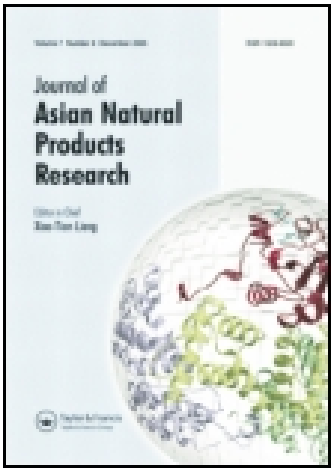

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A novel spinosin derivative from Semen Ziziphi Spinosae

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NOTE

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A novel spinosin derivative, 6‴-(4⁗-*O*-β-D-glucopyranosyl)-vanilloyl spinosin (**1**) was isolated from the methanol extract of Semen Ziziphi Spinosae, together with five known flavonoids, swertish (**2**), spinosin (**3**), 6‴-feruloylspinosin (**4**), isospinosin (**5**) and kaempferol 3-*O*-α-L-rhamnopyranosyl-(1 → 2)-*O*-[*O*-α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside (**6**), and two alkanoids, zizyphusine (**7**) and 6-(2′,3′-dihydroxyl-4′-hydroxymethyl-tetrahydro-furan-1′-yl)-cyclopentene[c]pyrrole-1,3-diol (**8**). The structure of compound **1** was elucidated by spectroscopic methods including UV, IR, ESI-TOF-MS, 1D, and 2D NMR techniques.

Keywords: Semen Ziziphi Spinosae; flavone-*C*-glycoside; alkaloid; spectroscopic methods

1. Introduction

Semen Ziziphi Spinosae, botanically from the dried seeds of *Ziziphus jujuba* Mill. var. *spinosa* (Bunge) Hues. H.F. Chou, has been used as a sedative-hypnotic drug for symptoms such as insomnia and dysphoria for thousands of years in Chinese medical practice. Chemical investigations on jujuba seeds resulted in the discovery of several groups of bioactive components including flavonoids, triterpenoid saponins, and alkaloids [1–9]. Experimental studies validated that they exhibited wide pharmacological effects, such as the inhibitory activities of the spontaneous activity, facilitating the hypnotic action of pentobarbital, and antagonizing the excitatory action of morphine and pentylenetetrazole [10,11]. Although some flavone C-glycosides such as spinosin, as well as saponins of jujubogenin were isolated from the seeds and leaves of this plant, the minor components have rarely been studied yet.

This study describes the isolation and structural elucidation of a new spinosin derivative, 6‴-(4⁗-*O*-β-D-glucopyranosyl)-vanilloyl spinosin (**1**), together with seven known compounds including five polyhydroxylated flavonoids (**2**–**6**) and two alkaloids (**7** and **8**).

2. Results and discussion

The MeOH extract of the seeds of *Z. jujuba* Mill. Var. *spinosa* was prepared by reflux method after degreased by petroleum ether. A new spinosin derivative, 6‴-(4⁗-*O*-β-D-glucopyranosyl)-vanilloyl spinosin (**1**), was isolated from the MeOH extract, together with seven known compounds including five polyhydroxylated flavonoids, swertish (**2**), spinosin (**3**), 6‴-feruloylspinosin (**4**), isospinosin (**5**), and kaempferol-3-*O*-α-L-rhamnopyrano-

