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Antioxidant Activity with Flavonoidal Constituents from *Aerva persica*

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A new flavanone Persinol (**1**) and the new flavanone glucosides persinosides A (**2**) and B (**3**), along with known flavonoids (**4** and **5**) have been isolated from the ethyl acetate soluble fraction of the whole plants of *Aerva persica*. Their structures were elucidated on the basis of extensive analysis of nuclear magnetic resonance (1D & 2D-NMR) spectral data. All of them showed profound antioxidative activities by DPPH and cytochrome-*c*-reduction assays using the HL-60 cell culture system.

Key words: *Aerva persica*, Amaranthaceae, Persinol, Persinoside A, Persinoside B, Antioxidant activities

INTRODUCTION

Aerva persica Burm is an annual herb belonging to the family Amaranthaceae. The genus comprises fifteen species, distributed in the warmer part of Asia and Africa. Six species are found in Pakistan. *Aerva persica* is common in Western Himalaya, Punjab, and Kashmir. The plant possesses the diuretic and demulcent properties (Garg *et al.*, 1979). Literature survey revealed that ascorbic acid and two flavonoids have so far been reported from *A. persica* (Garg *et al.*, 1980). Our studies on the ethylacetate-soluble fraction of the whole plant of *A. persica* resulted in the isolation and characterization of a new flavanone, persinol (**1**) and two new flavanone glucosides named as persinosides A (**2**) and B (**3**) along with known flavonoids **4** and **5**.

MATERIALS AND METHODS

General experimental procedures

UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer with tetramethylsilane (TMS) as an internal standard. The 2D-NMR spectra were recoded on a Bruker AMX 500 NMR spectrometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 and MAT 312 spectrometers and ions are given in *m/z* (%). TLC was performed on precoated silica gel F_{254} plates; the detection was done at 254 nm and by spraying with ceric sulphate reagent. Silica gel (E. Merck, 230-400 mesh) was used for column chromatography. Melting points were determined on a Gallenkemp apparatus and are uncorrected. For antioxidant assay all the chemicals were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.).

Materials

The whole plant of *Aerva persica* Burm was collected from Cholistan desert near district Bahawalpur (Punjab), Pakistan in October, 2003 and identified by Dr. Muhammad Arshad, Plant Taxonomist, Cholistan Institute of Desert Studies, Islamia University Bahawalpur, where a voucher specimen (01/CIDS/IUB/PK) has been deposited.

Extraction and isolation

The air dried whole plant (10 kg) was extracted with ethanol (3 × 25 L) at room temperature. The extract was evaporated to yield the residue (500 g), which was partitioned between *n*-hexane (75 g), chloroform (115 g),

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ethylacetate (95 g), *n*-butanol (45 g) and water (22 g) soluble fractions. The ethylacetate-soluble fraction was subjected to column chromatography over silica gel eluting with *n*-hexane-$CHCl_3$, $CHCl_3$, $CHCl_3$-MeOH in increasing order of polarity. The fractions which were obtained from *n*-hexane: chloroform (1:9) were combined and rechromatographed over silica gel eluting with *n*-hexane: chloroform (1.5:8.5) to afford 13 mg of liquiritigenin (**4**). The fractions which were obtained from $CHCl_3$: MeOH (9.5:0.5) were combined and rechromatographed over silica gel eluting with $CHCl_3$: MeOH (9:1) to afford persinol (**1**) (14 mg). The fractions which were obtained from $CHCl_3$:MeOH (8:2) were combined and rechromatographed over silica gel eluting with chloroform: methanol in increasing order of polarity. The fractions obtained from $CHCl_3$: MeOH (8.5:1.5) were subjected to preparative TLC ($CHCl_3$:MeOH:H_2O, (7:3:1) to afford persinoside A (**2**) (17 mg) and eriodictyol 5-*O*-β-D-glucoside (**5**) (14 mg). The fractions which were obtained from $CHCl_3$:MeOH (7:3) were combined and rechromatographed over silica gel eluting with $CHCl_3$:MeOH (7.2:2.8) to afford persinoside B (**3**) (20 mg).

