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Antioxidant and Vasorelaxant Activities of Flavonoids from *Amygdalus lycioides* var. *horrida*

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Abstract: *Amygdalus lycioides* var. *horrida* (Spach) Browicz (Rosaceae), also known as *Prunus lycioides* (Spach.) Schneid., is an endemic Iranian species of the genus *Amygdalus*. In Iranian traditional medicine, the aerial parts and roots of *A. lycioides* are used in the treatment of diabetes. Six flavonoids, i.e. quercetin 3-*O*-rhamnoside (1), luteolin 7-*O*-rhamnoside (2), isorhamnetin 3-*O*-rutinoside (3), kaempferol 3-*O*-rhamnoside (4), apigenin (5), and naringenin (6), have been isolated from the aerial parts of this plant. The structures of these compounds were elucidated by UV, MS, and NMR spectroscopic data analyses. While the antioxidant activity of these compounds was assessed by the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay, the vasorelaxant effect was determined using the rat aortic vascular smooth muscle. Compounds 1-6 displayed significant antioxidant activity, with the RC_{50} values ranging from 0.0033 to 0.5186 mg/ml. Compound 2 showed a considerable vasorelaxant activity on rat aortic vascular smooth muscle in a dose-dependent manner.

Key Words: *Amygdalus lycioides* var. *horrida,* Rosaceae, flavonoid, antioxidant, DPPH, vasorelaxant

Amygdalus lycioides var. *horrida* Bitkisinin Flavonitlerinin Antioksidant ve Vasorelaksant Aktiviteleri

Özet: : *Prunus lycioides* (Spach.) Schneid olarakta isimlendirilen *Amygdalus lycioides* var. *horrida* (Spach) Browicz (Rosaceae) *Amygdalus* genusunda İran'a ait endemic bir bitkidir. İran halk tebabetinde şeker hastalığının tedavisinde A. lycioides bitkisinin hava kökleri kullanılır. Bu bitkinin hava köklerinden kuersetin 3-*O*-rhamnosit (1), luteolin 7-*O*-rhamnosit (2), isorhamnetin 3-*O*-rutinosit (3), kaemferol 3-*O*-rhamnosit (4), apigenin (5) ve naringenin (6) adlı altı flavonit izole edilmiştir. Bileşiklerin yapısı UV, MS ve NMR spektroskopisi ile belirlenmiştir. Bileşiklerin antioksidant aktiviteleri 2,2-difenil-1-pikril-hidrazil (DPPH) ile, vasorelaksant etki ise rat aortic vasküler düz kası kullanılarak belirlenmiştir. Bileşikler önemli derecede antioksidant aktivite gösterirken, RC_{50} değerleri 0,0033 ile 0,5186 mg/ml arasında gözlenmiştir. 2 nolu Bileşik ise doza bağımlı olarak rat vasküler düz kası üzerine önemli derecede vasorelaksant aktivite göstermiştir.

Anahtar Sözcükler: *Amygdalus lycioides* var. *horrida*, Rosaceae, flavonoit, antioksidant, DPPH, vasorelaksant

Introduction

Amygdalus lycioides var. *horrida* (Spach) Browicz (Rosaceae), (*syn. Prunus lycioides* (Spach.) Schneid.), known as wild almond, is an endemic Iranian species of the genus *Amygdalus* (1,2). In Iranian traditional medicine, the aerial parts and roots of *A. lycioides* are used in the treatment of diabetes. Amygdalin, a characteristic compound of the genus *Amygdalus*, was previously reported from this plant (3). However, there are no reports available to date on any phytochemical or bioactivity studies on *A. lycioides*. As a part of our on-going studies on the Iranian flora (4-14), we now report the isolation, identification, and antioxidant and vasorelaxant properties of 6 flavonoids, namely quercetin

3-*O*-rhamnoside (1), luteolin 7-*O*-rhamnoside (2), isorhamnetin 3-*O*-rutinoside (3), kaempferol 3-*O*-rhamnoside (4), apigenin (5), and naringenin (6), from the aerial parts of *A. lycioides.*

