Flavonoid, Phenylethanoid and Iridoid Glycosides from *Globularia aphyllanthes*

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A new flavone glycoside, 6-hydroxyluteolin 7-*O*-[6‴-benzoyl-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside (aphyllanthoside, **1**) was isolated from the MeOH extract of the aerial parts of *Globularia aphyllanthes*. Besides this new compound, two flavonoid glycosides (6-hydroxyluteolin 7-*O*-[6‴-(*E*)-caffeoyl-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside and isoquercitrin), three phenylethanoid glycosides (verbascoside, rossicaside A, and trichosanthoside A), and 11 iridoid glycosides (aucubin, catalpol, 10-*O*-benzoylcatalpol, globularin, asperuloside, besperuloside, asperulosidic acid, daphylloside, scandoside, alpinoside and baldaccioside) were also obtained and characterized. Identification of the isolated compounds was carried out by spectroscopic analysis including 1D and 2D NMR experiments as well as HRMS.

Key words: *Globularia aphyllanthes*, Plantaginaceae, Flavonoids, Aphyllanthoside, Phenylethanoid and Iridoid Glycosides

Introduction

Globularia aphyllanthes Crantz (Plantaginaceae) is a perennial plant, distributed from northwest Turkey to central and southern Europe [1]. Some members of the genus *Globularia* are used as hypoglycaemic, laxative, and cholagogue agents and for the treatment of haemorrhoids in the folk medicines of some Mediterranean countries [2, 3]. *G. aphyllanthes* (Syn: *G. vulgaris* sensu Lam.) has only been investigated for its iridoid contents so far [4]. In the continuation of our work on secondary metabolites of the genus *Globularia* from Turkey [5 – 10], we have examined the chemical constituents of *G. aphyllanthes*. We describe herein the isolation and structure elucidation of a new flavone glycoside, aphyllanthoside (**1**), as well as 16 known metabolites (**2** – **17**).

Experimental Section

General experimental procedures

Optical rotations were measured on a JASCO DIP 1000 polarimeter. UV and IR spectra were recorded on a HP Agilent 8453 spectrophotometer and a Perkin-Elmer 2000 FT-IR spectrometer, respectively. NMR experiments were performed on a Bruker DRX-600 spectrometer equipped with a Bruker 5 mm TCI CryoProbe. All spectra were acquired in the phase sensitive mode, and the TPPI method was used for quadrature detection in the ω_1 dimension. The ^{1}H, gCOSY, gHSQC, and gHMBC NMR experiments were run under standard conditions at 300 K, dissolving each sample in 500 μL of 99.8 % CD_3OD (Carlo Erba) (^{1}H, δ = 3.34 ppm; ^{13}C, δ = 49.0 ppm). ESI-MS analyses were performed using a ThermoFinnigan LCQ Deca XP Max ion trap mass spectrometer equipped with XCALIBUR software. Samples were

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dissolved in MeOH (Baker) and infused in the ESI source by using a syringe pump; the flow rate was 3 μL min^{-1}. The capillary voltage was 43 V, the spray voltage was 5 kV, and the tube lens offset was 30 V. The capillary temperature was 280 °C. Exact masses were measured by a MALDI micro MX instrument (Waters), a high-performance matrix-assisted laser desorption/ionization time of flight mass spectrometer (MALDI-TOF-MS). A mixture of analyte solution and epigallocatechin gallate, glycyrrhizic acid and pentagalloylglucose was applied to the metallic sample plate and dried. Mass calibration was performed with the protonated ions from epigallocatechin gallate at 459.0927 Da, glycyrrhizic acid at 823.4116 Da and pentagalloylglucose at 941.1259 Da as internal standards. TLC analyses were carried out on silica gel 60 F254 precoated plates (Merck, Darmstadt), detection by 1 % vanillin/H_2SO_4. For medium-pressure liquid chromatographic (MPLC) separations, a Combi Flash Companion (Isco), was used with Redi step columns packed with LiChroprep C_{18} (130 g and 13 g, Teledyne Isco). Silica gel 60 (0.063 – 0.200 mm; Merck, Darmstadt), polyamide (Fluka) and Sephadex LH-20 (Fluka) were utilized for open column chromatography (CC).