Persinol (1)

Yellow amorphous powder; m.p. 205°C. $[\alpha]_D^{20}$ + 41° (*c* = 0.1 MeOH); CD curve $[\theta]_{333}$ + 6856 (max), $[\theta]_{298}$ -3208 (min), $[\theta]_{249}$ + 3609 (max), $[\theta]_{242}$ + 2023 (min); UV λ_{max}^{MeOH} nm: 329 (sh) (1.93), 286 (2.73); (+ $AlCl_3$) 369, 311; (+$AlCl_3$ + HCl) 369, 309; IR ν_{max}^{KBr} cm^{-1}; 3425 (OH), 2916 (OCH_3), 1647 (C=O), 1570, 1485, 1335, 1290, 1150; HREIMS *m/z*: 318.0702 (calcd for $C_{16}H_{14}O_7$: 318.0739); EIMS *m/z* 318 $(M)^+$, 303, 287, 271 255, 166, 152, 119; ^{1}H- and ^{13}C-NMR see Table I.

Persinoside A (2)

Light yellow amorphous powder; $[\alpha]_D^{20}$ -315° (*c* = 0.75, MeOH); UV λ_{max}^{MeOH} nm: 340 (sh); 313 (2.90), 273 (3.5); + $AlCl_3$: 300; + HCl: 300; 360 (sh); IR ν_{max}^{KBr} cm^{-1}; 3200); HRFABMS $[M+H]^+$ *m/z* 465.1362 (calcd for $C_{22}H_{25}O_{11}$, 465.1396). ^{1}H- and ^{13}C-NMR see Table I.

Persinoside B (3)

Light yellow powder; $[\alpha]_D^{20}$ -66° (*c* = 1.25, MeOH); UV λ_{max}^{MeOH} nm: 340 (sh), 313 (2.95), 273 (3.5). IR ν_{max}^{KBr} cm^{-1}; 3240-3480, 2850, 1652, 1565, 1375; 3422 (OH), 2919 (OCH_3), 1651 (C=O), 1570, 1480, 1339, 1290, 1151;

Table I. ^{1}H (400 MHz) and ^{13}C-NMR (100 MHz) data for compounds **1-3** (CD_3OD)

Position	1		2		3	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2	5.45 (dd, 13.1, 2.5)	79.2	5.51 (dd, 12.8, 2.9)	79.9	5.40 (dd, 12.5, 1.8)	79.1
3	2.82 (dd, 17.1, 2.5) 3.08 (dd, 17.1, 13.1)	43.0	2.69 (dd, 17.1, 2.9) 3.08 (dd, 17.1, 12.8)	43.8	2.60 (dd 17.0, 1.8) 3.12 (dd 17.0, 12.5)	43.4
4	-	197.8	-	198.5	-	198.1
5	-	164.9	-	166.6	-	165.9
6	6.40 (d, 2.0)	98.0	6.61 (d, 2.1)	98.2	6.78 (d, 1.9)	98.4
7	-	167.2	-	168.1	-	170.3
8	6.31 (d, 2.0)	95.5	6.50 (d, 2.1)	96.9	6.48 (d, 1.9)	94.4
9	-	162.9	-	163.3	-	162.7
10	-	105.2	-	104.8	-	105.5
1×	-	131.4	-	130.8	-	131.3
2×	6.95 (d, 1.9)	108.9	6.56 (d, 2.1)	112.8	7.10 (d, 2.1)	108.9
3×	-	141.9	-	146.8	-	141.7
4×	-	137.8	-	143.9	-	137.7
5×	-	141.9	7.34 (d, 8.8)	113.1	-	141.7
6×	6.95 (d, 1.9)	108.9	6.68 (dd, 8.8 2.1)	117.5	7.1 (d, 2.1)	108.9
1"	-	-	5.00 (d, 7.2)	103.6	5.19 (d, 7.1)	102.8
2"	-	-	3.24 (m)	74.9	3.25 (m)	74.6
3"	-	-	3.30 (m)	76.9	3.30 (m)	75.4
4"	-	-	3.40 (m)	70.8	3.35 (m)	70.0
5"	-	-	3.30 (m)	77.1	3.28 (m)	76.9
6"	-	-	3.70 (m)	62.0	3.62 (m)	61.8
OCH_3	3.41 (s)	54.9	3.52 (s)	55.4	-	-