Materials and Methods

General experimental procedures

UV spectra were obtained in methanol (MeOH) using a Shimadzu UV-1650PC UV-visible spectrometer. NMR spectra were recorded in CD_3OD on a Bruker 200 MHz NMR Spectrometer (200 MHz for ^1H and 50 MHz for ^{13}C) using residual solvent peak as internal standard. HPLC separation was performed in a Shimadzu photodiode-array detector (SPD-M20A). A Shim-Pack ODS preparative HPLC column (15 m, 250 mm × 20 mm) was used. A Sep-Pak Vac 35 cc (10 g) C_{18} cartridge (Waters) was used for pre-HPLC fractionation. FAB-MS analyses were performed on a Finnigan MAT95 spectrometer.

Plant material

The aerial parts of *Amygdalus lycioides* var. *horrida* (Spach) Browicz were collected during April-May 2006 from Naeine in Esfahan province in Iran. A voucher specimen (TUM-ADE 0241) has been retained in The Herbarium of the Faculty of Pharmacy, Tabriz University of Medical Sciences, Iran.

Extraction and isolation of compounds (1-6)

The dried and ground aerial parts of *A. lycioides* (100 g) were Soxhlet extracted with *n*-hexane, dichloromethane, and methanol (MeOH), successively (1.1 l each), 10 cycles each. The MeOH extract (2 g) was subjected to Sep-Pack fractionation using a step gradient of MeOH-water mixture (10:90, 20:80, 40:60, 60:40, 80:20, and 100:0). The preparative reversed-phase HPLC analysis (linear gradient: 30% to 70% methanol in water in 50 min, flow rate: 20 ml/min) of the 40% methanolic Sep-Pack fraction yielded 2 flavonoids: quercetin 3-*O*-rhamnoside (1, 18.3 mg, t_R = 28.1 min) and luteolin 7-*O*-rhamnoside (2, 17.4 mg, t_R = 36.5 min). Similar purification of the 60% methanolic Sep-Pack fraction (linear gradient: 50% to 90% ACN in water in 50 min, flow rate: 20 ml/min) afforded 4 flavonoids: isorhamnetin 3-rutinoside (3, 1.7 mg, t_R = 8.2 min), kaempferol 3-*O*-rhamnoside (4, 3.5 mg, t_R = 11.8 min), apigenin (5, 2.1 mg, t_R = 21.1 min), and naringenin (6, 7.3 mg, t_R = 21.9 min). All of the compounds (1-6) were identified by spectroscopic means.

Quercetin 3-O-rhamnoside (1): Yellow amorphous solid; 18.3 mg; UV l_{max} (MeOH): 256, 268 sh, 299 sh, 362; +$AlCl_3$: 275, 305 sh, 331 sh, 437; +$AlCl_3$/HCl: 268, 299 sh, 366 sh, 405; +NaOMe: 272, 327, 409; +NaOAc: 274, 324, 380; +NaOAc/H_3BO_3: 262, 298 sh, 378 nm; FAB-MS *m/z* 471 $[M + Na]^+$; ^1H-NMR (200 MHz, CD_3OD) and ^{13}C-NMR (50 MHz, CD_3OD) as in accordance with the published data (15-18).

Luteolin 7-O-rhamnoside (2): Yellow amorphous solid; 17.4 mg; UV l_{max} (MeOH): 255, 267 sh, 348; +$AlCl_3$: 274, 299 sh, 329, 432; +$AlCl_3$/HCl: 273, 293 sh, 358, 387; +NaOMe: 263, 300 sh, 394; +NaOAc: 259, 266 sh, 365 sh, 405; +NaOAc/H_3BO_3: 259, 372 nm; FAB-MS *m/z* 455 $[M + Na]^+$; ^1H-NMR (200 MHz, CD_3OD) and ^{13}C-NMR (50 MHz, CD_3OD) as in accordance with the published data (15-18).