Plant material

Globularia aphyllanthes Crantz was collected from Vize, Kırklareli, Turkey, in June 2007. A voucher specimen (Akaydın 11537) has been deposited at the Herbarium of the Faculty of Education, Hacettepe University, Ankara, Turkey.

Extraction and isolation

The air-dried and powdered aerial parts of *G. aphyllanthes* (240 g) were extracted two times with MeOH (1 L × 2.4 h) at 45 °C. The methanolic extracts were combined to a residue (50 g). The extract was suspended in H_2O (100 mL), and then extracted with equal volumes of $CHCl_3$ (100 mL × 3). The H_2O-soluble portion provided 36 g of an extract. An aliquot of the H_2O extract (30 g) was loaded onto a polyamide column eluting with H_2O/MeOH mixtures to yield seven fractions, A–G. Fraction A (11.5 g) was dissolved in H_2O (20 mL) and partitioned with *n*-BuOH (20 mL × 3). The *n*-BuOH layer was evaporated and lyophilized (2.64 g), and an aliquot of it (1.5 g) was subjected to C_{18}-medium-pressure liquid chromatography (C_{18}-MPLC, 130 g) eluting with stepwise H_2O-MeOH gradient (0 to 80 % MeOH) to obtain catalpol (**8**, 115 mg), aucubin (**7**, 24 mg), and asperuloside (**11**, 320 mg), in addition to four fractions, frs. A_{1-4}. Fraction A_1 (30 mg) was rechromatographed on a SiO_2 column ($CHCl_3$-MeOH-H_2O, 95 : 5 : 0 to 80 : 20 : 1) to give daphylloside (**14**, 4 mg) and alpinoside (**16**, 4 mg). Likewise, fraction A_2 (64 mg) was applied to a SiO_2 column ($CHCl_3$-MeOH-H_2O, 90 : 10 : 1 to 80 : 20 : 1) to yield 10-*O*-benzoylcatalpol (**9**, 12 mg) and alpinoside (**16**, 7 mg). Globularin (**10**, 23 mg) was purified from fraction A_3 (73 mg) by using SiO_2 CC ($CHCl_3$-MeOH-H_2O, 85 : 15 : 1 to 80 : 20 : 1). Similarly, fraction A_4 (36 mg) was subjected to a SiO_2 column using $CHCl_3$-MeOH-H_2O (90 : 10 : 1 and 85 : 15 : 1) to give besperuloside (**12**, 19 mg) and baldaccioside (**17**, 3 mg). Fraction C (900 mg) was separated by C_{18}-MPLC (130 g, 10 – 80 % MeOH-H_2O, gradient) to obtain frs. C_{1-9}. Rechromatography of fraction C_1 (40 mg) on a SiO_2 column ($CHCl_3$-MeOH-H_2O, 90 : 10 : 1 to 70 : 30 : 3) afforded scandoside (**15**, 8 mg). Likewise, fraction C_2 (26 mg) was applied to a SiO_2 column ($CHCl_3$-MeOH-H_2O, 90 : 10 : 1 and 80 : 20 : 2) to obtain catalpol (**8**, 13 mg). Asperulosidic acid (**13**, 5 mg) was purified from fr. C_3 (60 mg) by SiO_2 CC ($CHCl_3$-MeOH-H_2O, 90 : 10 : 0 and 80 : 20 : 1).

Table 1. ^{13}C and ^{1}H NMR spectroscopic data for aphyllanthoside (**1**), (CD_3OD, ^{13}C NMR: 150 MHz; ^{1}H NMR: 600 MHz; δ in ppm, J in Hz)[a].

