Positive ion LC-ESIMS *m/z* 517 ($(M+Na)^+$, calc. for $C_{24}H_{30}O_{11}$). ^1H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) data were superimposable with those reported in the literature[9].

Verbascoside {= acteoside, (β-(3,4-dihydroxyphenyl)-ethyl)-(3′-*O*-α-L-rhamnopyranosyl)-(4′-*O*-caffeoyl)-β-D-glucopyranoside} (5): UV (MeOH, λ_{max}, nm): 212, 332. IR (KBr, ν_{max}, cm^{-1}): 3689 (OH), 1708 (C=O), 1634 (C=C), 1604, 1515, 1385 (aromatic ring). Positive ion-LC-ESIMS *m/z* 647 ($(M+Na)^+$, calc. for $C_{29}H_{36}O_{15}$). ^1H NMR (300 MHz, DMSO-d_6) and ^{13}C NMR (75 MHz, DMSO-d_6) data superimposable with those reported in the literature[9].

Compound **1** was obtained as an amorphous powder. Its structure was identified as aucubin[15] by comparing its ^1H and ^{13}C NMR data with previously published data and by direct comparison with an authentic sample on a TLC plate[11].

Compound **2** was isolated as a yellow amorphous powder with the molecular formula $C_{15}H_{24}O_9$ (LC-ESIMS *m/z* 370.9 $(M+Na)^+$). Its UV spectrum suggested the presence of an iridoid enolether system (220 nm) and in its IR spectra absorption bands were typical for a hydroxyl group (3416 cm^{-1}) and a double bond (1656 cm−1). The ^1H and ^{13}C NMR spectra of **2** (*see* Table) are superimposible with those of ajugol[12]. Based on this evidence, compound **2** was identified as ajugol.

Compound **3** proved to have the molecular formula $C_{17}H_{26}O_{10}$, as seen from the positive-ion ESIMS (*m/z* 412 $(M+Na)^+$) combined with ^1H and ^{13}C NMR data (*see* Table). The UV and IR data of compound **3** showed that **3** consist of a non-conjugated enol-ether system. The ^1H NMR signals at δ_H 6.20 (*d*, *J* = 6.4 Hz), 4.70 (*d*, *J* = 6.4 Hz) were attributed to H-3 and H-4, respectively, whose chemical shift values and multiplicities indicated that C-5 was non-substituted. This assumption was also supported by the H-9 signal (δ_H 2.66, *m*). On the other hand, the multiplet signal at

δ_H 3.86 was attributed to an oxymetine proton at C-6 (δ_C 74.5), which was coupled to H_2-7 (δ_H 2.00, *dd*, *J* = 5.5/15.0 and 2.10, *d*, *J* =15.0 Hz) methylene protons. In the ^{1}H NMR spectrum of **3**, δ_C 170.0 and 22.0 signal patterns implied the presence of an acethyl group. Thus, the location of the acethyl group was ascertained from downfield acetylation shifts (ca. 9.3 ppm) observed for C-8 (δ_C 87.8) resonance comparing with that of ajugol (δ_C 78.5)[12]. Accordingly, the structure of **3** was determined to be ajugoside[13, 14].

Compound 4 was obtained as amorphous powder whose UV spectra indicated its non-conjugated enol-ether functional group. Its IR spectra showed absorption bands typical of conjugated carbonyl groups. The molecular formula of compound **4** was determined by LC-ESIMS, which exhibited a pseudomolecular ion at *m/z* 517 $(M+Na)^+$, and ^{1}H and ^{13}C NMR data as $C_{24}H_{30}O_{11}$. The ^{1}H NMR spectrum of **4** revealed the resonances of two olefinic protons, observed as an AX system, at δ_H 6.47 and 7.53 (*d*, J_{AX}= 16.0 Hz) and 5 aromatic protons at δ_H 7.34 (1H), 7.35 (2H) and 7.62 (2H), consistent with the presence of a *trans*-cinnamoyl moiety. The chemical shift values of both C-8 and H_3-10 indicated that the acyl group was attached at C-8. From the above findings and comparison with the published data, compound **4** was considered identical to harpagoside[9].

Compound **5** was also obtained as an amorphous powder. Its structure was identified as verbascoside[9] by comparing its ^{1}H and ^{13}C NMR data with previously published data and by direct comparison with the authentic sample on a TLC plate.

Conclusion

Concerning the iridoid and phenylethanoid glycosides of the genus *Verbascum,* the isolation of iridoid glucosides, aucubin[1], ajugol[2], harpagoside[4] and a phenylethanoid glycoside, verbascoside[5] from several other *Verbascum* species has been reported previously[5]. It is well known that these compounds are common iridoid and phenyethanoid glycosides and taxonomic markers in the genus *Verbascum* and family Scrophulariaceae. To the best of our knowledge, ajugoside[3] has been isolated from *Verbascum* species for the first time. Additionally, this is the first report on the isolation and characterization of all these compounds from *Verbascum pycnostachyum* as well as a *Verbascum* species from Group K of the ge-

nus (1), although several sterols such as β-sitosterol and stigmasterol were isolated from *V. pycnostachyum* in previous studies[15]. Our continuing studies will be of assistance in clarifying the chemotaxonomic classification of the genus *Verbascum.*

Results of our previous study had clearly demonstrated that the methanolic extract of the flowers of *Verbascum pycnostachyum* possess significant antinociceptive and anti-inflammatory activities which support the traditional utilization in Turkey[10]. The isolated compounds, aucubin[1] was also found to possess significant antinociceptive and anti-inflammatory activities, per os without inducing any apparent acute toxicity or gastric damage[8]. Harpagoside[4] and verbascoside[5], exhibited a dose-dependent inhibition of bioautographic and spectrophotometric DPPH activities[9, 16].

In connection with the role of aucubin as well as the roles of harpagoside and verbascoside which were identified as free radical scavengers of *V. pycnostachyum*, it seems that they could be synergistic with each other in the methanolic extract. In order to correlate the obtained data in the field, further examinations in different assays can be evaluated.

Summary

Secondary Metabolites from Bioactive Methanolic Extract of *Verbascum pycnostachyum* Boiss. & Helder Flowers

Four iridoid glucosides, aucubin[1], ajugol[2], ajugoside[3], harpagoside[4], and a phenylethanoid glycoside, verbascoside[5] were isolated from the flowers of bioactive methanolic extract of *Verbascum pycnostachyum* Boiss & Helder. The structures of the compounds were determined from spectral methods (UV, IR, 1D NMR and Mass Spec.). Ajugoside[3] is encountered for the first time from *Verbascum* species.

Keywords: Scrophulariaceae, *Verbascum pycnostachyum* Boiss & Helder, iridoid glucosides, phenylethanoid glycoside.

Özet

***Verbascum pycnostachyum* Boiss. & Helder Çiçeklerinin Biyoaktif Metanol Ektresinin Sekonder Metabolitleri**

Verbascum pycnostachyum Boiss & Helder'in çiçekli kısımlarının biyoaktif methanol ekstresinden, dört iridoit glukoziti, okubin[1], aju-

gol[2], ajugozit[3], harpagozit[4] ve bir feniletanoit glikoziti, verbaskozit[5] izole edilmiştir. Bileşiklerin yapıları spektral yöntemlerle (UV, IR, 1D NMR ve Mass Spektr.) tespit edilmiştir. Ajugozit[3] ilk defa *Verbascum* türlerinden elde edilmiştir.

Anahtar kelimeler: Scrophulariaceae, *Verbascum pycnostachyum* Boiss & Helder, iridoit glukozitleri, feniletanoit glikoziti.

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