Isorhamnetin 3-O-rutinoside (3)*:* Yellow amorphous solid; 1.7 mg; UV l_{max} (MeOH): 254, 266 sh, 305 sh, 356; +$AlCl_3$: 268, 279 sh, 300 sh, 369 sh, 402; +$AlCl_3$/HCl: 267, 275 sh, 300 sh, 359 sh, 399; +NaOMe: 271, 328, 413; +NaOAc: 272, 320, 396; +NaOAc/H_3BO_3: 254, 267 sh, 304 sh, 360 nm; FAB-MS *m/z* 647 $[M + Na]^+$; ^1H-NMR (200 MHz, CD_3OD) and ^{13}C-NMR (50 MHz, CD_3OD) as in accordance with the published data (15-18).

Kaempferol 3-O-rhamnoside (4): Yellow amorphous solid; 11.8 mg; UV l_{max} (MeOH): 264, 317, 345; +$AlCl_3$: 268, 300, 335, 395; +$AlCl_3$/HCl: 274, 300, 335, 396; +NaOMe: 267, 326; +NaOAc: 266, 315 sh, 345; +NaOAc/H_3BO_3: 264, 315 sh, 345 nm; FAB-MS *m/z* 455 $[M + Na]^+$; ^1H-NMR (200 MHz, CD_3OD) and ^{13}C-NMR (50 MHz, CD_3OD) as in accordance with the published data (15-18).

Apigenin (5): Yellow amorphous solid; 2.1 mg; UV l_{max} (MeOH): 267, 296 sh, 336; +$AlCl_3$: 276, 302, 348, 384; +$AlCl_3$/HCl: 276, 299, 340, 380; +NaOMe: 276, 324, 392; +NaOAc: 274, 301, 376; +NaOAc/H_3BO_3: 268, 302 sh, 338 nm; FAB-MS *m/z* 293 $[M + Na]^+$; ^1H-NMR (200 MHz, CD_3OD) and ^{13}C-NMR (50 MHz, CD_3OD) as in accordance with the published data (15-18).

Naringenin (6): Yellow amorphous solid; 7.3 mg; UV l_{max} (MeOH): 287, 326 sh; +$AlCl_3$: 311, 378; +$AlCl_3$/HCl: 310, 373; +NaOMe: 248, 325; +NaOAc: 286 sh, 326; +NaOAc/H_3BO_3: 291, 332sh nm; FAB-MS *m/z* 295 $[M + Na]^+$; ^1H-NMR (200 MHz, CD_3OD) and ^{13}C-NMR (50 MHz, CD_3OD) as in accordance with the published data (15-18).

Antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH), with the molecular formula of $C_{18}H_{12}N_5O_6$, was obtained from Fluka Chemie AG, Bucks, UK. Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma-Aldrich, UK. The method used by Takao et al. (19) was adopted with appropriate modifications (20,21). DPPH (4 mg) was dissolved in MeOH (50 ml) to obtain a concentration of 80 mg/ml.

Qualitative assay: Compounds 1-6 were applied to a precoated silica gel TLC plate (0.25 mm thickness) and sprayed with DPPH solution using an atomizer. It was allowed to develop for 30 min. The colour change (purple on white) was noted.

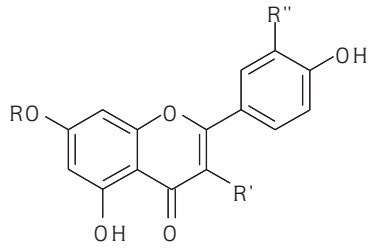
Quantitative assay: Compounds 1-6 were dissolved in MeOH to obtain a concentration of 0.5 mg/ml. Dilutions were made to obtain concentrations of 5 10^{-2}, 5 10^{-3}, 5 10^{-4}, 5 10^{-5}, 5 10^{-6}, 5 10^{-7}, 5 10^{-8}, 5 10^{-9}, and 5 10^{-10} mg/ml. Diluted solutions (1 ml each) were mixed with DPPH (1 ml) and allowed to stand for half an hour for any reaction to occur. The absorbance was recorded at 517 nm. The experiment was performed in triplicate and the average absorption was noted for each concentration. The same procedure was followed for the positive control, a well-known antioxidant Trolox®. The RC_{50} value, which is the concentration of the test material that reduces 50% of the free radical concentration, was calculated as mg/ml.

