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Sesquiterpene coumarins from *Ferula szowitsiana* and *in vitro* antileishmanial activity of 7-prenyloxycoumarins against promastigotes

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Abstract

Two new sesquiterpene coumarins, named szowitsiacoumarin A (**1**) and szowitsiacoumarin B (**2**), and a phenylpropanoid derivative, 2-epihelmanticine (**3**), together with nine known compounds, auraptene (**4**), umbelliprenin (**5**), galbanic acid (**6**), methyl galbanate (**7**), farnesiferol B (**8**), farnesiferol C (**9**), persicasulfide A (**10**), β-sitosterol and stigmasterol were isolated from the roots of *Ferula szowitsiana*. The structures of these compounds were elucidated by extensive spectroscopic methods including 1D-(^{1}H and ^{13}C) and 2D-NMR experiments (DQF-COSY, HSQC, HMBC, and ROESY) as well as HR-MALDI-MS analysis. Since the configuration of 2-epihelmanticine was previously only partly determined, a relative configurational analysis of its four stereocenters was carried out on the basis of the recently reported *J*-based method.

The inhibiting activity of prenylated coumarins, auraptene (**4**) and umbelliprenin (**5**), in addition to galbanic acid (**6**), as major component, and of the Me_2CO extract of *Ferula szowitsiana* (Apiaceae) roots has been evaluated against promastigotes of *Leishmania major*. Umbelliprenin and auraptene showed significant activity with IC_{50} values of 4.9 μg/ml (13.3 μM) and 5.1 μg/ml (17.1 μM) after 48 h incubation, respectively.

Keywords: *Ferula szowitsiana*; Szowitsiacoumarin A; Szowitsiacoumarin B; 2-Epihelmanticine; Auraptene; Galbanic acid; Umbelliprenin; *Leishmania major*; Promastigotes

1. Introduction

Leishmaniasis is a parasitic disease, in which the sand fly is the common vector of transmission. Species of the genus *Leishmania*, a protozoan member of the hemoflagelate group, are causative agents of human leishmaniasis, which has a reservoir in rodents, dogs and others in the wild animal population, and transmitted by mosquitoes of the genera *Lutzomia* and *Phlebotomus*. Members of the genus *Leishmania* differentiate from proliferative promastigotes in the sand fly vector gut to infective metacyclic promastigotes in the insect foregut. Parasites are inoculated by the vector as the flagellate promastigotes enter the mammalian host, where they infect macrophages, differentiating into nonmotile amastigotes and multiply as such (Carvalho et al., 2000). The term leishmaniasis comprises three clearly distinguishable clinical manifestations: generalized visceral infection, cutaneous leishmaniasis, and mucocutaneous leishmaniasis.

Leishmaniasis is regarded as a major public problem (WHO), causing significant morbidity and mortality in Africa, Asia and Latin America. With an estimated number of 500,000 new cases accruing annually, visceral

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leishmaniasis is still considered as one of the most severe affections by the World Health Organization (WHO. Fact sheet No 116).

The treatment of leishmaniasis is difficult because of the intramacrophagic location of the infectious form. In the absence of a vaccine, there is an urgent need for effective drugs to replace or supplement those in current use. The clinically used drugs, many of which are based on pentavalent antimony compounds, were developed before 1959. The toxicity of these agents and the persistence of side-effects even after modification of dose level and duration of treatment are, however, severe drawbacks. The search for antileishmanial agents has been exhaustive. Alternative drugs, such as amphotericin B and pentamidine, also have unpleasant side effects (Balana et al., 1998; Carvalho et al., 2000). On the other hand, plant extract or plant-derived compounds are likely to provide a valuable source of new medicinal agents (Kayser et al., 2001; Carvalho and Ferreira, 2001).

Recently, many natural products have been reported to show antileishmanial activity including naphthoquinones, lignans, neolignans, alkaloids, chalcones and triterpenoids (Kayser et al., 2000; Sauvain et al., 1996; Barata et al., 2000; Delorenzi et al., 2001; Torres-Santos et al., 1999; Camacho et al., 2000). Coumarins have been also reported to show antileishmanial activity (Oketch-Rabah et al., 1997; Bravo et al., 1999) and it was recently reported that auraptene can inhibit the growth of promastigotes of *Leishmania major* ($LD_{50} = 30\ \mu M$) (Napolitano et al., 2004).