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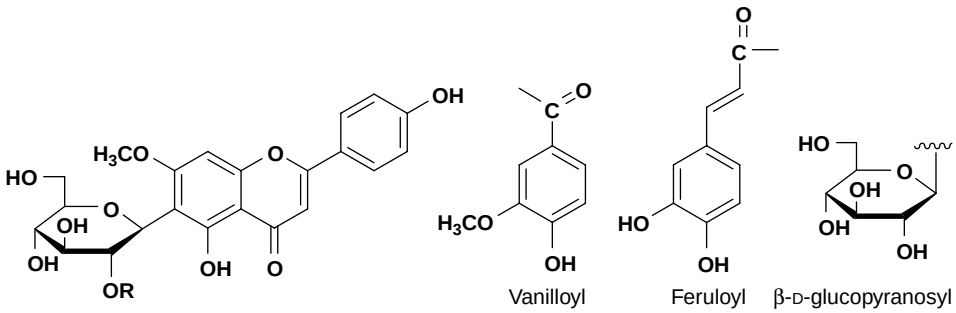
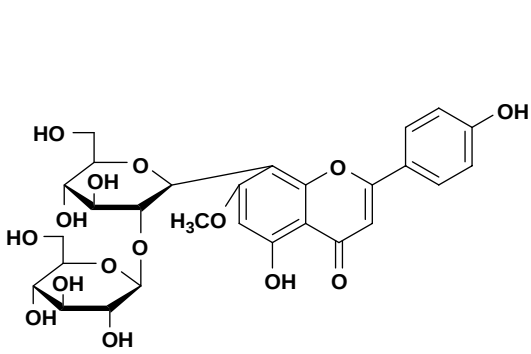
syl-(1 → 3)-*O*-[*O*-α-L-rhamnopyranosyl-(1 → 6)]-β-D-glucopyranoside (**6**), and two alkaloids, zizyphusine (**7**) and 6-(2′,3′-dihydroxyl-4′-hydroxymethyl-tetrahydrofuran-1′-yl)-cyclopentene[c]pyrrole-1,3-diol (**8**) (Figure 1). Among them, compound **1** is a new one, whose structure was elucidated using UV, IR, ESI-TOF-MS, 1D, and 2D NMR techniques. The known compounds were identified by comparing their NMR and MS data with the reported values [7,12–14].