HRFABMS $[M+H]^+$ *m/z* 467.1158 (calcd for $C_{21}H_{23}O_{12}$, 467.1138). ^1H- and ^{13}C-NMR see Table I.

Acid hydrolysis of 2 and 3

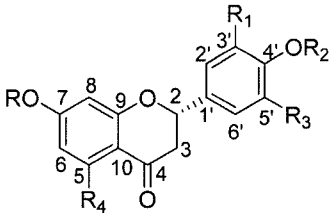
A solution of compound **2** (8 mg) in methanol in 5 mL 6% HCl was heated for 3 h. The reaction mixture was diluted and extracted with EtOAc. The EtOAc fraction crystallized from methanol, m.p 221-223°C. It could be identified as sternbin (**2a**) through comparison of physical and spectral data to those reported in literature (Wollenweber, 1981; Rani, 1986; Susan *et al.*, 1985; Gonzalez *et al.*, 1983). On the other hand, the aglycon in the case of **3** crystallized from methanol, mp 204-205°C and could be identified as 5,7,3',4',5'-pentahydroxyflavanone (**3a**) through comparison of physical and spectral data to those reported in literature (Forkman, 1983). In both cases the same glycon moiety was obtained and identified as D-glucose by sign of its optical rotation ($[\alpha]_D$+ 52.9° and 52.7°, respectively) and co-TLC with an authentic sample of D-glucose using solvent system *n*-BuOH-EtOAc-HOAc-H_2O (12:2:2:2). TLC was run three times in the same direction and spots were visualized with aniline phthalate reagent.

Antioxidant assay

DPPH (1,1-diphenylpicryl 2-hydrazyl) assay was performed essentially according to the modified method of Kirby and Schmidt (1997): 95 μL of 3.2×10^{-4} M of DPPH solution in absolute EtOH and 5*l* of sample solution in DMSO were mixed in a 96-well plate. The optical density was measured at 515 nm after incubation of the plate for 1 h at 37°C. The DPPH control contained no sample but was otherwise identical. The cytochrome-c-reduction assay was performed according to the method of sharma (Sharma *et al.*, 1994). HL-60 (human leckemic) cells were maintained in PRMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum, 1% penicillin streptomycin at 37°C in humidified atmosphere at 5% CO_2 in air. Differentiation was induced by 7 days treatment with 1.3% DMSO, and the cells were cultured in a 96 well plate (1×10^6 cells per well) in HBSS (Hanks buffered saline solution). After the addition of 8 mm TPA (12-*O*-tetradecanoylphorbol-13-acetate) to induce free radical formation, cytochrome-c (160 μm) and samples are added. The cells were incubated for 1h at 37°C and antioxidant activity was determined by monitoring absorbance at 550 nm. The same reaction mixture, without the HL-60 cells, was used as a blank control.

