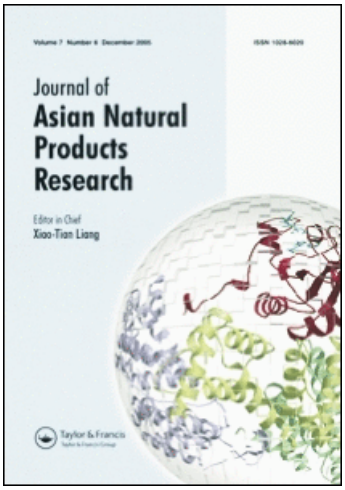

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Antioxidant constituents from *Cotoneaster racemiflora*

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A new lignan rhamnoside, racemiside (**1**), has been isolated from the ethyl acetate-soluble fraction of *Cotoneaster racemiflora*, along with scopoletin (**2**), 7,8-dimethoxy-6-hydroxycoumarin (**3**), 3,3′,4′-tri-*O*-methylellagic acid (**4**), and cereotagloperoxide (**5**), reported for the first time from this species. All of them showed profound antioxidative activities in the DPPH assay.

Keywords: *Cotoneaster racemiflora*; Rosaceae; antioxidant constituents

1. Introduction

The genus *Cotoneaster* belongs to the family Rosaceae and comprises well over 85 species widely distributed in Asia and Europe. The genus is represented in Pakistan by nine species [1]. Various species of the genus *Cotoneaster* are used as laxative, astringent, expectorant, and stomachic agents. Some are also used in the treatment of eye diseases, bronchitis, abdominal pain, stangury, itch, leucoderma, fever, wounds, piles, and urinary calculi [2–4]. *Cotoneaster racemiflora* commonly grows in Baluchistan province and northern areas of Pakistan. Although a variety of natural products have so far been reported from various species of the genus *Cotoneaster*, no phytochemical investigation has been carried out on *C. racemiflora*. The chemotaxonomic and ethnopharmacological significance of the genus *Cotoneaster* prompted us to carry out phytochemical studies on *C. racemiflora.* A methanolic extract of this plant showed strong toxicity in the brine shrimp lethality test [5]. Further pharmacological screening of the extract and its subsequent fractions revealed antioxidative activity that was most pronounced in the ethyl acetate-soluble fraction. Bioassay-directed isolation studies on this fraction have now resulted in the isolation of a new lignan rhamnoside named as racemiside (**1**) along with scopoletin (**2**) [6], 7,8-dimethoxy-6-hydroxycoumarin (**3**) [7], 3,3′,4′-tri-*O*-methylellagic acid (**4**) [8], and cereotagloperoxide (**5**) [9], reported for the first time from this species. All of them showed profound antioxidative activity in the DPPH assay.

2. Results and discussion

The ethyl acetate-soluble sub-fraction of the methanolic extract of *C. racemiflora* was subjected to a series of chromatographic techniques to obtain compounds **1**–**5**.

Racemiside (**1**) was obtained as a colorless amorphous solid, which gave a positive ferric chloride test for a phenolic moiety. The HREIMS gave the molecular ion peak at *m*/*z* 566.2358, corresponding to the molecular

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formula $C_{28}H_{38}O_{12}$, which was also confirmed by a positive-mode FABMS ion peak at *m/z* 567 $[M + H]^+$. The IR spectrum showed bands for hydroxyl (3450–3310 cm^{-1}) and aromatic (1610–1500 cm^{-1}) moieties. The UV absorption maxima at 206, 240, and 276 nm were typical for aryltetralin-type lignans [10]. Besides the signals of methylene and methine protons, the ^1H NMR spectrum showed four oxymethylene protons (δ 3.65, 3.60, 3.45, and 3.38) and four methoxyl groups were observed as singlets at δ 3.85 (3H), 3.73 (6H), and 3.34 (3H), respectively (Table 1). A pentasubstituted aromatic ring could be inferred by a one-proton singlet at δ 6.58, while protons of a further tetrasubstituted aromatic ring occurred together at δ 6.33. The anomeric proton of the sugar moiety resonated at δ 4.71 (IH, br s), while four other oxymethine protons were observed between δ 3.35 and 3.67 along with a methyl doublet at δ 1.19 ($J = 6.1$ Hz). Acid hydrolysis of **1** provided the sugar moiety, which could be identified as L-rhamnose through the sign of its optical rotation and the comparison of the retention time of its trimethylsilyl ether with that of standard in GC. The characteristic smaller coupling constant of the anomeric proton indicated α-linkage of the rhamnose moiety.