The exclusively old world genus *Ferula* belongs to the family Umbelliferae with about 130 species distributed throughout the Mediterranean area and central Asia, specially in the former USSR and neighboring countries such as Iran. This genus is well documented as a good source of biologically active compounds such as sesquiterpene derivatives (Ahmed et al., 2001; Ahmed, 1999; Valle et al., 1987). Sesquiterpene derivatives, especially sesquiterpene coumarins, were stored in the roots of the plants; therefore the roots are better source for isolating sesquiterpene coumarins than the aerial parts. *Ferula szowitsiana*, similar to other species of the genus *Ferula*, is a rich source of sesquiterpene coumarins (Murray et al., 1982). In the present study, we report the isolation and the structure elucidation of two new compounds, szowitsiacoumarin A, szowitsiacoumarin B, (**1**–**2**) along with the isolation and the NMR configurational assignment of 2-epihelmanticine (**3**). We also isolated persicasulfide A (**10**) and the two common well-known steroids, β-sitosterol and stigmasterol, for the first time from *F. szowitsiana*, together with auraptene (**4**), umbelliprenin (**5**), galbanic acid (**6**), methyl galbanate (**7**), farnesiferol B (**8**), farnesiferol C (**9**) (Fig. 1). On the basis of the antileishmanial effect of auraptene, a prenylated coumarin, we tested the growth inhibitory activity of umbelliprenin (**5**), galbanic acid (**6**) and of the Me_2CO extract of the roots against *L. major* promastigotes.

2. Results and discussion

The isolated coumarins (**4**–**9**) (Murray et al., 1982; Lee et al., 1998; Iranshahi et al., 2003b; Nabiev et al., 1982), persicasulfide A (**10**) (Iranshahi et al., 2003a) and β-sitosterol and stigmasterol (Goad and Akihisa, 1997) were identified by comparison of their NMR, IR, MS and melting point data with those previously described in the literature. This is the first report of persicasulfide A, a sulfur containing compound, β-sitosterol and stigmasterol from *F. szowitsiana*. Other components including six coumarins (**4**–**9**) were previously reported from the plant (Murray et al., 1982).

Szowitsiacoumarin A (**1**) showed a pseudomolecular ion peak in the HR-MALDI-MS spectrum at *m*/*z* of 383.2195 $[M + H]^+$, consistent with the molecular formula $C_{24}H_{31}O_4$ (exact mass calculated for $C_{24}H_{31}O_4$ 383.2222). The structure of **1** was established from analysis of the ^1H and ^{13}C NMR spectra (Table 1). Compound **1** displayed 24 carbon signals, nine being typical of an umbelliferone skeleton and the other 15 signals were ascribable to a sesquiterpene moiety. The downfield signal at δ_C 161.2 was assigned to the carbonyl carbon of the coumarin moiety, whereas the downfield signal at δ_C 212.5 was indicative of a ketone group belonging to the sesquiterpene unit. HSQC spectrum classified the carbon signals to four aliphatic methylenes at δ_C 23.6, 25.4, 32.3, and 41.4, to a primary alcoholic carbon at δ_C 75.9 characteristic for C-11′, to nine methines, five of them for umbelliferone moiety at δ_C 113.0 (C-3), 143.3 (C-4), 128.7 (C-5), 113.1 (C-6), and 101.2 (C-8) and to four methyls at δ_C 7.1, 14.4, 14.9, and 20.0. The ^1H NMR showed for four three-proton signals two singlets in accordance with two tertiary methyl groups and two doublets in accordance with two secondary methyl groups.

Long range HMBC correlations from the proton signal at δ_H 1.13 (Me-12′) to the carbon resonance at δ_C 39.5 (C-9′) and from the protons of the primary oxygenated carbon C-11′ to the same carbon C-9′ revealed the location of a tertiary methyl group (Me-12′) at C-9′.

A third HMBC correlation between the doublet methyl (Me-15′) at δ_H 1.03 and the carbon resonance at δ_C 39.5 (C-9′) allowed to assign a first doublet methyl at C-8′. The remaining methyl groups (Me-13′ and Me-14′) were established at C-4′ and at C-5′ carbons, respectively, from the COSY cross-peak between the quartet (H-4′) at δ_H 2.36 and the doublet methyl (Me-13′) at δ_H 0.93 and from the HMBC correlation between the carbon resonance at δ_C 58.3 (C-4′) and the proton signals at δ_H 0.82 (Me-14′). The down-field carbon chemical shift at δ_C 58.3 was consistent with a neighboring group effect from a C-3′ ketone.