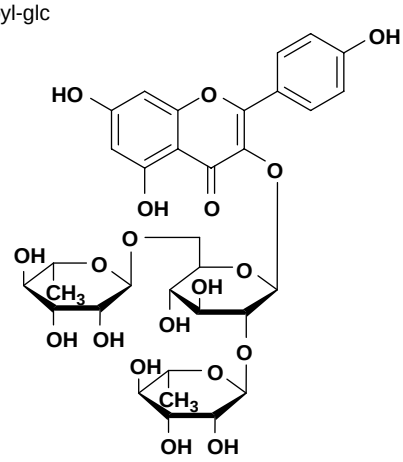
Compound **1** was isolated as a yellowish amorphous powder. The molecular formula was determined to be $C_{42}H_{48}O_{23}$ from the $[M-H]^-$ ion peak at *m/z* 919.2508 in the HR ESI-TOF-MS. The UV absorption maxima at 216, 262, and 343 nm revealed a flavone skeleton. A positive reaction in the $AlCl_3$ reagent suggested that compound **1** was a hydroxyl-substituted flavonoid. The IR spectrum showed absorption bands due to hydroxyl group (3401 cm^{-1}), carbonyl (1652 and 1701 cm^{-1}), and aromatic ring (1607, 1510, and 1443 cm^{-1}). The paper chromatography (PC) analysis of the acid hydrolysis of compound **1** exhibited only the presence of glucose. The ^1H NMR spectrum (600 MHz, DMSO-d_6) of **1** indicated six aromatic proton signals due to aglycone moiety, i.e., a characteristic singlet signal at δ 6.67/6.64 due to the H-3 proton, a singlet at δ 6.55/6.42 (1H, s, H-8) indicating A-ring with three substituents, an AA′BB′ aromatic proton system appearing at δ 7.80/7.70 (d, $J = 8.4$, 9.0 Hz, H-2′, 6′) and 6.91/6.87 (d, $J = 8.4$, 9.0 Hz, H-3′, 5′) indicating a C-4′ substituted B-ring [15], and a singlet at δ 3.86 (3H, s) due to methoxyl protons. The resonances for the carbons and protons of the aglycone and the sugar moiety, except for those of C-5‴ and C-6‴, as well as the related protons (Table 1), had a close resemblance to those of spinosin (**3**), and they were assigned according to the ^1H and ^{13}C NMR spectral data [7] for spinosin as well as its own HSQC spectrum. The signals of two anomeric protons at δ 4.67 (1H, d, $J = 9.6$ Hz, H-1″) and 4.29 (1H, d, $J = 7.8$ Hz) and 10 protons at δ 3.73–3.06 together with the ^{13}C NMR spectral data indicated the presence of two β-D-glucosyl moieties [16]. The linkage between the anomeric C-1″ and C-6 and the interglucosidic linkage between C-2″ and C-1‴ were determined by the HMBC correlations of H-1″/C-6 and H-1‴/C-2″. The chemical shift value of the anomeric carbon C-1″ and HMBC correlation between H-1″ (δ 4.67) and C-6 (δ_C 108.8/108.7) indicated that the anomeric carbon (C-1″, δ_C 70.8) of β-glucose was connected to C-6 through a *C*-linkage. The similar NMR phenomenon to that of spinosin, i.e. the appearance of serial separate signals, was observed. It is proposed that rotational isomers produced by the rotational barriers 7-OCH_3 in flavones-6-C-glycoside must exist in compound **1** [7]. The downfield shift of C-6‴ (δ_C 62.9, + 2.2 ppm) and the upfield shift of C-5‴ (δ_C 73.2, −3.6 ppm) relative to the corresponding signals of spinosin revealed the acylation of C-6‴, which is also supported by the corresponding signals of 6‴-feruloyspinosin (**4**) [7]. The spinosin moiety accounted for a partial molecular formula of $C_{28}H_{31}O_{15}$. Thus, the remaining molecular formula should be $C_{14}H_{17}O_8$ as a substituent attached to the spinosin group at C-6‴. The resonances for the carbons and protons of the substituent in the ^1H and ^{13}C NMR as well as HSQC spectra included an ABX aromatic proton system appearing at δ 6.98 (1H, d, $J = 9.0$ Hz, H-5⁗; δ_C 112.4/112.0), 7.10 (1H, dd, $J = 9.0$, 1.2 Hz, H-6⁗; δ_C 122.7) and 7.20 (1H, d, $J = 1.2$ Hz, H-2⁗; δ_C 114.2/114.4), a methoxyl (δ 3.66, 3H, s; δ_C 55.4), and a carbonyl (δ_C 165.3). The IR absorption bands at 1701 cm^{-1} also proved the existence of the carbonyl group. These data together with the HMBCs between H-6⁗ and C-7⁗, H-2⁗and C-7⁗ as well as the proton of methoxyl and C-3⁗ indicated the presence of a vanilloyl moiety



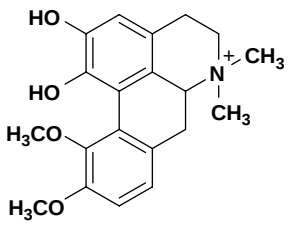
6‴-(4⁗-*O*-β-D-glucopyranosyl)-vanilloyl spinosin (**1**)	6-(4-*O*-β-D-glc-vanilloyl)-glc
swertish (**2**)	H
spinosin (**3**)	-glc
6‴-feruloylspinosin (**4**)	6-feruloyl-glc



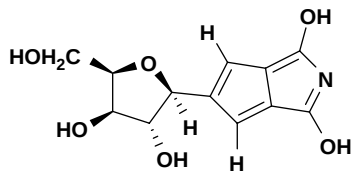
Isospinosin (**5**)



Kaempferol-3-*O*-α-L-rhamnpyranosyl-(1→3)-*O*-[*O*-α-L-rhamnpyranosyl-(1→6)]-β-D-glucopyranoside (**6**)



Zizyphusine (**7**)



6-(2',3'-dihydroxyl-4'-hydroxyl-ethyl-tetrahydrofuran)-cyclopentene [c] pyrrole-1,3-diol (**8**)

Figure 1. Structures of compounds **1**–**8**.