The ^{13}C NMR spectra of **1** showed 28 signals comprising five methyl, three methylene, 11 methine, and nine quaternary carbon atoms. The oxygenated aromatic carbons resonated at δ 149.0, 148.7, 147.5, 138.9, and 134.6, respectively. The oxymethylene carbons were observed at δ 69.7 and 66.3, and the anomeric carbon occurred at δ 101.9. The oxymethine carbons of the rhamnose moiety gave signals at δ 73.9, 72.6, 72.4, and 70.1, and the methyl group resonated at δ 17.9. The NMR spectral data of the aglycone moiety

Table 1. ^1H and ^{13}C NMR spectral data and HMBC correlations of compound **1** (CD_3OD).

No.	δ_H	δ_C	HMBC (H → C)
1	–	130.1	–
2	6.58 (s)	107.8	1, 3, 4, 7
3	–	148.7	–
4	–	138.9	–
5	–	147.5	–
6	–	126.0	–
7	2.73 (dd, 15.0, 4.6) 2.57 (dd, 15.0,11.6)	33.6	1, 2, 6, 8, 9
8	1.64 (m)	41.0	1, 7, 9, 7′, 8′
9	3.65 (m) 3.45 (m)	66.3	7, 8, 8′
1′	–	139.1	–
2′, 6′	6.33 (s)	106.7	1′, 4′, 7′
3′, 5′	–	149.0	–
4′	–	134.6	–
7′	4.31 (d, 5.4)	42.9	5, 6, 8, 1′, 2′, 6′, 8′, 9′
8′	2.07 (m)	46.4	6, 7, 8, 1′, 7′, 9′
9′	3.60 (m) 3.38 (m)	69.7	8, 7′, 8′, 1″
1″	4.71 (br s)	101.9	9′, 2″, 3″, 5″
2″	3.67 (m)	72.6	1″, 3″, 4″
3″	3.78 (m)	72.4	1″, 2″, 4″, 5″
4″	3.35 (m)	73.9	2″, 3″, 5″, 6″
5″	3.61 (m)	70.1	1″, 3″, 4″, 6″
6″	1.19 (d, 6.1)	17.9	4″, 5″
3-OMe	3.85 (s)	56.6	3
5-OMe	3.34 (s)	60.1	5
3′, 5′-OMe	3.73 (s)	56.8	3′, 5′

showed a close resemblance to those of linguresinol [10] except for the variation in the chemical shift and coupling constant (5.4 Hz) of H-7′ and downfield shift of C-9′ by about 3 ppm. The larger coupling constant of H-7′ in **1** compared to linguresinal indicated β and axial orientation. The downfield shift of C-9′ confirmed the glycosidation at C-9′. The HMBC correlations (Table 1) were in conformity with the assigned structure of racemiside (**1**), particularly the anomeric proton at δ 4.71 showed ^{3}J correlation with C-9′ (δ 69.7) and H_2-9′ at δ 3.60 and 3.38 showed ^{3}J correlation with the anomeric carbon (δ 101.9). The H-7′ at δ 4.31 showed ^{2}J correlations with C-1′ (δ 139.1), C-6 (δ 126.0), and C-8′ (δ 46.4), and ^{3}J correlations with C-5 (δ 147.5), C-9′ (δ 64.7), C-2′/C-6′ (δ 106.7), C-8 (δ 41.0), and C-1 (δ 130.1). The relative stereochemistry was established through nuclear Overhauser enhancement and exchange spectroscopy in which cross-peaks were observed between H-7′ (δ 4.31) and H-8′ (δ 2.07), H-8′ (δ 2.07) and H-8 (δ 1.64), and H-8 (δ 1.64) and H-7, β in conformity with the all cis orientation of these protons. On the basis of these evidences, the structure of racemiside (**1**) could be assigned as 7′-epilinguresinol-9′-*O*-α-L-rhamnoside (Figure 1).

For the screening and evaluation of antioxidant activity of the isolated compounds, DPPH scavenging assay was adopted. All compounds exhibited profound antioxidant activity compared with the positive control (Table 2).

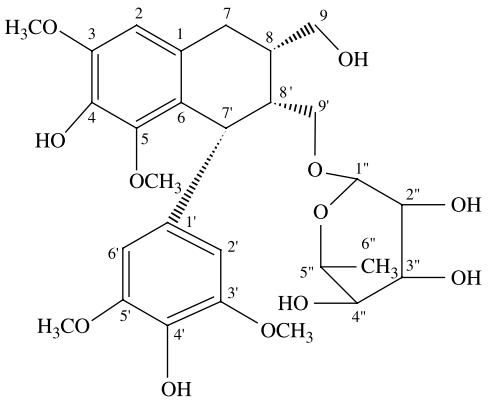

Figure 1. Structure of racemiside **1**.