Position	δ_C	δ_H (J)
Aglycone		
2	166.8	–
3	103.5	6.56 s
4	184.5	–
5	147.8	–
6	132.8	–
7	152.3	–
8	97.2	7.00 s
9	151.1	–
10	108.0	–
1′	123.7	–
2′	114.3	7.39 d (1.8)
3′	147.0	–
4′	151.0	–
5′	116.6	6.92 d (8.3)
6′	120.5	7.40 dd (8.3, 1.8)
Glucose		
1″	102.4	5.15 d (7.7)
2″	83.8	3.83 dd (9.1, 7.7)
3″	77.2	3.74 t (8.9)
4″	71.5	3.51 t (8.9)
5″	78.3	3.55 m
6″	62.4	3.96 dd (12.1, 2.0)
		3.78 dd (12.1, 3.8)
Glucose (T)		
1‴	105.8	4.84 d (8.1)
2‴	75.8	3.38 dd (9.1, 8.1)
3‴	77.6	3.51 t (8.9)
4‴	70.8	3.50 t (8.9)
5‴	75.7	3.72 m
6‴	64.8	4.38 m
Benzoyl		
1⁗	131.0	–
2⁗/6⁗	130.5	7.90 dd (8.3, 1.2)
3⁗/5⁗	129.3	7.32 t (7.7)
4⁗	133.9	7.45 t (7.5)
C=O	167.8	–

[a] Assignments based on 1D TOCSY, 2D COSY, HSQC and HMBC experiments.

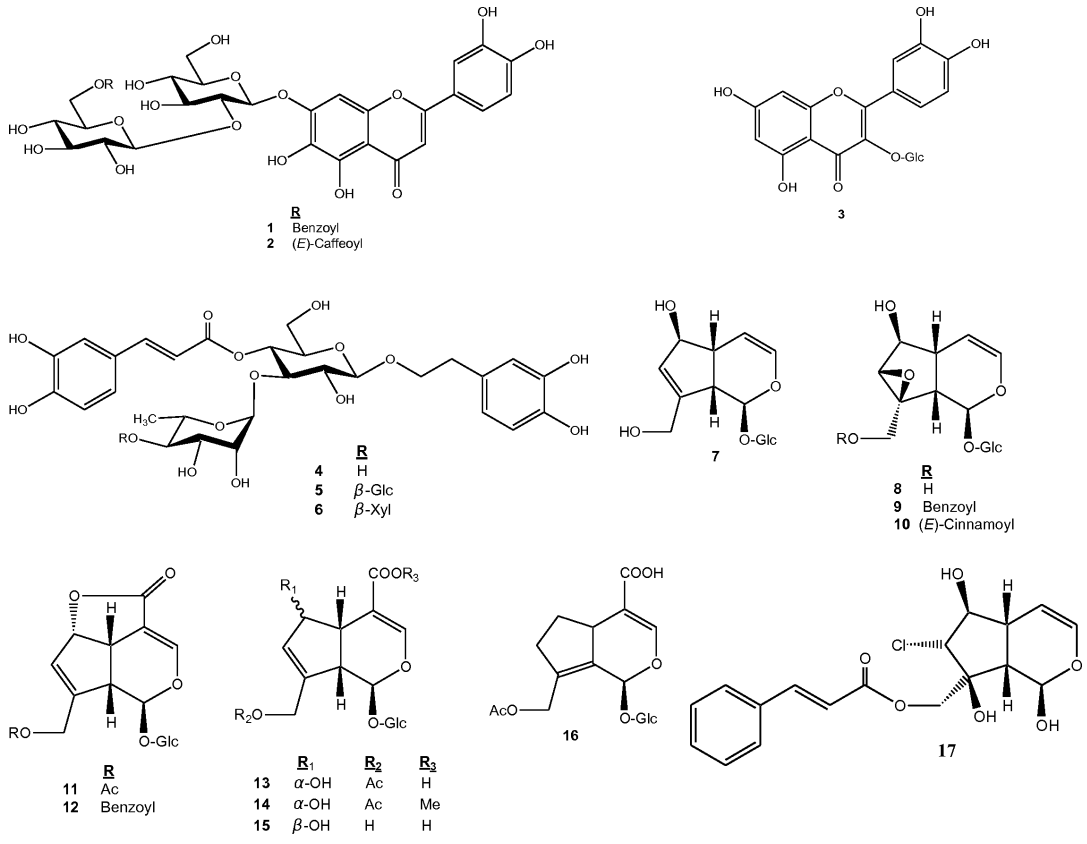

Fig. 1. Secondary metabolites (**1** – **17**) of *G. aphyllanthes*.