The ROESY experiment supported the relative configuration of the stereogenic centres at C-4′, C-5′, C-8′, C-9′, and C-10′.

In particular ROEs H-11′/Me-15′, H-10′/Me-15′ established an α-orientation for both H-10′ and Me-15′, and a β-orientation for Me-12′. On the other hand, H-10′ showed correlation with H-4′, whereas Me-14′ with H-7′β at δ_H 1.97 which in turn exhibited correlation with Me-12′. These

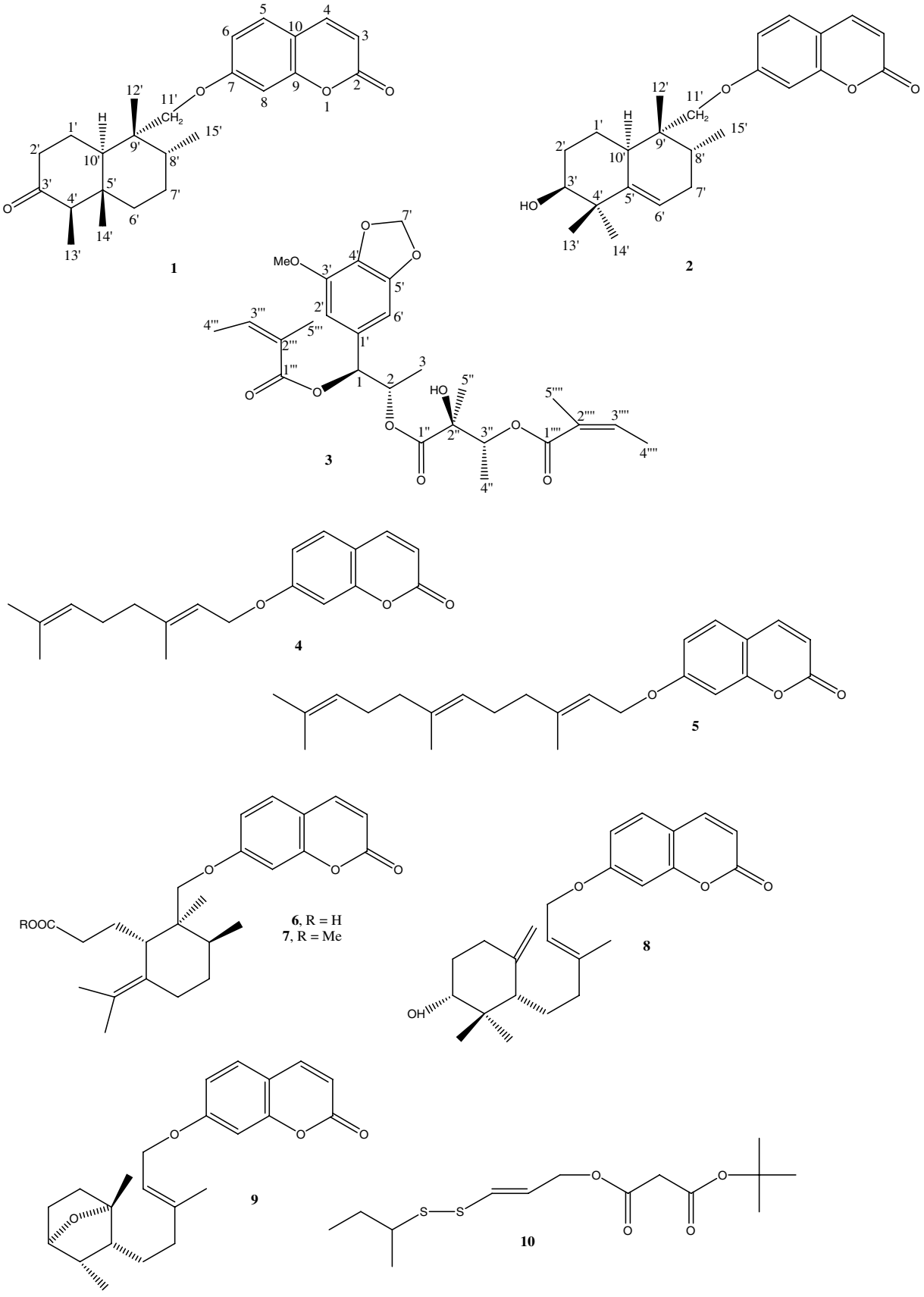


Fig. 1. Compounds **1**–**10** isolated from *Ferula szowitsiana*.