RESULTS AND DISCUSSION

Persinol (**1**) was obtained as a pale yellow amorphous solid and gave a positive ferric chloride test for a phenolic moiety. The high resolution EIMS of **1** exhibited the molecular ion peak at *m/z* 318.0702 corresponding to the molecular formula $C_{16}H_{14}O_7$. The UV spectrum in methanol showed absorption maxima at 286 and 329 (sh) nm, which was typical of a flavanone derivative (Mabry *et al.*, 1970). On addition of $AlCl_3$ and $AlCl_3$/ HCl, a bathochromic shift of 40 nm was observed suggesting the presence of a chelated hydroxyl group at C-5 (Mabry *et al.*, 1970). The IR absorption bonds at 3425 and 1647 cm^{-1} indicated the presence of hydroxyl and carbonyl functions, respectively. The ^1H-NMR spectrum of **1** revealed a D_2O exchangeable downfield signal at δ 12.60 assignable to the chelated hydroxyl group at C-5. An ABX system with resonances at δ 5.45 (1H, dd, J = 13.1, 2.5 Hz), 3.08 (1H, dd, J = 17.1, 13.1 Hz) and 2.82 (1H, dd, J = 17.1, 2.5 Hz) was characteristic of H-2, H-3*ax* and H-3*eq* of the flavanone moiety. Two meta coupled doublets (J = 2.0 Hz), each integrating for one proton at δ 6.40 and 6.31, were diagnostic for a C-6 and C-8 dioxygenated ring A. A typical AA' system at δ 6.95 integrating for two protons (J = 1.9 Hz, H-2' and H-6') established the presences of a 3', 4', 5'-trioxygenated ring B (Horowitz *et al.*, 1960). The presence of one methoxyl group as singlet was also observed at δ 3.41 (3H, s). The *retro* Diels-Alder fragmentation at ring C yielded the diagnostic peaks at *m/z* 166 and 152, confirming the presence of one hydroxyl and one methoxyl group in ring A, and three hydroxyl groups in ring B, respectively. The DEPT spectrum revealed the presence of one methoxyl, one methylene, five methine and nine quaternary carbons. The downfield signal at δ 197.8 was assigned to the carbonyl carbon, while signals of methoxyl and methylene were respectively observed at δ 54.9 and 43.0. The application of HMQC and HMBC experiments (Fig. 2) led to full assignments of the ^1H- and ^{13}C-NMR chemical shifts of **1**. The absolute stereochemistry at C-2 was established as '*S*' based on



	R	R_1	R_2	R_3	R_4
1	-Me	-OH	-H	-OH	-OH
2	-Me	-OH	-β-D-glu	-H	-OH
2a	-Me	-OH	-H	-H	-OH
3	-β-D-glu	-OH	-H	-OH	-OH
3a	-H	-OH	-H	-OH	-OH
4	-H	-H	-H	-H	-H
5	-H	-H	-H	-OH	-β-D-glu