Fr. E (850 mg) was further chromatographed by C_{18}-MPLC (130 g, 20 – 80 % MeOH-H_2O, gradient) and yielded three fractions, frs E_{1-3}. Fraction E_1 (100 mg) was purified by SiO_2 CC ($CHCl_3$-MeOH-H_2O, 90 : 10 : 1 to 70 : 30 : 3) to give rossicaside A (**5**, 6 mg). Similarly, fr. E_2 (200 mg) was applied to a SiO_2 column ($CHCl_3$-MeOH-H_2O, 85 : 15 : 1 to 70 : 30 : 3) to give trichosanthoside A (**6**, 43 mg) in addition to fr. E_{2a} (40 mg). The latter was purified by C_{18}-MPLC (13 g, 20 – 40 % MeOH-H_2O, gradient) to yield verbascoside (**4**, 15 mg). Fr. G (1 g) was separated by C_{18}-MPLC (130 g, 40 – 80 % MeOH-H_2O, gradient) to obtain frs. G_{1-3}. Fr. G_1 (110 mg) was purified by chromatography on C_{18}-MPLC (130 g, 20 – 80 % MeOH-H_2O, gradient) to obtain **2** (41 mg). Fr. G_2 (85 mg) was subjected to a SiO_2 column (CH_2Cl_2-MeOH-H_2O, 85 : 15 : 1 to 70 : 30 : 3) to give isoquercitrin (**3**, 3 mg). Compound **1** (aphyllanthoside, 9 mg) was isolated by SiO_2 CC (CH_2Cl_2-MeOH-H_2O, 85 : 15 : 1 to 70 : 30 : 3) and Sephadex CC (MeOH) successively.

Aphyllanthoside (**1**): Amorphous powder; $[\alpha]_D^{24} = -22.9$ (c = 0.20, MeOH). – UV (MeOH). – λ = 217 (sh), 289, 335 nm. – IR (KBr): ν = 3400, 2935, 1699, 1662, 1604, 1516, 1452, 1359, 1283 cm^{-1}. – HRMS (MALDI-TOF): *m/z* = 731.6402 (calcd. 731.6411 for $C_{34}H_{35}O_{18}$, $[M+H]^+$). – ESI-MS: *m/z* = 729 $[M-H]^-$, 607 $[M-H-121]^-$, 445 $[M-H-121-162]^-$. – ^{1}H NMR (600 MHz, CD_3OD): Table 1. – ^{13}C NMR (150 MHz, CD_3OD): Table 1.

Results and Discussion

The H_2O soluble portion of the MeOH extract of *G. aphyllanthes* was fractionated by a polyamide column. Further chromatographic separation of these fractions led to the isolation of a total of 17 compounds (see Fig. 1), one of which is the new compound **1**.

Compound **1** was obtained as an amorphous yellow powder. Its molecular formula was determined to be $C_{34}H_{34}O_{18}$ on the basis of the ion peak at *m/z* = 731.6402 $[M+H]^+$ (calcd. 731.6411 for $C_{34}H_{35}O_{18}$) in the HR-MALDI-TOFMS spectrum. The ^{1}H NMR spectrum of **1** (see Table 1) showed the characteristic signals arising from the 1,3,4-trisubstituted ring B [three aromatic protons as an *ABX* system at δ_H = 7.40 (dd, J = 8.3 and 1.8 Hz), 7.39 (d, J = 1.8 Hz)

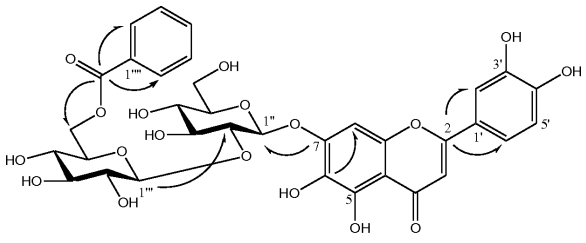

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Fig. 2. Key HMBC (C → H) for **1**.