Vasorelaxant activity

The vasorelaxant activity of compounds 1, 2 and 4 was assessed in triplicate following the method described by Chan et al. (22) and Kim et al. (23). Rings of rat (Wistar 250-300 g) thoracic aorta 3-5 mm in length were trimmed from adjacent tissues. Two stainless steel triangular hooks were introduced through the lumen of the ring. One hook was fixed to the bottom of the organ bath and the other was connected to a force-displacement transducer. Each aortic ring was set up in a 10 ml bath containing modified Krebs Ringer–bicarbonate solution of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$, $7H_2O$ 1.2, $CaCl_2$ 2.5, glucose 11.1. The solution was equilibrated with a mixture of 95% O_2 and 5% CO_2 to give a pH of 7.3 to 7.4. Temperature was held at 37 °C. The optimal resting tension was adjusted to 2 g, which was obtained in preliminary tests and maintained throughout the experiments. Tissues were allowed to attain a steady level of tension during a 60 min accommodation period before being tested. During this time, the bathing solution was changed every 15 min. Functional integrity of the endothelium was confirmed routinely at the beginning of the experiment by the presence or absence of relaxation induced by acetylcholine (6 µM) on contraction induced by phenylephrine (0.1 µM). Changes in isometric tension were recorded on a computer assisted data acquisition system (ADInstrument, Power Lab/4SP) with force displacement transducers (LETICA, Spain). In the various experiments, the vascular rings were contracted with prostaglandin F2 (PGF2 , 10 µM). When contraction was stable, the test compound (highest concentration: 800 M) was applied to the bath. Relaxation was expressed as percentage reversal of contraction induced by vasoactive agents.

Results and Discussion

Reversed-phase preparative HPLC analysis of the methanol extract of the aerial parts of *A. lycioides* var. *horrida* produced 6 flavonoids, which were identified as quercetin 3-*O*-rhamnoside (1), luteolin 7-*O*-rhamnoside (2), isorhamnetin 3-*O*-rutinoside (3), kaempferol 3-*O*-rhamnoside (4), apigenin (5), and naringenin (6), on the basis of UV, MS, and NMR data analyses (Figure 1).



	R	R'	R''
1	H	*O*- Rhamnosyl	OH
2	Rhamnosyl	H	OH
3	H	*O*- Rutinosyl	OMe
4	H	*O*-Rhamnosyl	H
5	H	OH	H

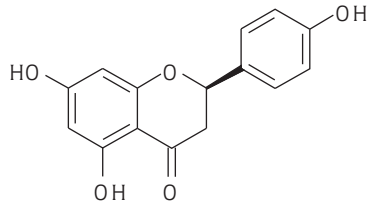


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Figure 1. Structures of the flavonoids isolated from *A. lycioides* var. *horrida.*

The UV absorption spectra in MeOH of 1-6 were typical for flavonoids (15). Further UV analyses using various shift reagents confirmed the presence/absence of free hydroxyls as well as the site of conjugations in these compounds (15). The FABMS spectra of all compounds (1-6) established the molecular mass and thereby the molecular formula of these compounds. The UV, ^{1}H-, and ^{13}C-NMR spectroscopic data of 1-6 were identical to the literature data for quercetin 3-*O*-rhamnoside (1), luteolin 7-*O*-rhamnoside (2), isorhamnetin 3-*O*-rutinoside (3), kaempferol 3-*O*-rhamnoside (4), apigenin (5), and naringenin (6) (15-18).