Table 1
NMR data for compounds **1** and **2**[a]

Position	**1**		**2**	
	^{1}H	^{13}C	^{1}H	^{13}C
1	–	–	–	–
2	–	161.2	–	161.2
3	6.25 d (9.4)	113.0	6.24 d (9.4)	112.9
4	7.63 d (9.4)	143.3	7.63 d (9.4)	143.4
5	7.37 d (8.4)	128.7	7.34 d (8.4)	128.6
6	6.86 dd (8.4, 2.4)	113.1	6.85 dd (8.4, 2.4)	113.0
7	–	162.2	–	162.9
8	6.82 d (2.4)	101.2	6.82 d (2.4)	101.4
9	–	155.9	–	155.9
10	–	112.6	–	112.4
1′	β 1.86 m α 1.83 m	23.6	α 1.67 m β 1.45 m	29.6
2′	β 2.37 m α 2.46 m	41.4	α 1.98 m β 1.74 m	23.1
3′	–	212.5	3.47 t (2.4)	76.5
4′	2.36 q (6.6)	58.3	–	42.1
5′	–	41.9	–	142.5
6′	α 1.54 β 1.47	32.3	5.5 brt (3.3)	119.8
7′	β 1.97 m α 1.41 m	25.4	β 2.22 m α 1.81[b] m	31.5
8′	1.90 m	35.7	1.88 m	37.9
9′	–	39.5	–	38.4
10′	2.09 dd (11.1, 4.5)	44.0	2.35 dd (11.8, 3.9)	31.7
11′	3.80 d (9.0) 3.77 d (9.0)	75.9	3.83 d (8.8) 3.88 d (8.8)	72.5
12′	1.13 s	20.0	1.10 s	20.7
13′	0.93 d (6.6)	7.1	1.10 s	24.5
14′	0.82 s	14.4	1.06 s	26.0
15′	1.03 d (7.0)	14.9	0.95 d (6.6)	15.2

[a] Assignments were made by 2D-COSY, TOCSY, HSQC and HMBC data.
[b] Signals overlapped are not labeled with multiplicity.

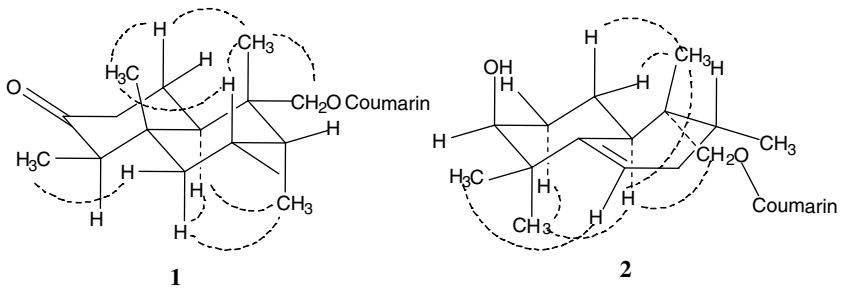
last correlations allowed defining a relative β-orientation for the Me-13′ and Me-14′. The main ROEs effects observed are depicted in Fig. 2.

Szowitsiacoumarin A represents an example of rare sesquiterpene coumarin, with one of the geminal methyls at C-4′ shifted to C-5′. Szowitsiacoumarin A is a diastereoisomer of lehmannolone isolated from *F. lehmannii* (Xinyu et al., 1995). Two similar compounds have been isolated from *F. sinaica* (Ahmed, 1999), *F. asafoetida* (Hofer et al., 1984).