(4-hydroxy-3-methoxy benzoyl) which accounted for a partial molecular formula of $C_8H_6O_3$ [17]. In addition, the signals of an anomeric proton at δ 5.05 (d, $J = 7.2$ Hz, H-1⁗) and five protons at δ 3.73–3.06 as well as the ^{13}C NMR data

Table 1. ^{1}H and ^{13}C NMR spectroscopic data for compound **1** in DMSO-d_6.

Position	δ_C[a]	δ_H[b] (*J* in Hz)
2	164.0 (163.6)	
3	102.9	6.67/6.64, s
4	182.4 (181.8)	
5	159.6	
6	108.7 (108.9)	
7	165.0 (164.8)	
8	90.6 (90.0)	6.55/6.42, s
9	157.1 (156.8)	
10	104.6 (104.0)	
1′	122.7 (122.5)	
2′, 6′	128.6 (128.4)	7.80/7.70, d (8.4)
3′, 5′	116.0	6.91/6.87, d (8.4)
4′	161.7 (160.9)	
7-OCH3	56.5 (56.2)	3.86, s (3.81, s)
1″	70.8	4.67, d (9.6)
2″	81.8	
3″	78.7	
4″	70.4	
5″	82.1	
6″	61.6	
1‴	105.8 (105.1)	4.29, d (7.8)
2‴	74.6	
3‴	76.3	
4‴	69.8	
5‴	73.2	
6‴	62.9 (62.5)	3.86, s (3.81, s)
1⁗	123.0 (122.8)	
2⁗	114.4 (114.2)	7.20, d (1.2)
3⁗	148.5 (148.4)	
4⁗	150.6 (150.5)	
5⁗	112.4 (112.0)	6.98, d (9.0)
6⁗	122.7 (122.5)	7.10, dd (1.2, 9.0)
7⁗	165.3	
3⁗-OCH3	55.4 (55.2)	3.66, s (3.52, s)
1⁗′	99.9 (99.7)	5.05 (7.2)
2⁗′	74.6	
3⁗′	77.3	
4⁗′	70.8 (70.5)	
5⁗′	78.9 (78.7)	
6⁗′	62.9 (62.5)	

[a] Measured separately at 150 MHz.
[b] Measured separately at 600 MHz.

indicated the presence of a β-D-glucosidic moiety in the substituent. The correlation between the proton at δ 5.05 (H-1⁗′) and the carbon at δ_C 150.6/150.5 (C-4⁗) in the HMBC experiment suggested that the β-D-glucosyl group should be attached to the C-4⁗ position of the vanilloyl moiety. Thus, the substituent was confirmed as 4⁗-*O*-β-D-glucopyranosyl-vanilloyl [18] (Figure 2).

After mild alkaline hydrolysis of **1** with 0.05 N NH_4OH in 50% MeOH, a mixture of two aromatic compounds were recovered from the ethyl acetate extract of the hydrolysate. They were separated and one of the hydrolysis products was identified as spinosin. The above evidence suggested

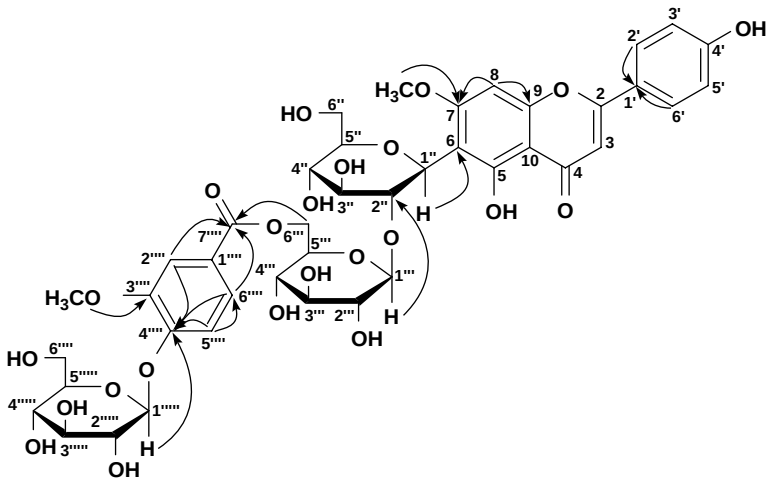


Figure 2. Selected HMBC correlations of **1**.

