Isotamarixen – A New Antioxidant and Prolyl Endopeptidase-Inhibiting Triterpenoid from *Tamarix hispida*

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Abstract

A new pentacyclic triterpenoid, 3α-(3″,4″-dihydroxy-*trans*-cinnamoyloxy)-D-friedoolean-14-en-28-oic acid (**1**) has been isolated along with two known compounds, rhamnocitrin (**2**) and isorhamnetin (**3**) from the aerial parts of *Tamarix hispida* Willd. Compound **1** was found to be a potent antioxidant. In addition, compounds **1** – **3** showed significant inhibitory activity against prolylendopeptidase (PEP).

The leaves of the plant *Tamarix hispida* Willd (Tamaricaceae) are used in traditional medicine in the treatment of dysentery, rheumatism, and ulcers [1]. Our phytochemical investigation on *T. hispida* led to isolation of a new triterpenoid with antioxidant and enzyme inhibiting potential. It is the first report of isolation of a triterpene from this species.

Compound **1** was isolated as a colorless amorphous solid with IR absorptions at 1259 (α,β-unsaturated ester) and 3411 (hydroxy) cm^{-1}. The UV spectrum showed absorptions at 216, 244, 255, 305 and 372 nm. The ^{1}H-NMR signals of seven 3H singlets at δ = 0.87 – 0.98 represented the seven tertiary methyls. An olefinic proton appeared as a double doublet at δ 5. = 5.57 (1H, dd, J = 8.0 Hz, J = 3.5 Hz, H-15). The chemical shift, multiplicity, coupling constants of the olefinic proton and chemical shifts of the tertiary methyl signals were in agreement with those of taraxer-14-en skeleton [2]. The H-3 (δ = 4.69) was deduced to be in a β conformation from the coupling constant (J = 2.5 Hz) of its signal. The coupling relationship between aromatic proton signals, appearing at δ = 7.03 (1H, d, J = 2.0 Hz, H-2″), 6.76 (1H, d, J = 8.0 Hz, H-5″) and 6.92 (1H, dd, J = 8.0 Hz, J = 2.0 Hz, H-6″), indicated a 1,3,4-trisubstituted aromatic ring. Two 1H doublets at δ = 6.26 and 7.51 (J = 16.0 Hz) represented the *trans*-substituted olefinic H-2′ and H-3′, which indicated the presence of *O*-3″,4″-dihydroxycinnamoyl group in **1**. The α-position of dihydroxycinnamoyl group at C-3 was deduced by the small $^3J_{H,H}$ (2.5 Hz) of the equatorial H-3 signal. The ^{13}C-NMR spectrum of **1** showed seven methyls, ten methylenes, ten methine and twelve quaternary carbon signals. The signals for eight olefinic carbons (C-2′, C-3′, C-1″, C-2″, C-3″ to C-6″), an ester carbonyl (C-1′), a carboxyl carbon (C-28) and one double bond carbon (C-14 and C-15) resonated at δ = 115.5, 146.5, 127.4, 115.0, 146.4, 149.2, 117.7, 122.7, 168.0, 182.2, 161.3 and 116.2, respectively. The position of the COOH-moiety at C-17 was deduced by a quaternary carbon signal at δ = 52.1 which was assigned to C-17. The complete ^{1}H- and ^{13}C-NMR chemical shifts and multiplicities of each protonated carbon are listed in Table **1**. The HMBC long-range coupling, data in which H-18 exhibited an interaction with C-28 (δ = 182.2), further confirmed the location of COOH at C-17. The H-3 (δ = 4.69) showed a long-range correlation with the C-1′ (δ = 168.0), indicating that the 3″,4″-dihydroxycinnamoyl group was located at C-3. The complete carbon-hydrogen connectivities are presented in Table **1**. To confirm the po-

Table **1** ^{1}H- and ^{13}C-NMR data of **1** (CD_3OD, $CDCl_3$)