Szowitsiacoumarin B (**2**) showed the same molecular formula of szowitsiacoumarin A $C_{24}H_{30}O_4$ by HRMALD-IMS (*m*/*z* of 383.2164 $[M + H]^+$). The NMR spectral data of **2** were similar to those of **1**, the main differences between the two compounds being the signals assigned to the sesquiterpene unit. Regarding this portion and with respect to szowitsiacoumarin A, HSQC spectrum showed the occurrence of three tertiary and one secondary methyls, of one olefinic methine in place of a methylene and one oxygenated methine, while the HMBC spectrum revealed the occurrence of one olefinic quaternary carbon. The replacement of the keto group at C-3′ with an oxymethine was established from the HMBC spectrum: the secondary alcoholic H-3′ (δ_H 3.47) showed correlations to δ_C 23.1 (C-2′), δ_C 42.1 (C-4′), and to δ_C 142.5 (C-5′).

The relative configurations of the stereogenic centres of **2** were easily deduced from the observation of ROESY correlations between CH_2-11′ and Me-15′, CH_2-11′ and H-10′, H-10′ and Me-14′. Furthermore, the occurrence of the ROE correlation between Me-14′ and H-2′α and the small coupling constant value for H-3′ (t, $J = 2.4$ Hz) allowed us to assign an α-orientation to H-3′ (see Fig. 2). Microlobidene, a sesquiterpene coumarin skeleton with different relative configurations at C-8′, C-9′, and C-10′ and without configurational assignment for C-3′ has previously been reported from *F. microloba* (Nabiev and Malikov, 1983a,b).

The structure of compound **3** was established by comparison of its NMR data (Table 2) with those of 2-epihelmanticine, a phenylpropanoid isolated from the roots of *F. rigidula* (Miski and Jakupovic, 1990; De Pascual et al., 1985). Because the previous configurational assignment of the C1–C2 pair of adjacent stereocenters were based on comparison of the limited spectral data (δ and J) of H-1, H-2 and Me-3 signals with those of laserine and laserine oxide and because no attempt was made to assign the relative configuration of the C-2″ and C-3″ stereocenters (Pinar et al., 1982) we re-examine the proposed 1*S*, 2*S* configurational attribution for the C1–C2 pair and carried out a configurational study on the C-2″ and C-3″ stereocentres by the recently reported *J*-based method (Matsumori et al., 1999; Bassarello et al., 2001). Other phenylpropanoids found in umbelliferous plants and containing linked to C-3″ different ester moieties have been reported recently, but few NMR details about the configurational assign-



1 **2**

Fig. 2. Important ROESY NMR correlations observed for **1** and **2**.

Table 2
NMR data for compound **3**[a]

Position	**3**	
	^{1}H	^{13}C
1	5.76 d (6.6)	76.2
2	5.21 quintet (6.6)	73.9
3	1.14 d (6.6)	16.2
1′	–	131.0
2′	6.54 s	101.2
3′	–	143.3
4′	–	135.4
5′	–	148.9
6′	6.54 s	107.0
7′	5.97 s	101.6
OMe	3.91	56.5
1″	–	173.9
2″	–	76.6
3″	5.06 q (6.1)	73.7
4″	1.29 d (6.1)	13.6
5″	1.33 s	21.5
1‴	–	166.4
2‴	–	127.1
3‴	6.12 q (7.0)	139.7
4‴	1.99 d (7.2)	15.9
5‴	1.91 s	20.4
1⁗	–	166.4
2⁗	–	127.5
3⁗	6.05 q (7.0)	138.7
4⁗	1.95 d (7.0)	15.7
5⁗	1.82 s	20.5

[a] Assignments were made by 2D-COSY, TOCSY, HSQC and HMBC data.

ments at C-2″ and C-3″ were supplied (Saouf et al., 2006). The *J*-based configuration analysis, relying on the extensive use of $^{3}J_{H,H}$/$^{2,3}J_{C,H}$ couplings in combination with ROE/NOE data, has been widely applied in the elucidation of relative configurations of natural compounds featuring acyclic chains bearing substituents such as hydroxyl, alkoxy, methyl groups. This approach allows the determination of the predominant rotamer among the six main staggered conformers (three for each relative *threo* and *erythro* stereochemical arrangement) of each two-carbon fragment in which a molecule with consecutive and alternating stereogenic centers can be ideally divided (Matsumori et al., 1999; Bassarello et al., 2001).