Fig. 1. Structures of compounds **1-5**

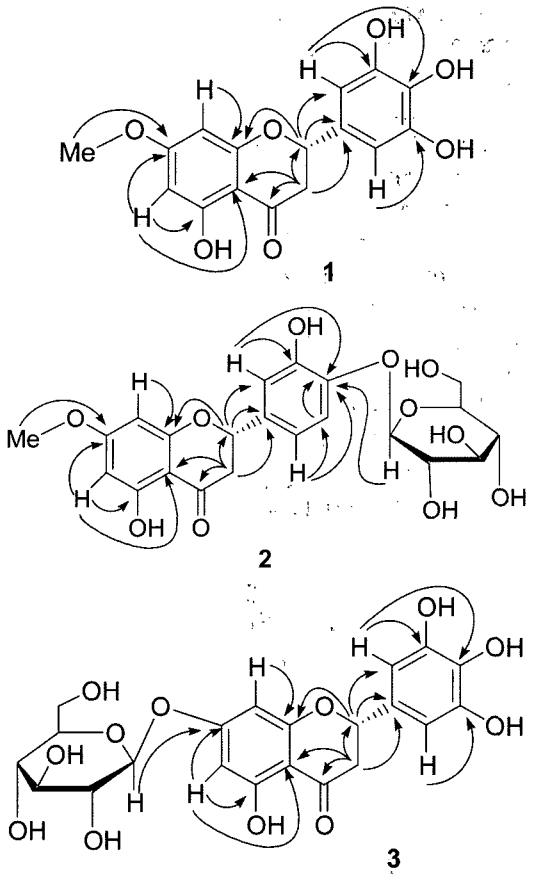


Fig. 2. Important HMBC correlations of compounds **1-3**

the observation of positive Cotton effect at 333 nm and a negative Cotton effect at 298 nm in its circular dichroism spectrum (Gaffield, 1970; Santzke *et al.*, 1973). Accordingly, the structure of persinol (**1**) (Fig. 1) was assigned as 7-methoxy-5,3',4',5'-tetrahydroxyflavanone.

Persinoside A (**2**) was isolated as a light yellow amorphous solid having molecular formula $C_{22}H_{24}O_{11}$ (HRFABMS $[M+H]^+$ at *m/z* 465.1362; calcd for $C_{22}H_{25}O_{11}$, 465.1396). Its UV absorption maxima at 340, 313 and 273 nm were characteristic of a flavanone *O*-glycoside (Mabry *et al.*, 1975). The IR spectrum exhibited absorption bands at 3200-3500, 2840, 1668, 1580, and 1370 cm^{-1} revealing the presence of hydroxyl, conjugated carbonyl and aromatic functionalities, respectively. A signal at δ 12.80 for a chelated hydroxyl group and two meta coupled aromatic protons at δ6.61 (1H, d, J = 2.1 Hz) and δ6.50 (1H, d, J = 2.1 Hz) were diagnostic for a C-5 and C-7 deoxygenated ring A. This was further supported by the mass fragment being observed at *m/z* 166 for ring A. The ^{1}H-NMR spectrum showed three aromatic protons forming an ABX system [δ7.34 (1H, d, J = 8.8 Hz), 6.68 (1H, dd, J = 8.8, 2.1 Hz) and 6.56 (1H, d, J = 2.1 Hz)] which suggested the presence of two oxygenated functionalities in ring B. The appearance of three doublets of doublet at δ5.51 (1H, J = 12.8, 2.9 Hz, H-2), δ3.08 (1H, J = 17.1, 12.8 Hz, H-3), and δ2.69 (1H, J = 17.1, 2.9 Hz, H-3) were characteristic for ring C. The presence of the sugar unit in δ configuration was evident by the signals of an anomeric proton at δ 5.00 (1H, d, J = 7.2 Hz). The ^{13}C-NMR spectrum (BB and DEPT) revealed the presence of one methyl, two methylene, ten methine and eight quaternary carbons. The downfield signal at δ198.5 was assigned to the carbonyl group, while signals of methoxyl and two methylenes were observed at δ55.4, 43.8 (C-3) and 62.0 (C"-6) respectively. The ^{1}H- and ^{13}C-NMR data of flavanone skeleton was in complete agreement to those of sternbin (**2a**) (Wollenweber, 1981; Rani, 1986; Susan *et al.*, 1985; Gonzalez *et al.*, 1983). Persinoside A (**2**) is, therefore, a glycoside of sternbin. The sugar moiety was identified as D-glucose by comparing its ^{13}C-NMR signals with the reference data (Pfeffor *et al.*, 1979; Gorin *et al.*, 1975). Acid hydrolysis provided sternbin (**2a**) (Wollenweber, 1981) and the sugar moiety, the latter being confirmed as D-glucose through sign of its optical rotation ($[\lambda]_D$ + 52.9°) and Co-TLC with an authentic sample. The position of glucose moiety was established by comparison of the UV and ^{1}H-NMR spectra of **2** and **2a**. Both absorption spectra showed bathochromic shifts on the addition of $AlCl_3$ and $AlCl_3$/HCl. Thus, the 5-hydroxyl group was free in **2** and hence the glucose moiety must be either at C-3' or C-4'. The ^{1}H-NMR spectrum of **2** showed that due to glycosylation, the proton signal of C-5' shifted downfield compared to those of C-2' and C-6', indicating that the *O*-glycosylation should be at C-4'. Accordingly, persinoside A (**2**) (Fig. 1) could be assigned the structure as 5, 3'-dihydroxy-7-methoxyflavanone 4'-*O*-β-D-glucoside. The HMBC (Fig. 2) and HMQC spectra were in complete agreement to the assigned structure.