C/H No	*Compound 1*		*HMQC connectivity*
	δ_C	δ_H	
1	36.4	2H m 1.30	H-25
2	23.5	2H m 1.95	(3)[c]
3	79.4	4.69 t (2.5)	H-23, H-24
4	38.3	–	H-3, H-23, H-24
5	51.7	1H 1.35	H-23, H-24, H-25
6	19.4	2H m 1.40; 1.60	(5,7)[c]
7	42.0	2H m 1.40; 1.95	H-26
8	40.0	–	H-26
9	50.2	1H 1.55	H-26
10	37.1	–	H-25
11	18.2	2H m 1.55; 1.65	(12)[c]
12	34.1	2H m 1.6; 1.76	H-27
13	37.6	–	H-27
14	161.3	–	H-26, H-27
15	116.2	5.57 dd (3.5;8)	(16)[c]
16	32.8	2H m 1.96; 2.37	(15)[c]
17	52.1	–	–
18	42.7	1H m 2.37	H-27
19	34.6	2H m 1.1; 1.24	H-29, H-30
20	30.4	–	H-29, H-30
21	34.3	2H m 1.05; 1.6	H-29, H-30
22	32.1	2H m 1.5; 1.7	(21)[c]
23	28.4	3H s 0.87	H-24
24	22.2	3H s 0.92	H-23
25	15.7	3H s 0.97	–
26	26.6	3H s 0.98	–
27	22.8	3H s 0.94	–
28	182.2	–	–
29	32.5	3H s 0.96	H-30
30	29.3	3H s 0.93	H-29
1′	168.0	–	H-3
2′	115.5	6.26 d (16)	(3')[c]
3′	146.5	7.51 d (16)	(2')[c]
1″	127.4	–	H-2, H-6
2″	115.0	7.03 d (2)	H-1
3″	146.4	–	H-2
4″	149.2	–	H-2, H-5, H-6
5″	117.7	6.76 d (8)	(H-6)[c]
6″	122.7	6.92 dd (2,8)	(H-5)[c]

[c] Proton cross-peaks in ^{1}H-^{1}H COSY-45° spectrum.

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sition of the cinnamoyl moiety, basic hydrolysis of compound **1** was carried out. The HREI of **1** showed the highest peak at *m/z* = 438.3507 corresponding to the formula $C_{30}H_{46}O_2$ (calc. 438.3497). The FAB-MS (-ve) showed the pseudomolecular ion at *m/z* = 617.432 corresponding to the molecular formula $C_{39}H_{53}O_6$. The mass difference between HREI- and FAB-MS data was due to the loss of a 3″,4″-dihydroxycinnamoyl group ($C_9H_7O_4$). The characteristic fragments at *m/z* = 248, 203, 189 and 133 resulting from the retro-Diels-Alder cleavage suggested a traxer-14-ene skeleton with a carboxylic group. On the basis of all spectral observations, compound **1** was deduced to be 3α-(3″,4″-dihydroxy-*trans*-cinnamoyloxy)-D-friedoolean-14-en-28-oic acid (**1**).

The compound 3α-hydroxytaraxer-14-en-28-oic acid (**1a**) is the hydrolyzed product of **1**. The EI-MS of **1a** (*m/z* $[M]^+$ = 456) corresponds to the molecular formula $C_{30}H_{48}O_3$. In the ^{1}H-NMR spectrum of **1a**, H-3 appeared as a broad singlet at δ = 4.41 ($W_{1/2}$ = 2.56 Hz) which indicated that H-3 was equatorially oriented. A triterpene, 3α-acetyltaraxer-14-en-28-oic acid, previously isolated from *Phytolacca acinosa* is structurally related to **1** except that the 3α-acetyl group is present at C-3 [3]. Compounds **2** and **3** were identified as rhamnocitrin and isorhamnetin, respectively, by comparison of their spectral data with the literature values [4], [5], [6], [7].