The large value of $^{3}J_{H–H}$ and the pattern of $^{2,3}J_{C–H}$ couplings, extracted from HETLOC spectra allowed to rule out all the 3D arrangements with H1 and H2 in a *gauche* relationship, leaving out only two H1–H2 *anti*-conformers with opposite relative configurations. The ROESY spectrum contained a key dipolar coupling that permitted us to confirm unequivocally the correct relative configuration (1*S*, 2*S*) of this molecular segment (Table 3).

Concerning the C2″–C3″ fragment, the application of this methodology was not free from difficulties, because the presence of the quaternary C-2″ stereocenter implies the loss of the critical $^{3}J_{HH}$ coupling value. Nevertheless, the small values found for all three $^{2,3}J_{C–H}$-coupling constants measured by PFG-PS-HMBC left two possible rotamers with opposite relative configurations, while ROESY crosspeaks between Me-5⁗–Me-3 and Me-5⁗–H-2 suggested, with a high level of confidence, that the configuration of the C-2″–C-3″ segment was of the *syn*-type (Table 4).

Since Bravo et al. (1999) reported that coumarins showed inhibiting activity against *L. amazonensis*, *L. braziliensis* and *L. donovani*, and Napolitano et al. (2004) found that auraptene caused significant growth inhibition against the tropical parasite *L. major*, we tested the antileishmanial activity of the prenylated coumarins, umbelliprenin (**5**) and galbanic acid (**6**) and of the acetone extract of *F. szowitsiana* roots in comparison to that of auraptene (**4**) The inhibiting activity was evaluated against promastigotes of *L. major* after an incubation of 48 h. The antileishmanial effect of the compounds is summarized in Fig. 3. All of the evaluated compounds (**4**, **5**, and **6**) and of the extract had significant activity against *L. major* promastigotes compared to the negative control (DMSO). As shown in Fig. 3, *L. major* promastigote growth was significantly inhibited by auraptene and umbelliprenin, with IC_{50} of

Table 3
$^{3}J_{HH}$, $^{2,3}J_{HC}$ and ROESY data of the **C1**–**C2** fragment

syn- (1*S*, 2*S*)

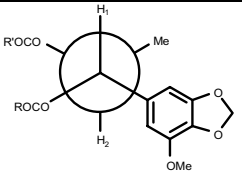


Table 3: $^{3}J_{HH}$, $^{2,3}J_{HC}$ and ROESY data of the **C1-C2** fragment

Coupling constants (Hz)				ROESY
$^{3}J_{H1-H2}$	$^{3}J_{H1-Me}$	$^{2}J_{H1-C2}$	$^{2}J_{H2-C1}$	
6.6	2.3	-3.8	-3.0	H2′/H6′ (Ph)-CH_3 (m)[a]

[a] The intensity of dipolar effects (ROESY) is expressed in terms of three categories: *s* = strong, *m* = medium, *w* = weak.

Table 4
$^{2,3}J_{HC}$ and ROESY data of the **C2″–C3″** fragment

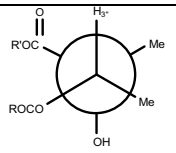
syn- (2"*S*, 3"*R*)	

Table 4: $^{2,3}J_{HC}$ and ROESY data of the **C2"-C3"** fragment

Coupling constants (Hz)			ROESY
$^{3}J_{H3"-Me}$	$^{3}J_{H3"-C=O}$	$^{2}J_{H3"-C2"}$	
< 1.6	< 1.6	< 1.6	Me5"" (ROCO)-Me3 (R'OCO) (wm) Me5"" (ROCO)- H2 (R'OCO) (w)