To our knowledge, this is the first report on the occurrence of flavonoids 1-6 in the aerial parts of *A. lycioides* var. *horrida*. The flavonoids found predominantly in the genus *Prunus*, which has a close taxonomical relation to *Amygdalus*, are isorhamnetin 3-*O*-rutinoside, isorhamnetin 3-*O*-glucoside, kaempferol 3-*O*-rutinoside, quercetin 3-*O*-galactoside, and isorhamnetin 3-*O*-galactoside (24-26). The co-occurrence of similar flavonoids both in *A. lycoides* and in *Prunus* species might have some chemotaxonomic implications; at least, this finding justifies the synonym *Prunus lycioides* for *Amygdalus lycioides*.

All compounds (1-6) exhibited significant levels of antioxidant activity in the DPPH assay. The RC_{50} (the concentration of the test material that reduces 50% of the free radical concentration, calculated as mg/ml) values of these compounds are presented in the Table. Among these flavonoids (1-6), quercetin 3-*O*-rhamnoside (1) and luteolin 7-*O*-rhamnoside (2) were the most potent ones (RC_{50} = 0.0035 and 0.0033 mg/ml), while naringenin (6) was the least potent (RC_{50} = 0.5186 mg/ml). The antioxidant properties of 1-6 indicated that not only the phenolic hydroxyls but also the C2-C3 double bond, as in compounds 1-5, was an important contributor to the antioxidant activity of these compounds. However, generally the antioxidant activity of 1-6, like that of other natural phenolic compounds, is a consequence of the presence of the phenolic moieties in the structures. The antioxidant activity of phenolic natural products is predominantly owing to their redox properties, i.e. the ability to act as reducing agents, hydrogen donors, and singlet oxygen quenchers, and to some extent could also be due to their metal chelation potential (21). The presence of these antioxidant compounds (1-6) in *A. lycioides* var. *horrida* might be significant in relation to this plant's various medicinal uses.

Due to the paucity of the samples, only compounds 1, 2, and 4 were assessed for their possible vasorelaxant activity. None of the flavonoids tested, except for luteolin 3-*O*-rhamnoside (2), showed any vasorelaxant property at the test concentrations (highest concentration: 800 M). Luteolin 3-*O*-rhamnoside (2) relaxed rat aortic vascular smooth muscle in a dose-dependent manner (Figure 2). This finding was in agreement with the results of previous studies on flavonoids conducted by other researchers (22,23,27,28). It is interesting to note that, although compounds 1, 2, and 4 are structurally quite similar, luteolin 3-*O*-rhamnoside (2) lacks any *O*-glycosylation at C-3. Thus, it is possible that *O*-glycosylation at C-3 rendered compounds 1 and 4 inactive.

Table. Antioxidant properties of compounds 1-6 in the DPPH assay.

Compounds	RC_{50}* value (mg/ml)
Quercetin 3-*O*-rhamnoside (1)	0.0035
Luteolin 7-*O*-rhamnoside (2)	0.0033
Isorhamnetin 3-*O*-rutinoside (3)	0.0403
Kaempferol 3-*O*-rhamnoside (4)	0.1605
Apigenin (5)	0.0043
Naringenin (6)	0.5186
Positive control: Trolox®	0.0026

* The concentration of the test material that reduces 50% of the free radical concentration was calculated as mg/ml

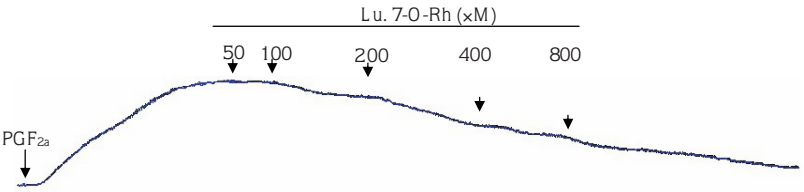

Figure 2. Vasorelaxant effect of luteoline 7-*O*-rhamnoside (Lu. 7-*O*-Rh) (2) in rat aortic rings precontracted by PGF_2 (10 M) in normal Krebs' solution.

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