PERGAMON

Phytochemistry 56 (2001) 93–97

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*

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Received 2 June 1999; received in revised form 7 July 2000

Abstract

The bisdesmoside oleanolic acid saponin, 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl-oleanolate, along with nine known compounds (two diterpenic acids, one chromene, three triterpenes, one steroidal glycoside, and two monodesmoside oleanolic acid saponins), were obtained from *Viguiera decurrens* roots. The chemical structure of the bisdesmoside oleanolic saponin was determined by chemical and NMR spectral evidence. A mixture of monodesmoside saponins displayed cytotoxic activity against P388 and COLON cell lines ($ED_{50} = 2.3$ and 3.6 μg/ml, respectively). Two of the known compounds showed insecticidal activity against the Mexican bean beetle larvae (*Epilachna varivestis*). © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Viguiera decurrens*; Asteraceae; Root; Oleanolic acid; Bisdesmoside; Saponin; Chikusetsusaponin methyl ester; Cytotoxic and insecticidal activity

1. Introduction

Chemical investigation of several species belonging to *Viguiera* (Asteraceae) have yielded diterpenes (Delgado et al., 1984a), sesquiterpene lactones (Alvarez et al., 1985) and flavonols (Delgado et al., 1984b) as the major constituents of their aerial parts. The following contribution is based upon our continuing study of the genus *Viguiera* and our search for bioactive natural products. *Viguiera decurrens* (Asteraceae) is a perennial herb endemic to the upper desert grasslands and lower oak-pine forests of the eastern Sierra Madre Occidental of western Chihuahua. The thick, elongated roots known to the Tarahumara Indians as "nakárori" ('naka' = ear, 'rori' = movement) are used medicinally and in fishing (Brambila, 1976). The distinctive decurrent leaves and winged stems (over one meter tall with yellow terminal flowers) appear as ears when the plant is blown by the wind. Upon placing the crushed roots (fresh or dried) in stream pools, the fish rise to the surface and are collected by the Indians as food. A poultice from the pungent root is also used to treat infections, wounds and boils, and an infusion of the root or leaves is drunk to alleviate gastric ulcers (Pennington, 1963; Bye, 1985).

This paper describes the bioactivity-isolation of oleanolic acid saponins from the roots of *V. decurrens*, the structure elucidation of the new saponin, and the cytotoxic and insecticidal evaluations of some of the isolated compounds.

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PII: S0031-9422(00)00283-1

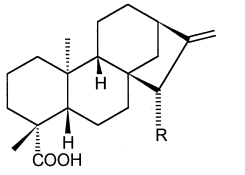
2. Results and discussion

Using a bioassay-directed fractionation of the roots of *V. decurrens*, three cytotoxic fractions and three non-cytotoxic fractions were obtained. Chromatographic purification of each cytotoxic fraction led to the isolation of two kaurenoic diterpenes, namely *ent*-kaur-16-en-19-oic acid (**1**) and 15α-angeloyloxy-*ent*-kaur-16-en-19-oic acid (**2**); three saponins: β-D-glucopyranosyl-oleanolate (**3**) (Yoshikawa et al., 1996a), β-sitosteryl-3-*O*-β-D-glucopyranoside, and oleanolic acid-3-*O*-methyl-β-D-glucuronopyranosiduronoate (**4**) (Sakai et al., 1994), and one chromene, 8-hydroxy-6-acetyl-2,2-dimethylchromene (**5**) (Bohlmann and Jakupovic, 1978). From the non-active fractions, the triterpenes friedelin, friedelan-3β-ol and oleanolic acid (**6**) as well as the oleanolic acid bisdesmoside saponin 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl oleanolate (**7**) were also obtained.

