Chemistry of Natural Compounds, Vol. 48, No. 3, July, 2012 [Russian original No. 3, May–June, 2012]

CHEMICAL CONSTITUENTS AND CYTOTOXIC ACTIVITY OF *Ranunculus pedatus* subsp. *pedatus*

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UDC 547.972

The genus *Ranunculus* belongs to the family Ranunculaceae. In Turkey, it is represented by 94 native taxa and about 85 species [1, 2]. In traditional medicine, several plants belonging to this genus are used for wound healing [3], as antihemoroid [4, 5], for maturation of abscess [6, 7], treatment of jaundice [7], and against rheumatism [6]. Flavonoids [8–10], saponins [11], alkaloids [12], and fatty acids and organic acids [13] were isolated in previous phytochemical studies of this genus.

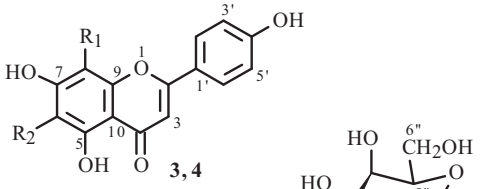
To the best of our knowledge, no previous work has been reported on the phytochemical investigations and cytotoxic activity of *R. pedatus* Waldst. & Kit. We report here the isolation and structure elucidation of four known compounds, **1–4**, which were isolated for the first time from this plant. The structures of these compounds were deduced by comparison of their spectral data with those reported in the literature.

General Procedures. The ^{1}H NMR spectra were recorded using a Bruker DRX-400 (400 MHz) spectrometer, while the ^{13}C NMR spectra were recorded at 100 MHz on the same instrument. IR spectra were measured on a Jasco FT-IR-300 spectrometer in a KBr pellet. UV spectra were recorded on a Shimadzu UV-160A spectrometer. Mass spectra were performed on a Finnigan MAT TSQ 700 mass spectrometer. Optical rotations were determined on a Perkin–Elmer polarimeter. Column chromatography (CC) was carried out on silica gel GF-254 preparative plates (20 × 20 cm, 0.25 mm thick, Mecrk) and the spots were detected under UV light (254 and 366 nm). The compounds were detected by UV fluorescence and by spraying with Neu (1% methanolic solution of the β-aminoethyl ester of diphenylboric acid).

Plant Material. *R. pedatus* Waldst & Kit. subsp. *pedatus* was collected from Manisa in May 2005. The plant was identified by M. Ali Onur, and a voucher specimen (No. 1364) is deposited in the Herbarium of Ege University, Faculty of Pharmacy.

Extraction and Isolation. The dried and powdered plant material (6.5 kg) was first extracted with petroleum ether (yield 0.29%) to remove the lipophilic compounds and then with ethanol (96%) in a Soxhlet apparatus. The concentrated ethanol extract was diluted with water and extracted with benzene (yield 0.02%) and ethyl acetate (yield 0.08%) for fractionation.

Chromatographic analysis has shown the presence of flavonoids in the ethyl acetate extract. For the isolation and purification of the flavonoids column, preparative column and preparative thin layer chromatography were used. The total concentrated ethyl acetate extract (115 g) was fractionated using silica gel column chromatography (CC, 3.5 cm × 100 cm) with toluene:ethanol mixtures of increasing polarity (0 to 100%). Fraction 7 (0.28 g) was chromatographed on silica gel CC eluted with toluene–ethanol–acetonitrile (8.5:1:0.5). *p*-Hydroxybenzoic acid (3.2 mg) and *p*-coumaric acid (4.2 mg) were purified on a preparative TLC plate (silica gel 60 F_{254}) using toluene–chloroform–ethanol–methanol (4:4:1:0.5) and then acetone for crystallization.



3: R_1 = X, R_2 = H
4: R_1 = H, R_2 = X

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TABLE 1. LC_{50} Values of Ethyl Acetate Extract of *R. pedatus* subsp. *pedatus* and Two Fractions

Sample	Concentration, ppm	LC_{50}, μg/mL	SE, % (n = 3)
Ethyl acetate extract	1000:100:10	823.61	0.21
Fr.7	1000:100:10	101.47	0.42
Fr. 11	1000:100:10	108.81	0.15
Umbelliferone	500:50:5	377.02	
Colchicine	500:50:5	0.0009	

LC_{50}: lethal concentration 50%. SE: standard error.

Fraction 11 (1.32 g) was chromatographed on a silica gel CC eluted with toluene–acetonitrile–water (2:7:1). Vitexin (10.1 mg, toluene–acetonitrile–ethyl acetate–buthanol–water, 2:6:1:0.5:0.5) and isovitexin (124 mg, toluene–acetonitrile–ethyl acetate–buthanol, 2:6:1:1) were isolated from these column fractions by using repeated CC and preparative thin-layer (TLC) chromatographic techniques.

All isolated compounds were identified by comparison with authentic substances and with spectroscopic data (UV, IR, NMR, MS).