that compound **1** is a derivative of spinosin, with 4-*O*-β-D-glucopyranosyl-vanilloyl as a substituent attached to the sugar moiety. The HMBC correlations between H-6‴ (δ 3.84, m and δ 4.02, m) and the carbonyl carbon (δ_C 165.0/165.3) suggested a linkage between the 4-*O*-β-D-glucopyranosyl-vanilloyl moiety and C-6‴ of glucose. Hence, compound **1** was assigned as 6‴-(4⁗-*O*-β-D-glucopyranosyl)-vanilloyl spinosin, a new flavonoid glycoside. The possibility of **1** as an artifact was ruled out by the fact that the HPLC analysis confirmed its presence in the extract from the outset.

3. Experimental

3.1 General experimental procedures

Melting points were measured using a micro melting point apparatus and are uncorrected (Yanamoto Manufactory Co., Kyoto, Japan). Optical rotations were recorded on a Perkin-Elmer 241MC automatic polarimeter. UV spectra were obtained on a Shimadzu UV-2201 spectrophotometer, and IR spectra were obtained on a Bruker IFS-55 infrared spectrometer. 1D and 2D NMR spectra were recorded separately on a Bruker ARX-600 or ARX-300 spectrometer. ESI-TOF-MS were acquired on a Micro TOF Bruker Daltonics mass spectrometer with a resolution of 25,000 (10% Valley). Preparative HPLC was carried out using a Shimadzu's LC-8A solvent delivery pump and Shimadzu's SPD-10AVP detector. Column chromatography was carried out on silica gel (200–300 mesh, Qingdao Haiyang Chem. Group Co., Qingdao, China), Sephadex LH-20 (GE Healthcare, Uppsala, Sweden), octadecylsilyl silica (ODS) (YMC Co., Ltd, Kyoto, Japan), MDS-5 reverse-phase packing (200–300 mesh, Beijing Medicine Technology Center, Beijing, China), and Waters C_{18} column (7.8 × 250 mm, 6 μm, Waters Co., Ltd, Milford, MA, USA).

3.2 Plant material

The seeds of *Z. jujuba* were obtained from Chaoyang, Liaoning Province of China, in May 2008, and authenticated by Prof. Dan Yuan, Shenyang Pharmaceutical University. A voucher specimen (No. 080518) has been maintained in the Department of Traditional Chinese Medicines, Shenyang Pharmaceutical University.

3.3 Extraction and isolation

The air-dried seeds (2.0 kg) of *Z. jujuba* were ground into powder (particle size