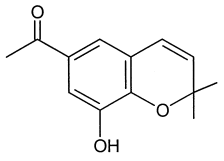
Compound **7** was obtained as a colorless powder (mp 216–218°C). In the positive mode FABMS, the quasi molecular ion-peak was observed at *m/z* 809 $M+H^+$, indicating a molecular formula of $C_{43}H_{68}O_{14}$. On acid hydrolysis compound **7** afforded oleanolic acid, glucose and 6-methylglucuronosiduronoate. The ^1H NMR ($CDCl_3$-DMSO-d_6) (see experimental) and ^{13}C NMR (see Table 1) spectra of (**7**) showed signals assignable to an oleanolic acid moiety [δ 5.26 (1H, *br t*, *J* = 3.5 Hz, H-12), 3.34 (1H, *m*, H-3), 2.83 (1H, *dd*, *J* = 13.5, 4.0 Hz, H-18], a β-D-methylglucuronopyranosiduronoate moiety [δ 4.35 (1H, *d*, *J* = 7.5 Hz, H-1′), 3.74 (3H, *s*, OCH_3], and a β-D-glucopyranosyl moiety [δ 5.43 (1H, *d*, *J* = 8.0 Hz, H-1″].

In order to clarify the type of cyclization of the sugar moieties, ^1H and ^{13}C NMR spectroscopic analysis was undertaken on the heptaacetyl derivative **8** by means of HMBC and HMQC experiments, and all the proton signals due to the sugar moieties were assigned. The profiles of these signals indicated that both methyl glucuronosiduronoate and glucose were β-pyranoses.

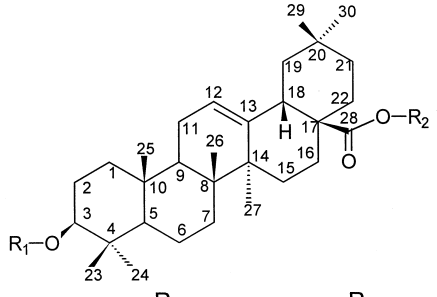
The bisdesmoside nature of 8 was demonstrated by the HMBC experiment which showed cross peaks between H-1′ of the methyl glucuronopyranosiduronoate moiety and C-3 of the oleanolic acid moiety, and between H-1″ of the β-D-glucopyranosyl moiety and C-28 of the genin. Similarly, the detection of all possible two- and three-bond correlations of the anomeric pro-



1 R = H
2 R = OAng



5



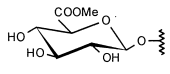
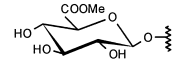
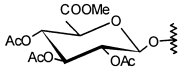
	R 1	R 2
3	H	β-D-Glcp
4		H
6	H	H
7		β-D-Glcp
8		β-D-Glcp

Table 1
^{13}C NMR spectral data for **7** ($CDCl_3$-DMSO-d_6) and **8** ($CDCl_3$) at 125 MHz, δ values

	7	8			7	8
	Sapogenol moiety				Sugar moieties	
1	38.6	38.9	6 Methyl-glucuronosiduronoate moiety	1′	104.8	102.9
2	27.1	27.7		2′	73.0	71.9
3	88.8	90.6		3′	71.8	72.9
4	39.6	39.4		4′	70.8	68.1
5	54.6	55.5		5′	76.3	72.5
6	17.3	18.1		6′	168.9	167.2
7	32.9	33.8		OCH_3	51.41	52.7
8	39.6	41.0				
9	46.7	47.6	Glcp moiety	1″	93.3	91.6
10	35.8	36.7		2″	74.4	71.6
11	24.9	23.4		3″	76.3	72.0
12	121.5	122.9		4″	69.4	69.6
13	142.7	142.9		5″	75.9	70.0
14	39.1	41.7		6″	62.9	61.6
15	29.8	29.7				
16	22.5	22.9				
17	45.7	45.8				
18	40.8	41.7				
19	45.7	46.8				
20	31.1	31.8				
21	32.9	33.8				
22	32.2	32.9				
23	27.1	27.7				
24	14.6	16.3				
25	15.8	15.3				
26	16.1	16.9				
27	26.6	25.6				
28	175.4	175.6				
29	32.2	32.9				
30	24.9	25.5				

tons confirmed the glycosidic linkages and the assignments for proton and carbon resonances.