and 6.92 (d, J = 8.3 Hz)]. Moreover, two singlets at δ_H = 7.00 and 6.56 together with the quaternary carbon resonance which appeared at δ_C = 132.8 (C-6) in the ^{13}C spectrum were consistent with the 6-hydroxyflavone skeleton as the aglycone [11]. The aromatic region of the ^{1}H NMR spectrum of **1** also contained resonances at δ_H = 7.90 (2H), 7.45 (1H) and 7.32 (2H) which were typical of a benzoyl moiety. The two anomeric proton and carbon resonances (δ_H = 5.15 d, J = 7.7 Hz; δ_C = 102.4 and δ_H = 4.84 d, J = 8.1 Hz; δ_C =105.8) observed in the ^{1}H and ^{13}C NMR spectra indicated the diglycosidic structure of **1**. The complete assignments of all proton and carbon resonances of the disaccharide unit based on the 1D TOCSY, COSY, HSQC and HMBC (see Fig. 2) experiments revealed the presence of two β-glucopyranosyl units. A 1 → 2 interglycosidic linkage was established on the basis of the downfield shift for C-2″ (δ_C = 83.8) of the inner glucose which was further confirmed by the cross-peak at δ = 3.83/105.8 (H-2″/C-1‴) in the HMBC spectrum. The glycosidation site of the sophorose moiety was found to be C-7(OH) of the aglycone due to the long-range correlation between the H-1″ (δ_H = 5.15) of the inner glucose and the C-7 (δ_C = 152.3) of the aglycone in the HMBC spectrum. The above NMR data of **1** were found to be similar to those of 6-hydroxyluteolin 7-*O*-sophoroside [12], except for the additional aromatic signals arising from the benzoyl unit contained in **1**. The esterification site of the benzoic acid was found to be C-6‴(OH) of the terminal glucose, on the basis of the deshielding of H_2-6‴ (δ_H = 4.38 m) and C-6‴ (δ_C = 64.8) as well as the cross-peak at δ = 4.38/167.8 (H_2-6‴/C=O) in the HMBC spectrum. Thus, the structure of compound **1** was determined to be a 6-hydroxyluteolin 7-*O*-[6‴-benzoyl-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside and named as aphyllanthoside.

The known compounds were identified as 6-hydroxyluteolin 7-*O*-[6‴-(*E*)-caffeoyl-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside (**2**) [12], isoquercitrin (**3**) [13], verbascoside (**4**) [14], rossicaside A (**5**) [15], trichosanthoside A (**6**) [5], aucubin (**7**) [16], catalpol (**8**), 10-*O*-benzoylcatalpol (**9**) [17], globularin (**10**) [18], asperuloside (**11**) [19], besperuloside (**12**) [20], asperulosidic acid (**13**) [19], daphylloside (**14**) [21], scandoside (**15**) [22, 23], alpinoside (**16**) [24], and baldaccioside (**17**) [4] by comparison of their spectroscopic data with those of published values.

6-Hydroxyluteolin diglucosides have been reported from various *Globularia* species [10 – 12]. Aphyllanthoside (**1**) differs from these flavonoids with its benzoyl moiety. The chlorinated iridoid glycoside, baldaccioside (**16**), was isolated for the first time from the genus *Globularia*, while its C-7 epimer was reported from *G. alypum* [25]. Baldaccioside has recently been reported from *Wulfenia baldaccii* as a new iridoid [4]. Iridoid glucosides, being a significant chemotaxomic marker, are utilized to support the phylogenetic relationships found by the DNA sequence analysis. Thus, the close relationship between *Globularia* and *Wulfenia*, which were recently transferred to the new Plantaginaceae family [26], is corroborated again by the common occurrence of rare chlorinated iridoids in these two genera. This work also constitutes the first report of a chlorinated iridoid from the *Globularia* species growing in the flora of Turkey. On the other hand, the iridoid glucoside, daphylloside is new for the genus *Globularia*. Regarding the chemical constituents of *Globularia* species, it can be deduced that the iridoid and phenylethanoid composition of the *G. aphyllanthes* is similar to those of *G. trichosantha* [5, 6].

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