20–40 mesh) and refluxed with 16 liters of petroleum ether (60–90°C) two times (each for 1 h). After removing the petroleum ether under reduced pressure, the residue was refluxed with 15 liters of methanol twice to obtain the total extract of jujuba seeds with a yield of 6.5%. The condensed MeOH extract (130 g) was subjected to silica gel column chromatography eluting with $CHCl_3$–MeOH in a stepwise manner to furnish five major fractions: fraction 2 ($CHCl_3$–MeOH, 7:1, 1.5 g), fraction 3 ($CHCl_3$–MeOH, 6:1, 0.2 g), fraction 5 ($CHCl_3$–MeOH, 6:1, 1.8 g), fraction 7 ($CHCl_3$–MeOH, 4:1–3:1, 3.0 g), and fraction 9 ($CHCl_3$–MeOH, 2:1, 8 g). Fraction 2 (1.5 g) was passed through a Sephadex LH-20 column eluted with MeOH to give compound **8** (50 mg). Fraction 3 (200 mg) was further separated by open column chromatography using an MDS-5 reverse-phase packing and eluting with a linear gradient of MeOH–H_2O (30:70–95:5) to yield four major fractions (F3-1, F3-2, F3-3, and F3-4). F3-4 (80 mg) eluted with MeOH–H_2O (95:5) was further subjected to Sephadex LH-20 column (1.0 × 40 cm) and eluted with MeOH to give compound **4** (50 mg). Fraction 5 (5.3 g) was repeatedly recrystallized with MeOH to give compound **3** (450 mg). The mother-liquor was subjected to silica gel column chromatography eluted with $CHCl_3$–MeOH gradient. The fraction 7 (3.0 g) was further separated with an open column chromatography packed with MDS-5 reverse-phase packing particles eluted with MeOH–H_2O (40:60) to yield compound **5** (20 mg) and compound **2** (5.6 mg). Fraction 9 (8 g) was subjected to MDS-5 open column chromatography eluting with a linear gradient of MeOH–H_2O (10:90–95:5) to yield nine major fractions (F9-1–F9-8). F9-3 (537 mg) eluted with MeOH–H_2O (50:50) was purified using ODS open column chromatography to give compound **7** (39 mg). Fraction 9-5 (153 mg) eluted with MeOH–H_2O (60:40) was further subjected to a Sephadex LH-20 column eluting with MeOH to yield subfractions F9-5-1–F9-5-10. F9-5-5 (55 mg) was further purified by ODS open column chromatography eluted with MeOH–H_2O (30:70) to afford compound **1** (10.4 mg). F9-5-10 (15 mg) was further separated with preparative HPLC (Waters C_{18} column, 7.8 × 300 mm, 6 μm) eluting with MeOH–H_2O (30:70) at a flow rate of 2 ml/min to give compound **6** (9.4 mg), and the detective wavelength was set at 254 nm.

*3.3.1 6‴-(4⁗-O-β-D-Glucopyranosyl)-vanilloyl spinosin (**1**)*

A yellowish amorphous powder, m.p. 215–218°C. $[\alpha]_D^{24}$ − 54.2 ($c = 0.2$, MeOH). UV (MeOH) λ_{max} nm: 217, 262, and 343. IR (KBr) ν_{max} cm^{-1}: 3401, 2924, 1701, 1652, 1607, 1510. ^1H and ^{13}C NMR spectral data, see Table 1. ESI-TOF-MS (negative) *m/z*: 919.2508 $[M - H]^-$ (cald for $C_{42}H_{47}O_{23}$, 919.2502).

3.4 HPLC analysis

HPLC analysis was carried out on a Waters 510 HPLC instrument equipped with photodiode array detector. The separation was carried out on a Hypersil ODS2 column (250 × 4.6 mm i.d., 5 μm, Elite Analysis Instruments, Dalian, China). The temperature of column was set at 35°C. The mobile phase consisted of a gradient system of (A) water and (B) acetonitrile with a gradient elution as followed: linear gradient from eluent A–B (100:0, v/v) to eluent A–B (50:50, v/v) in 60 min at the flow rate of 1.0 ml/min. The detective wavelength was set at 334 nm.

3.5 Mild alkaline hydrolysis of 1

Solution of compound **1** (1 mg) in 0.05 N NH_4OH-50%MeOH (2 ml) was stirred at room temperature for 1 h. The reaction mixture was neutralized with

Dowex HCR-W2 (H^+ form) and the resin was removed by filtration. The hydrolysate was extracted with EtOAc. After purification of the extract by Varian BOND ELUT® C_{18} column (1.0 × 4.0 cm, GL Science, Tokyo, Japan) eluting with a MeOH–H_2O gradient (20% MeOH → 50% MeOH → MeOH), an aromatic compound was obtained, which was identified as spinosin by co-chromatograph (TLC silica gel) in $CHCl_3$–MeOH–H_2O (7:3:1, R_f 0.35), EtOAc–MeOH–H_2O (4:1:1, R_f 0.45) with an authentic sample.

3.6 *Acid hydrolysis of 1*

Solution of compound **1** (1 mg) was refluxed at 100°C for 1 h in 2 N HCl in MeOH (10 ml). The acid hydrolysate was extracted with EtOAc, and the sugar in the aqueous layer was identified as glucose by co-PC (*n*-BuOH–AcOH–H_2O 4:1:5, R_f 0.19, aniline phthalate spray).

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