Based on the above mentioned evidence and comparison of the ^{13}C NMR (Table 1) data for **7** and **8** with those for oleanolic acid 3, 28-*O*-bisdesmosides (Yoshikawa et al., 1995), the structure of this compound was confirmed as 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl oleanolate (**7**).

The demethyl derivative of **7** has been reported as a natural product of *Aralia elata* (Lin et al., 1976), *Talinum triangulare* (Kohda et al., 1992), *Aralia elata* SEEM. (Yoshikawa et al., 1993), *Panax pseudoginseng* (Ida et al., 1994), and *Dolichos lablab* L. (Yoshikawa et al., 1998) and named chikusetsusaponin IVa; therefore, chikusetsusaponin IVa methyl ester (**7**) is a natural saponin from *V. decurrens*. The NMR spectral data of its heptaacetyl derivative (**8**) have not previously been recorded in the literature (see Table 1).

Although **7** could be considered an artifact due to the fact that MeOH was originally used for the isolation procedure, this possibility was ruled out from the following observations: (a) the corresponding acid was not isolated, and it is well known that the use of MeOH does not allow per se the formation of esters; (b) compound **7** was isolated from an EtOH extract of additional material of *Viguiera decurrens* roots (3.4 mg of compound) obtained from big root tissue (data not shown) and (c) the presence of methyl β-D-glucuronopyranosiduronoate triterpenoidal saponins as natural products, has been well documented recently in the literature (Sang et al., 1998; Zhang et al., 1999).

None of the six main fractions displayed antimicrobial activity against *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and *Trycophyton mentagrophytes,* at concentrations up to 400 μg/ml.

The mixture of compounds **3**, **4** and β-sitosteryl-3-*O*-β-D-glucopyranoside obtained from fraction V (see Table 2) showed cytotoxic activity against the P388 cell line ($ED_{50} = 2.3$ μg/ml); however, the pure compounds were not toxic ($ED_{50} > 4$ μg/ml) (Geran et al., 1972).

Compounds **1**, **3**, **5**, **7** and friedelin were also evaluated for their insecticidal activity at different concentrations against *Epilachna varivestis* larvae, the Mexican bean beetle (Coleoptera: Coccinellidae), an important pest in Mexico (MacGregor and Gutiérrez, 1983). The results of these experiments indicated that friedelin and compounds **1** and **5** were not toxic ($LC_{50} > 5000$ μg/ml), while saponins **3** and **7** displayed toxicity ($LC_{50} = 1380$ and 80 μg/ml (calculated), respectively).

The presence of oleanolic acid mono- and bidesmoside saponins in the roots of *Viguiera decurrens* is noteworthy, and chemically validates the Tarahumara people's use of the roots to stupefy fish (Bye, 1985).

Recently, **4** has been reported to have in vivo inhibitory effects on ethanol absorption (Yoshikawa et al., 1996b), as well as hypoglycemic activity (Yoshikawa et al., 1996c), along with other oleanolic acid glycosides, isolated from *Aralia elata* and *Beta vulgaris*.

This is the first report of oleanolic acid saponins from the roots of a *Viguiera* species.

Table 2
Evaluation of cytotoxic activity

Fractions	ED_{50}[a] KB[b]	P388[c]	OVCAR[d]	COLON[e]	UISO[f]
I	>20	7.24	–	–	–
II	>20	3.3	–	–	–
III	>20	9.1	21.4	>20	22.4
IV	>20	12	25.1	>20	>20
V	>20	2.3	8.9	>20	3.02
VI	>20	5.7	47.9	>20	>20
Mixture of **3**, **4** and β-sitosteryl-3-*O*-β-D-Glcp	>4.0	2.3	–	3.6	–
3	>4.0	>4.0	–	–	–
4	>4.0	>4.0	–	–	–

[a] (ED_{50} μg/ml).
[b] KB, human epidermal carcinoma.
[c] P388, murine leukemia.
[d] OVCAR, ovarian cancer.
[e] COLON, colon carcinoma.
[f] UISO, uterine-cervix cancer.