Compound **1** having a caffeoyl moiety exhibited significant antioxidant activity by scavenging the DPPH and superoxide radicals (Table **1**). The IC_{50} value of compound **1** is almost equal to both the standards indicating its effectiveness as a free radical scavenger. On the other hand, compound **1a** which does not have a caffeoyl moiety was found to be inactive. It has been reported that the phenolic groups contribute to the free radical scavenging ability of chemical compounds, the radical scavenging potential of **1** may therefore very well be due to the presence of the *o*-dihydroxy groups present at the caffeoyl moiety. Compounds **1** – **3** demonstrated significant inhibitory activities against PEP as compared to the positive control bacitracin [8] (Table **1**). This is the first report of a triterpene that showed potent inhibitory activity against PEP (EC 3.4.21.26). This enzyme has recently gained pharmaceutical interest since its specific inhibitors showed antiamnesic effects [9], [10], [11]. The hydroxy function at C-7 in compounds **2** and **3** seems to be responsible for the inhibitory activity. Cinnamic acid, methyl caffeate and pyrocatechol were also screened and only methyl caffeate showed inhibition with IC_{50} = 341.37 μM. These results indicate that the activity of compound **1** is probably due to the triterpenoid moiety itself or the ester linkage between caffeic acid and the triterpenoidal moiety.

Material and Methods

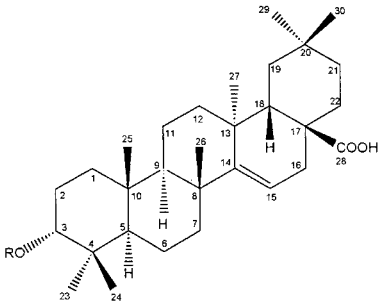
Thin layer chromatography was carried out on precoated silica gel sheets (E. Merck, 60 F_{254}). Column chromatography was also performed using silica gel (230 – 400 mesh) and spots were detected at 254 and 366 nm and also by using ceric sulphate spraying reagent. UV spectra were recorded on a Hitachi UV 3200 spectrophotometer, while IR spectra were recorded on a Jasco 302-A spectrophotometer. Optical rotation was measured on a Schmidt + Haensch Polartronic D. Melting points were measured on a Gallenkamp apparatus. FAB-MS was measured on a Jeol HX 110 mass spectrometer. EI-MS and HREI-MS were recorded on a Varian MAT 311A mass spectrometer. The ^{1}H-NMR spectra were recorded on Bruker AM 500 MHz spectrometer, while ^{13}C-NMR spectra were recorded at 125 MHz on the same instrument.

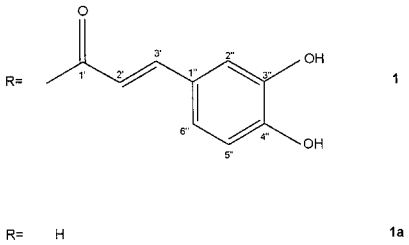
The aerial parts (3 kg) of *T. hispida* Willd. were collected from the southern parts of Kazakhstan in September 2000. The plant was identified by Dr. Lapshina and a voucher specimen (# 5239) was deposited at Department of Botany, Al-Farabi Kazakh National University, Almaty.

Dried and crushed plant material of *T. hispida* (2 kg) was macerated in 50% acetone-H_2O (2 × 5 L) at room temperature. The extract was filtered and concentrated. The concentrated extract (50 g) was dissolved in water (1 L) and defatted with petroleum ether, followed by chloroform and ethyl acetate extractions. The ethyl acetate extract (10 g) which was found to be most active in the DPPH free radical assay, was chromatographed over silica gel (900 g) with gradient eluents (CH_2Cl_2, 2.5 L ; 0.5 – 100% MeOH in CH_2Cl_2, each 2.5 L) to afford frs. 1 – 4. Fr. 2 (3.0 g) obtained from the 5% MeOH was chromatographed over silica gel (240 g) using hexane/EtOAc (0 – 100% EtOAc, each 100 mL) as eluents to yield four fractions (frs. 2.1 – 2.4). Fr. 2.2 (500 mg) obtained from the 20% EtOAc was rechromatographed over silica gel (40 g) and eluted with hexane/EtOAc (9 : 1 → 8 : 2 → 7 : 3, each 10 mL) afforded compound **2** (10 mg) and fr. 2.2a on elution with hexane/EtOAC (9 : 1). The fr. 2.2a (50 mg) was chromatographed over silica gel (4.5 g) and eluted with hexane-EtOH-BuOH (8 : 1.5 : 0.5, 5 mL) to afford a partially purified compound, which was purified by preparative TLC using pre-coated silica gel plates with solvent system CH_2Cl_2/MeOH (9.8 : 0.2) to furnish compound **1** (20 mg). Fr. 2.4 (250 mg) obtained from the hexane/EtOAc (6 : 4) was chromatographed over a flash silica gel (15 gm) column and eluted with hexane/EtOAc (8 : 2 → 7 : 3 → 6 : 4, each 10 mL) to afford three frs. 2.4a – 2.4c. Fr. 2.4b (20 mg) obtained from the hexane/EtOAc (7 : 3) was purified by preparative TLC using pre-coated silica gel plates with solvent system MeOH/$CHCl_3$ (3 : 7) to afford compound **3** (12 mg).