Table 2. Antioxidant activities of compounds **1**–**5**.

Compound	DPPH assay (IC_{50})[a]
1	11.1 ± 0.48
2	3.8 ± 0.23
3	1.8 ± 0.19
4	15.1 ± 0.51
5	5.9 ± 0.39
BHA*	44.2 ± 0.2

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

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Jasco Dip-360 digital polarimeter using a 10 cm tube. The UV spectrum was recorded on a Hitachi UV-3200 spectrometer. The IR spectrum was recorded on a Jasco 320-A spectrometer. The NMR spectra were recorded in CD_3OD on a Bruker AM-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) using tetramethylsilane as an internal standard. Chemical shifts δ are expressed in ppm and coupling constant *J* in Hz. The 2D NMR spectra were obtained on a Bruker AM-500 NMR spectrometer. The mass spectra were measured 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 (E. Merck, Darmstadt, Germany). The detection was done at 254 nm and by spraying with ceric sulfate reagent. Silica gel (E. Merck, 230–400 mesh) was used for column and flash chromatography. For the antioxidant assay, all chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

3.2 Plant material

The whole plant of *C. racemiflora* Desf was collected from Swat (Pakistan) in July 2004 and identified by Prof. Dr Jahandar Shah,

Plant Taxonomists, Islamia College Peshawar, University of Peshawar, where a voucher specimen (CR-119-04) is deposited.

3.3 Extraction and isolation

The shade-dried whole plant of *C. racemiflora* (15 kg) was extracted with MeOH (3 × 50 L) at room temperature. The combined methanolic extract was evaporated under reduced pressure to obtain a thick gummy residue (500 g). It was suspended in water and successively extracted with *n*-hexane, EtOAC, and *n*-BuOH. The EtOAc fraction (40 g) was subjected to column chromatography over silica gel eluting with *n*-hexane, *n*-hexane–EtOAc, EtOAc, and EtOAc–MeOH in the increasing order of polarity. The fractions obtained from *n*-hexane:EtOAc (8:2) gave two major spots on TLC, which on final purification by flash chromatography using solvent system *n*-hexane:EtOAc (7.8:2.2) afforded scopoletin (**2**) (11 mg) from the top fractions and 7,8-dimethoxy-6-hydroxycoumarin (**3**) (12 mg) from the tail fractions, respectively. The fraction obtained from *n*-hexane:EtOAc (6:4) was a binary mixture of compounds which on flash chromatography using solvent system *n*-hexane:EtOAc (5.7:4.3) provided cereotagloperoxide (**5**) (18 mg) and 3,3′,4′-tri-*O*-methylellagic acid (**4**) (25 mg) from the top and tail fractions, respectively. The fractions obtained from *n*-hexane:EtOAc (1:9) was rechromatographed over silica gel using *n*-hexane:EtOAc (0.7:9.3) as an eluent to obtain racemiside (**1**) (22 mg).

The compounds **2**–**5** were identified by comparing their physical and spectral data with the literature values [6–9].

3.3.1 Racemiside (*1*)

A colorless amorphous solid. $[\alpha]_D^{20}$ +4.8 (c 0.00062, MeOH). UV (MeOH) λ_{max} nm (log ε): 206 (3.8), 240 (4.02), 276 (3.9). IR (KBr) ν_{max} cm^{-1}: 3450–3310 (OH), 1610–1500 (aromatic). ^1H NMR (400 MHz, CD_3OD), ^{13}C NMR (100 MHz, CD_3OD) spectral data and HMBC correlations: see Table 1. HREIMS *m/z* 566.2358 $[M]^+$ (calcd for $C_{28}H_{38}O_{12}$, 566.2363). FABMS *m/z*: 567 $[M + H]^+$.

3.3.2 Acid hydrolysis of *1*

A solution of **1** (8 mg) in MeOH (5 ml) containing 1 N HCl (4 ml) was refluxed for 4 h, concentrated under reduced pressure, and diluted with H_2O (8 ml). It was extracted with EtOAc and the residue from the organic phase was a mixture of products which was not worked up due to paucity of material. The aqueous phase was concentrated and L-rhamnose was identified by the sign of its optical rotation $[\alpha]_D^{20}$ −8.1 and also confirmed by the comparison of the retention time of its trimethysilyl ether (8.6 min) with a standard.

3.4 Antioxidant assay

The DPPH (1,1-diphenyl picryl-2-hydrazyl) assay was performed essentially according to the reported protocol [11]: 95 μl of 3.2×10^{-3} M of DPPH solution in absolute EtOH and 5 μl 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.

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