3. Experimental

Mps are uncorr. NMR instrumentation: Varian VXR-200 and Varian Unity Plus-500. MS: JEOL JMS-AX 505 HA.

3.1. Plant material

The roots of *Viguiera decurrens* A. Gray were obtained in the eastern Sierra Tarahumara, Chihuahua, Mexico, on 12 October 1991 and identified by R. Bye. The herbarium voucher specimen (R. Bye 18338) is deposited in the National Herbarium of Mexico (MEXU).

3.2. Bioactivity-directed isolation of compounds

Dried and powdered roots (1.07 kg) of *V. decurrens* were extracted by percolation using a gradient of *n*-hexane–EtOAc–MeOH, yielding six fractions: Fr. I (100:0:0, 18.0 g); Fr. II (9:1:0, 1.6 g); Fr. III (7:3:0, 4 g); Fr. IV (1:1:0, 2.8 g), Fr. V (0:100:0, 12.0 g); Fr. VI (0:3:7, 30.6 g). These fractions were evaluated for their antimicrobial and cytotoxic activities (Table 2). Fractions I, II and V showed cytotoxic activity. Fr. I after filtration afforded 2.23 g of *ent*-kaur-16-en-19-oic acid (**1**, 0.1151%, mp 170–174°C). Fr. II was ground with *n*-hexane to yield 68 mg of 15α-angeloyloxy-*ent*-kaur-16-

en-19-oic acid (**2**, 0.104%). Fr. III was methylated with diazomethane and the residue was chromatographed on a silica gel column using mixtures of *n*-hexane–EtOAc; fractions 1–31 eluted with 95:5 (*n*-hexane–EtOAc) yielded 105 mg of friedelin (0.0098%, mp 242–245°C), from fractions 15–46 eluted with *n*-hexane–EtOAc 9:1 crystallized 50 mg of friedelan-3β-ol (0.0046%, mp 249–253°C), fractions 55–60 (8:2 *n*-hexane–EtOAc) were combined and the residue (700 mg) was subjected to silica gel column chromatography eluted with (CH_2Cl_2–MeOH 9:1) to yield 60 mg of oleanolic acid methyl ester (0.0056%, mp 198–200°C). Fr. IV was applied to a silica gel column using a gradient system of CH_2Cl_2–MeOH to yield 35.8 mg of oleanolic acid (**6**, 0.0033%, mp 196–198°C). Fr. V was subjected to chromatography with mixtures of CH_2Cl_2–MeOH, to obtain a cytotoxic fraction (see Table 2) which was composed of a mixture of saponins. The mixture was further separated by repeated column chromatography to yield 47 mg of β-D-glucopyranosyl olean-12-en-28-oate (**3**, 0.0043%, mp 235–237°C), 60 mg of β-sitosteryl-3-*O*-β-D-glucopyranoside (0.0056%, mp 267°C), 125 mg of oleanolic acid 3-*O*-methyl-β-D-glucuronopyranosiduronoate (**4**, 0.011%, amorphous powder), and 22 mg of 8-hydroxy-6-acetyl-2,2-dimethyl chromene (**5**, 0.002%, mp 135–137°C) (lit. mp 132°C, Bohlmann and Jakupovic, 1978). Fr. VI (30.6 g) was subjected to repeated CC on silica gel, eluting with EtOAc–MeOH (7:3), CH_2Cl_2–MeOH (95:5) and CH_2Cl_2–MeOH (85:15) respectively to afford 80 mg of 3-*O*-(methyl-β-D-glucuronopyranosiduronoate)-28-*O*-β-D-glucopyranosyl oleanolate (**7**, 0.0074%).