***p*-Hydroxybenzoic Acid (1)**. White needle crystal. UV (MeOH, λ_{max}, nm): 249. ESI-MS *m/z* 137 $[M - H]^-$ [15].

***p*-Coumaric Acid (2)**. White needle crystal. UV (MeOH, λ_{max}, nm): 249. ESI-MS *m/z* 163 [M – H] [15].

Vitexin (3). Yellow powder. UV (MeOH, λ_{max}, nm): 336, 302, 270. IR (KBr, ν_{max}, cm^{-1}): 3378, 3251, 1661, 1508. ESI-MS (*m/z*, I_{rel}, %): 431 $[M - H]^-$ (6), 413 $[(M - H) - 18]^-$ (1), 341 $[(M - H) - 90]^-$ (12), 311 $[(M - H) - 120]^-$ (100). ^1H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.71 (1H, s, H-3), 6.21 (1H, s, H-6), 7.98 (2H, d, J = 8.8, H-2′, H-6′), 6.88 (2H, d, J = 8.8, H-3′, H-5′), 4.73 (1H, d, J = 9.8, H-1″), 3.79 (1H, d, H-2″), 3.28 (1H, dd, H-3″), 3.46 (1H, t, H-4″), 3.25 (1H, d, H-5″), 3.74 (1H, dd, J = 11.9, 5.8, H-6″a), 3.53 (1H, dd, J = 11.9, 2.4, H-6″b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 163.85 (C-2), 102.44 (C-3), 182.01 (C-4), 156.01 (C-5), 98.35 (C-6), 163.29 (C-7), 104.62 (C-8), 161.23 (C-9), 104.64 (C-10), 121.62 (C-1′), 128.93 (C-2′), 115.89 (C-3′), 160.44 (C-4′), 115.89 (C-5′), 128.93 (C-6′), 73.46 (C-1″), 79.91 (C-2″), 78.71 (C-3″), 70.55 (C-4″), 81.82 (C-5″), 61.30 (C-6″) [11].

Isovitexin (4). Yellow powder. UV (MeOH, λ_{max}, nm): 271, 336. IR (KBr, ν_{max}, cm^{-1}): 3403, 1661, 1607, 1508. ESI-MS (*m/z*, I_{rel}, %): 431 $[M - H]^-$ (10), 413 $[(M - H) - 18]^-$ (20), 341 [(M – H) – 90] (25), 311 $[(M - H) - 120]^-$ (100). ^1H NMR (400 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.56 (1H, s, H-3), 6.42 (1H, s, H-8), 7.96 (1H, d, J = 8.0, H-2′), 6.89 (1H, d, J = 8.0, H-3′), 6.89 (1H, d, J = 8.0, H-5′), 7.96 (1H, d, J = 8.0, H-6′), 4.73 (1H, d, J = 8.0, H-1″), 3.89 (1H, dd, J = 8, 9.5, H-2″), 3.32 (1H, t, H-3″), 3.50 (1H, t, H-4′), 3.30 (1H, t, H-5″), 3.73 (1H, dd, J = 11.6, 5.4, H-6″a), 3.54 (1H, dd, J = 11.6, 2.8, H-6″b). ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 163.48 (C-2), 102.85 (C-3), 182.85 (C-4), 160.90 (C-5), 108.87 (C-6), 163.95 (C-7), 97.35 (C-8), 156.51 (C-9), 104.60 (C-10), 121.15 (C-1′), 128.53 (C-2′), 116.27 (C-3′), 160.89 (C-4′), 116.27 (C-5′), 128.53 (C-6′), 73.33 (C-1″), 70.91 (C-2″), 76.8 (C-3″), 70.55 (C-4″), 81.86 (C-5″), 61.02 (C-6″) [11].

Cytotoxic Activity. The ethyl acetate extracts, fraction 7 and fraction 11, were tested against *Artemia salina* Leach. larvae using the methodology proposed by McLaughlin and collaborators [14]. The 50% lethal concentration (LC_{50}) and the standard error were calculated by Probit analysis [15]. Sea salt (3.8 g) was dissolved in 100 mL water and filtered. Brine shrimp (*Artemia salina*) (San Fransisco Bay Brand Inc., Newark, CA 94560, USA) eggs were placed into the water and left to incubate for 48 h at 28°C in a small tank (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). Each extract was tested at 1000, 100, and 10 ppm. Twenty milligrams of plant extract was dissolved in 2 mL chloroform (20 mg/2 mL). From this solution, 500, 50, or 5 μL was transferred to vials corresponding to 1000, 100, or 10 ppm, respectively. Vials including chloroform and extraction solvents (500 μL) were prepared as controls. After incubation, 10 brine shrimp larvae (nauplii) were introduced into the vials containing graded concentrations (ranging from 10 to 1000 ppm) of the extracts. After 24 h, the number of surviving shrimps at each concentration of the extracts was counted and the data analyzed with the Finney computer program to determine the LC_{50} at a 95% confidence interval. Sea salt (Sigma 9883) was used in activity tests. The cytotoxic activity of all the extracts was compared with umbelliferone and colchicine as cyctotoxic substances [15]. The ethyl acetate extract, fraction 7 and fraction 11, showed cytotoxic activity (Table 1).

In this work, the cytotoxic activity and the constituents of *R. pedatus* Waldst.&Kit. subsp. *pedatus* have been investigated for the first time.

The cytotoxic activity of the ethyl acetate extract, fraction 7 and fraction 11, was determined by the brine shrimp lethality bioassay. The crude extract and its fractions showed cytotoxic activity against brine shrimp. The fractions showed greater activity than the crude extract. The brine shrimp bioassay was used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds [14].

Vitexin and isovitexin, previously found to be cytotoxic [16], may be responsible for the observed brine shrimp cytotoxicity of some extracts.

ACKNOWLEDGMENT

This research was supported by Ege University Research Fund (No. 05/ECZ/013), Izmir, Turkey. The authors also thank Prof. Dr. Alessandra Braca for NMR and MS spectral measurements.

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