3α-(3″,4″-Dihydroxy-trans-*cinnamoyloxy)*-D-*friedoolean-14-en-28-oic acid* (**1**), $C_{39}H_{54}O_6$; amorphous solid; Rf (CH_2Cl_2:MeOH, 9.7 : 0.3): 0.3, (EtOAc:hexane, 4 : 6): 0.5; m.p. 196 – 198 °C; UV (MeOH): λ_{max} = 216, 244, 255, 305, 372 nm; $[\alpha]_D^{27}$: –22° (*c* 0.04, MeOH); IR (KBr): ν_{max} = 3411 (OH), 1259 (α,β-unsaturated ester), 1691 (COOH), 1600, 812 (C = C) cm^{-1}; FAB-MS (–ve): *m/z* = 617.432 $[M - 1]^-$. EI-MS: *m/z* = 438, 248, 203, 189, 133; ^{1}H-NMR (500 MHz, mixture of CD_3OD and CD_3Cl_3) and ^{13}C-NMR (125 MHz, mixture of CD_3OD and $CDCl_3$), see Table **1**.

The compound **1** (6 mg) was refluxed in a solution of 3% KOH in MeOH (5 mL) for 2 hours at 60 °C. Work-up in the usual way afforded a product that was chromatographed over a silica-gel column using $CHCl_3$ as eluent to afford 3α-hydroxytaraxer-14-en-28-oic acid (**1a**) (3 mg) which was identified on the basis of IR, EI-MS and ^{1}H-NMR data [3]. EI-MS: *m/z* = 456.36 [$C_{30}H_{48}O_3$], $[M - H_2O]^+$, 438, 248, 203, 189, 133; IR (KBr): ν_{max} = 3448 (OH); 1690 (COOH), 1460, 1386, 1061, 993 (C = C) cm^{-1}; $[\alpha]_D^{27}$: –13.5° (*c* 0.04, $CHCl_3$); m.p. 270 – 272 °C.

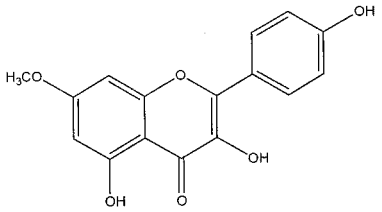




1

R= H

1a



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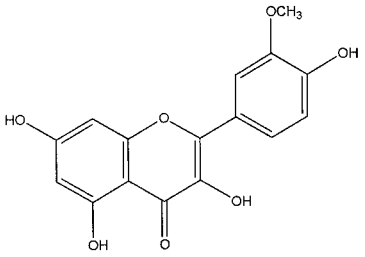

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Table **2** *In vitro* antioxidant and PEP inhibitory activities of compounds **1** – **3**

Compound	*IC50 (μM)**		
	DPPH Scavenging activity	*Superoxide scavenging activity*	*PEP Enzyme inhibition*
1	29 ± 0.5	306 ± 1.4	0.250 ± 0.021
1a	×	×	–
2	–	–	32.64 ± 0.84
3	–	–	18.94 ± 0.25
Bacitracin** for PEP	***	***	129.26 ± 3.28
PG**	30 ± 0.27	106 ± 1.7	***
BHA**	44 ± 2.00	***	***

* IC_{50} values are the mean ± standard mean (SEM) error of three assays. ** Used as standard. *** Not used as standard in these assays.
× Not active. - Not determined. PG = propyl gallate. BHA = 3-*t*-butyl-2-hydroxyanisole.

Antioxidant activities were determined by using the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) scavenging and NADH/PMS (β-nicotinamide adenine dinucleotide/phenazine methosulphate) superoxide anions scavenging assays [12], [13] while the PEP inhibitory activities were measured by the method of Yoshimoto et al. [14].

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