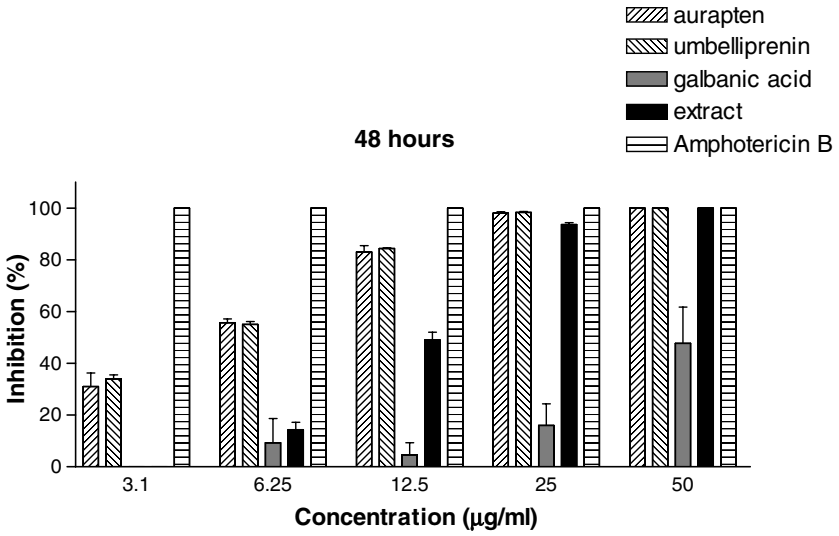


Fig. 3. Antileishmanial activity of compounds **4**–**6** isolated from the roots of *Ferula szowitsiana*, together with acetonic extract and the conventional antileishmanial drug amphotericin B. Each bar represents means ± SD of four independent experiments. Promastigotes were cultivated in the presence of different concentrations of the compounds and the extract and counted after 48 h. The height of the bars indicates the percentage of growth inhibition at each concentration compared to the control experiment containing only the solvent DMSO. The IC_{50} value for amphotericin B is 0.3 µg/ml (Judith et al., 2000).

17.1 µM and 13.3 µM, respectively. The extract also significantly inhibited promastigote growth with an IC_{50} of 11.8 µg/ml. However, galbanic acid, the major component of the extract (44% w/w), showed a weak inhibitory effect against promastigotes ($IC_{50} = 164.8$ µM). These results suggest the probable occurrence of other bioactive components in the extract.

3. Experimental

3.1. General experimental procedures

Melting points were determined on a Electrothermal 9100 apparatus and are uncorrected.The optical rotation was measured on Polarimeter Polax-2L ATAGO. UV spectra were recorded on a UV-2101PC Shimadzu UV–VIS Scanning Spectrophotometer. NMR measurements were performed on a Bruker DRX-600 and Avance-300 MHz spectrometers at $T = 300$ K. All spectra were acquired in the phase-sensitive mode and the TPPI method was used for quadrature detection in the ω_1 dimension. NMR samples were prepared dissolving each samples **1** (2.9 mg), **2** (2.4 mg), and **3** (2.0 mg) in $CDCl_3$ (Carlo Erba, 99.95% D). The spectra were calibrated using the solvent signal as internal standard (^{1}H, $\delta = 7.27$ ppm; ^{13}C, $\delta = 77.0$ ppm). For compound **3**, $^{2,3}J_{C-H}$ values were obtained from phase-sensitive PFG-PS-HMBC spectrum (Boyd et al., 1992; Davis et al., 1992) with a total of 32 scans/t_1, acquiring 4 K points in ω_2, and with a t_{1max} value of 4.7 ms. The delay for long-range coupling evolution (Δ) was set at 50 ms. Upon

2D-FT, zero-filling (4 K × 1 K) was carried out in ω_2 and ω_1. Phase-sensitive PFG-HETLOC spectrum was acquired for compound **3** with a total of 64 scans/t_1, 4 K points in ω_2, a spin lock of 50 ms and a t_{1max} value of 43 ms (Uhrín et al., 1998). The data matrices were zero-filled to 4 K × 1 K affording a digital resolution of 1.2 Hz in ω_2.

For compounds **1**, **2**, and **3** the ROESY spectra (Bax and Davis, 1985) were executed with a number of scans/t_1 ranging from 8 to 32, a t_{1max} values in the range of 15–75 ms and a mixing time of 400 and 600 ms.

The NMR data were processed on a Silicon Graphic Indigo2 workstation using the Bruker XWIN-NMR software.

Exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by matrix assisted laser desorption ionization (MALDI) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragments 18–39) at 2465.1989 Da and Angiotensin III at 931.5154 Da as internal standard. Column chromatography was conducted with Silica gel 230–400 mesh, Merck. Preparative TLC was performed on silica gel 60 GF_{254} plates (Merck) and observation of plates was carried out under UV CAMAG spectrometer (254 nm).

3.2. Plant material

The roots of *F. szowitsiana* were collected from the mountains of Golestan forest, Golestan province, Iran, on June 2003.

The plant material was identified by Dr. Hossein Akhani, Department of Botany, Faculty of Sciences, Tehran University.