Compounds **1**, **2**, **6**, β-sitosteryl-3-*O*-β-D-glucopyranoside, friedelin, friedelan-3-β-ol, were identified by direct comparison (IR, TLC) with authentic samples, while compounds **3**–**5** and **7** were characterized by means of physicochemical evidence.

3.2.1. 3-O-(Methyl-β-D-glucuronopyranosiduronoate)-28-O-β-D-glucopyranosyl oleanolate (7)

Colorless powder (MeOH, mp 216–218°C) (lit. mp 209–212°C, Sakai et al., 1994), $\alpha_D + 5.7$ ($CHCl_3$, *c* 0.28); IR ν_{max} cm^{-1}: 3420, 1720, 1700; positive FABMS *m*/*z*: 831 $[M+Na]^+$; high-resolution positive FABMS *m*/*z*: Calcd for $C_{43}H_{68}NaO_{14}$: 831.4506; found: 831.4478 $(M+Na)^+$, 809 $[M+H^+]$, 663 [M- Glc + Na^+], 475 [M-(Glu-Me)-Glc + Na]$^+$, 207 $[Glu]^+$, 179 $[Glc]^+$; ^{1}H NMR spectral data (500 MHz, $CDCl_3$-DMSO-d_6): δ 5.43 (1H, *d*, *J* = 8.0 Hz, H-1''), 5.26 (1H, *br t*, *J* = 3.5 Hz, H-12), 4.35 (1H, *d*, *J* = 7.5 Hz, H-1′), 3.78 (1H, *t*, *J* = 9.0 Hz, H-2″), 3.74 (3H, *s*, OCH_3), 3.72 (1H, *dd*, *J* = 11.0, 5.0 Hz, H-6a″), 3.63 (1H, *t*, *J* = 8.5 Hz, H-4′), 3.56 (1H, *dd*, *J* = 11.0, 5.5 Hz, H-6b″), 3.48 (1H, *t*, *J* = 8.5 Hz H-3″), 3.46 (1H, *d*, *J* = 9.0 Hz, H-5′), 3.44 (1H, *t*, *J* = 9.0 Hz, H-4″), 3.40 (1H, *t*, *J* = 8.5 Hz, H-3′), 3.36 (1H, *m*, H-5″), 3.34 (1H, *m*, H-2′), 2.83 (1H, *dd*, *J* = 13.5, 4.0 Hz, H-18), 1.11 (3H, *s*, H-30), 1.02 (3H, *s*, H-23), 0.92 (3H, *s*, H-29), 0.90 (3H, *s*, H-27), 0.89 (3H, *s*, H-24), 0.81 (3H, *s*, H-25), 0.71 (3H, *s*, H-26); ^{13}C NMR spectral data (125 MHz, $CDCl_3$-DMSO-d_6): see Table 1.