Persinoside B (**3**) was isolated as a light yellow amorphous solid. The molecular formula was assigned $C_{21}H_{23}O_{12}$ on the basis of HRFABMS showing $[M+H]^+$ peak at *m/z* 467.1158 (calcd 467.1138). The UV and IR spectra were almost identical to those of **2**. The ^{1}H-NMR spectrum showed the signals at δ13.10 (chelated hydroxyl group), δ6.78 (1H, d, J = 1.9 Hz, H-6), δ6.48 (1H, d, J = 1.9 Hz, H-8) and two aromatic protons forming an AA' system at δ 7.10 (2H, d, J = 2.1 Hz, H-2' and H-6'). Moreover, a one proton doublet (J = 7.1 Hz) was observed at δ5.19, indicating the presence of a sugar moiety in β-configuration. The ^{13}C-NMR spectrum showed twenty one signals, sorted by DEPT experiments into two methylene, ten methine and nine quaternary signals. The sugar moiety was identified as D-glucose by comparing its carbon signals in ^{13}C-NMR spectrum with the reference data (Pfeffor *et al.*, 1979; Gorin *et al.*, 1975) and further confirmed through acid hydrolysis which provided 5,7,3',4',5'-pentahydroxyflavanone (**3a**) (Forkman, 1983) and the sugar

Table II. Antioxidant activities of compounds **1-5** from *Aerva persica*

Compound	DPPH assay (IC_{50})[a]		Cytochrome-c reduction Assay (IC_{50})[a]	
	μg	μM	μg/mL	μM
1	16.9	24.2	16.5	21.1
2	18.1	21.4	17.6	18.8
3	14.4	20.0	14.0	19.1
4	19.9	28.1	18.8	26.5
5	16.1	25.9	17.5	23.7
Gallic acid*	3.6	21.2	3.0	17.6

[a]Results are expressed as IC_{50} values (μg/mL and μM).
Data for active compounds were mean of triplicates.
*Positive control.

moiety. The latter could be confirmed as D-glucose through sign of its optical rotation ($[\alpha]_D$ + 52.7°) and Co-TLC with an authentic sample. On addition of $AlCl_3$ both **3** and **3a** showed bathochromic shifts. However, on addition of sodium acetate the bathochromic shift could only be observed for **3a**, indicating the presence of a -*O*-β-D-glucose moiety at C-7. Thus the structure of persinoside B (**3**) (Fig. 1) was assigned as 5,3',4',5'-tetrahydroxyflavanone 7-*O*-β-D-glucoside. The HMBC (Fig. 2), HMQC and ^1H-^1H COSY spectra were in complete agreement to the assigned structure.

The compounds **4** and **5** were identified by comparing their physical and spectral data with the literature values (Oliveira *et al.*, 1972; Gujer *et al.*, 1986).

For the screening and evaluation of antioxidant activity of pure compounds, DPPH scavenging assay and cytochrome-c-reduction assay were adopted. All compounds exhibited profound antioxidant activity compared with the positive control against TPA-induced free radical formation in a HL-60 cell culture system and showed free radical scavenging activity in the DPPH assay (Table II). Substitution patterns in the B ring markedly affect the antioxidant potencies of the flavonoids (Arora *et al.*, 1998) particularly the di-OH substitution at C-3' and C-4' seems to be particularly important to the oxygen radical absorbing activity of flavonoids (Cao *et al.*, 1997; Sharma *et al.*, 1994).

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