A voucher specimen (No. M1001) has been deposited at the Department of Pharmacognosy and Biotechnology, Faculty of Pharmacy, Mashhad University of Medical Sciences.

3.3. Extraction and isolation

The air-dried roots (250 g) were ground into powder and extracted exhaustively by maceration at room temperature with acetone. After filtration, the extract was concentrated under vacuum to yield 20 g of a brownish residue.

Part of the extract (15 g) was subjected to column chromatography on silica gel (5 × 50 cm) using petroleum ether with increasing volumes of acetone [petrol–Me_2CO (100:0), (95:5), (90:10), (85:15), (80:20), (75:25), (70:30), (60:40), (50:50), and (0:100)]. The fractions were compared by TLC (Silica gel using petrol–EtOAc as solvent), and those giving similar spots were combined. Eight fractions were finally obtained. Fraction 1 was subjected to silica gel PLC (heptane–EtOAc, 85:15) to give **10** (16 mg). Fraction 3 afforded 300 mg compound **5** as white crystals. Fraction 4 afforded 18 mg white crystals of a mixture of β-sitosterol and stigmasterol. After separation of the sterols, the residue was subjected to silica gel PLC (heptane–EtOAc, 75:25) to give **4** (90 mg), **5** (46 mg) and **7** (50 mg). Fraction 5 was also chromatographed on silica gel PLC (petrol–EtOAc, 70:30) to yield **3** (45 mg), **7** (37 mg) and **9** (43 mg). Fraction 6 was subjected to silica gel PLC (petrol–EtOAc, 70:30) to give **1** (8 mg) and **2** (8 mg). Fraction 7 was further purified by silica gel PLC (petroleum ether–EtOAc, 70:30) to yield **8** (20 mg). Fraction 8 was also further purified by silica gel PLC (petrol–EtOAc, 70:30) to give 6716 mg amorphous powder as compound **6**.

3.4. Szowitsiacoumarin A (*1*)

Amorphous powder; $[\alpha]_D^{25}$: +103.4° ($CHCl_3$; $c = 0.29$); UV ($CHCl_3$) λ_{max} nm ($\log\varepsilon$): 325 (4.02), 235 nm (3.52); for ^1H and ^{13}C NMR spectra, see Table 1; HR-MALDI-MS m/z383.2195 (calcd. for $C_{24}H_{31}O_4$ 383.2222).

3.5. Szowitsiacoumarin B (*2*)

Amorphous powder; $[\alpha]_D^{25}$: +76.4° ($CHCl_3$; $c = 0.29$); UV ($CHCl_3$) λ_{max} nm ($\log\varepsilon$): 325 (3.92), 239 (3.55); for ^1H and ^{13}C NMR spectra, see Table 1; HR-MALDI-MS m/z 383.2164 (calcd. for $C_{24}H_{31}O_4$ 383.2222).

3.6. Biological activity

Leishmania major strain MRHO/IR/75/ER were maintained with passage in BALB/c female mice. The amastigotes were isolated from the lesions of infected BALB/c mice and transformed to promastigotes on NNN medium then subcultured in RPMI 1640 (Sigma) containing 10% v/v heat inactivated FCS, 2 mM glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin sulfate (RPMI-FCS) at 25 °C. Antileishmanial assays were conducted using stationary-phase promastigotes.

Leishmania major promastigotes in stationary phase were seeded at 40,000 parasite/400 μl/well in 24-well plate in RPMI-FCS. The extract and pure compounds were dissolved in DMSO and added in further 400 μl/well to give final concentrations of 1 mg/ml and serial two-fold dilutions thereof. Promastigotes were incubated over a period of 48 h at 25 °C and the amount of the parasites in each well determined on day 2 of experiment using Neubar chamber (Hemocytometer) under a microscope. Amphotericin B (3.1, 6.25, 12.5, 25, and 50 μg/ml) was used as positive control, culture media was used as negative control, and DMSO alone was used as solvent control (Jaafari et al., 2006).

Statistical analysis was carried out using one-way ANOVA and multiple comparison Tukey–Kramer test was used to compare the means of different treatment groups. The IC_{50} was determined by Litchfield and Wilcoxon method. CRC for the extract and pure compounds were plotted and the experimental data from the CRC were adjusted by non-linear, least squares, curve fitting program (PRISMA).

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