3.2.2. Compound (8)

Obtained by usual acetylation of **7**: powder, mp 99–101°C; positive FABMS *m*/*z*: 1125 $[M+Na]^+$. High-resolution positive FABMS *m*/*z*: calcd for $C_{57}H_{82}NaO_{21}$: 1125.5246; found: 1125.5318 $[M+Na]^+$, 769 [M- (Glu-Me)Ac_3 + H]$^+$, 741 [M- (Glu-Me)Ac_3 + K]$^+$, 331 [(Glc)Ac_4]$^+$; ^{1}H NMR spectral data (500 MHz, $CDCl_3$): δ 5.58 (1H, *d*, *J* = 8.0 Hz, H-1″), 5.31 (1H, *br t*, *J* = 3.5 Hz, H-12), 5.24 (1H, *t*, *J* = 9.5 Hz, H-3′), 5.22 (1H, *t*, *J* = 9.5 Hz, H-4′), 5.21 (1H, *t*, *J* = 9.5 Hz, H-3″), 5.19 (1H, *t*, *J* = 9.0 Hz, H-2″), 5.12 (1H, *t*, *J* = 8.5 Hz, H-4″), 5.04 (1H, *t*, *J* = 9.0 Hz, H-2′), 4.58 (1H, *d*, *J* = 8.0 Hz, H-1′), 4.27 (1H, *dd*, *J* = 12.5, 4.0, Hz, H-6a″), 4.04 (1H, *dd*, *J* = 12.5, 2.0 Hz, H-6b″), 3.98 (1H, *d*, *J* = 9.5 Hz, H-5′), 3.78 (1H, *ddd*, *J* = 10.5, 4.5, 2.5 Hz, H-5″), 3.77 (3H, *s*, OCH_3), 3.10 (1H, *dd*, *J* = 8.0, 4.5 Hz, H-3), 2.81 (1H, *dd*, *J* = 16.0, 4.5 Hz, H-18), 1.10 (3H, *s*, H-27), 0.90 (6H, *s*, H-23, H-30), 0.89 (6H, *s*, H-24, H-29), 0.73 (3H, *s*, H-25), 0.72 (3H, *s*, H-26); ^{13}C NMR spectral data (125 MHz, $CDCl_3$): see Table 1.

3.2.3. Acid hydrolysis of 7

Compound **7** (25 mg) was refluxed with 10% HCl-methanol (5 ml) for 2 h. The aglycone was extracted with EtOAc and identified by comparison with an authentic sample. The aqueous layer was adjusted to pH 6 with $NaHCO_3$. After evaporation to dryness, the sugars were extracted with pyridine and analyzed by TLC on silica gel with EtOAc–MeOH–H_2O–AcOH (11:2:2:2); detection was accomplished with *p*-anisidine phthalate.

3.3. Biological activity

3.3.1. Antimicrobial activity

Evaluations were performed with cultures of *Staphylococcus aureus* (ATCC 6538), *Salmonella typhimurium* (ATCC 06539), *Escherichia coli* (ATCC 8937), *Pseudomonas aeruginosa* (ATCC 9027); *Candida albicans* (ATCC 10231) and *Trycophyton mentagrophytes* (NRRL 1942), using the conventional disc assay procedure (Vanden Berghe and Vlietinck, 1991); nystatin (5–160 μg/ml) and gentamicin (2–128 μg/ml) were included as controls.

3.3.2. Cytotoxic activity

These studies were performed using the cell cultures listed in Table 2 according to Geran and Greenberg's screening protocols (Geran et al., 1972).

3.3.3. Insecticidal activity

This assay was carried out following the published protocol (Kubo, 1991), using first instar larvae of *Epilachna varivestis* (Coleoptera: Coccinellidae) which were

fed with different concentrations of compounds **1**, **3**, **5**, **7** and friedelin in contaminated leaf-discs. Leaves were obtained from greenhouse bean plants (criollo variety) free of pests and chemical insecticides. Concentrations ranged from 100 to 6000 μg/ml. The toxicity data were analyzed by the *Probit method* (Finney, 1962) and expressed as the LC_{50}.

Acknowledgements

We thank M. Sc. María Isabel Chávez (Instituto de Química de la UNAM) for her assistance in obtaining NMR spectra. Partial financial support from SUPERA (ANUIES) to S.M. is also acknowledged. R.B. acknowledges the generosity of the Tarahumara Indians in sharing their knowledge, the assistance of Francisco Basurto, Elia Herrera, David Martínez and Lucía Nava in the field and herbarium studies, the academic encouragement of C.W. Pennington, as well as the financial support of field work from United States Agency for International Development and Universidad Nacional Autónoma de México (Dirección General de Apoyo a Personal Académico; Jardín Botánico del Instituto de Biología).

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