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CHEMICAL INVESTIGATION AND ANTI-INFLAMMATORY ACTIVITY OF *VITEX NEGUNDO* SEEDS

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ABSTRACT.—The $CHCl_3$ extract of the defatted seeds of *Vitex negundo* exhibited anti-inflammatory activity and yielded four triterpenoids: 3β-acetoxyolean-12-en-27-oic acid [**1**], 2α,3α-dihydroxyoleana-5,12-dien-28-oic acid [**2**], 2β,3α-diacetoxyoleana-5,12-dien-28-oic acid [**3**], and 2α,3β-diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid [**5**]. This is the first report of the isolation of compounds **2**, **3**, and **5** from a natural source.

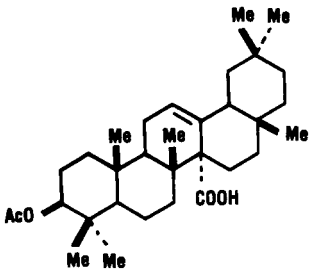
In the Indian system of medicine, *Vitex negundo* L. (Verbenaceae) has been used for the treatment of rheumatoid arthritis (1). Continuing our investigation of the seeds of *V. negundo*, the $CHCl_3$ extract has yielded four triterpenoids. Their characterization and biological activity are reported in this paper.

RESULTS AND DISCUSSION

The $CHCl_3$ extract of defatted seeds of *V. negundo* exhibited 34.8% anti-edema activity at 500 mg/kg p.o. in the carrageenan-induced paw edema test in albino rats. The extract was fractionated by chromatographic methods followed by crystallization, yielding compounds **1**–**3** and **5**.

Spectral characteristics of compound **1** and of its hydrolysis product identified the compound as 3β-acetoxyolean-12-en-27-oic acid (2–4).

Compound **2** did not show absorption above 224 nm in the uv spectrum. The ir spectrum showed bands at 3200 cm^{-1} (-OH), 1700 cm^{-1} (C=O, -COOH), and 1390 and 1375 cm^{-1} (*gem*-dimethyl). The ^{1}H-nmr spectrum gave a multiplet at δ 5.27 for two vinylic protons. The multiplet at δ 3.87 and a doublet at δ 3.34 (d, $J = 3.0$ Hz), one proton each, were assigned to H-2β and H-3β, respectively. The spectrum also gave singlets at δ 1.14, 1.09, 0.98, 0.96, 0.92, 0.84, and 0.81, corresponding to seven methyl groups, which suggested the oleanane skeleton. The eims gave a molecular ion peak at *m/z* 470 (1%) and fragments at *m/z* 452 $[M - H_2O]^+$ (1%), 434 $[M - 2 \times H_2O]^+$ (2%), 425 $[M - COOH]^+$ (2%), 248 (100%), 203 (65%), and 189 (20%). Retro-Diels-Alder fragmentation was observed in the mass spectrum of **2**, with ions at *m/z* 248 and 203, indicative of a triterpenoid having Δ^{12}-unsaturation. The higher intensity of peak *m/z* 203 suggested an oleanane skeleton with a 28-carboxyl group (5,6).



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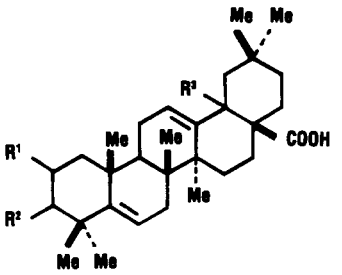


2 $R^1=R^2=\alpha$-OH, $R^3=H$
3 $R^1=\beta$-OAc, $R^2=\alpha$-OAc, $R^3=H$
4 $R^1=\beta$-OH, $R^2=\alpha$-OH, $R^3=H$
5 $R^1=\alpha$-OAc, $R^2=\beta$-OAc, $R^3=OH$

Compound **2** formed an acetonide (7), and its eims gave peaks at *m/z* 510 $[M]^+$ (1%), 464 $[M-H-COOH]^+$ (1%), 452 $[M-MeCOMe]^+$ (1%), 406 (5%), 248 (100%), and 203 (48%). Formation of an acetonide supported the cis configuration or the diequatorial conformation of the hydroxyl groups (8).

Acetylation of compound **2** yielded the product as a gum. The occurrence of two sharp three-proton singlets at δ 2.11 and 1.96 in the ^{1}H-nmr spectrum revealed the presence of two acetyl groups. Other signals were observed at δ 5.28 (2H, vinylic protons), 4.98 (H-2), and 4.70 (H-3). The ^{1}H-nmr spectrum also showed seven C-Me singlets at δ 1.25, 1.18, 1.12, 1.04, 0.97, 0.87, and 0.76. The similarity in chemical shifts of the seven methyl groups in the ^{1}H-nmr spectrum of **2** and its diacetate suggested that the two oxygen functionalities in the molecule were both α. If the hydroxyl groups were β, the chemical shifts of the C-24 and C-25 methyls of **2** would appear downfield in comparison to those of the diacetate (5,8).

Methylation of **2** with CH_2N_2 yielded a crystalline methyl ester, mp 285–286°. Thus compound **2** was shown to be 2α,3α-dihydroxyoleana-5,12-dien-28-oic acid. This is the first report of the isolation of compound **2** from a natural source.

The uv spectrum of compound **3** did not show absorption above 225 nm. The ^{1}H-nmr spectrum of **3** gave signals at δ 5.28 (m, 2H, vinylic protons), 4.72 (1H, H-2α), and 4.64 (1H, H-3β). The sharp three-proton singlets at δ 2.05 and 1.99 revealed the presence of two acetyl groups. The spectrum displayed C-Me singlets at δ 1.26 (3H), 1.07 (6H), 0.90 (9H), and 0.76 (3H), suggesting an oleanane skeleton. The eims of **3** gave the molecular ion peak at *m/z* 554 (1%) and other major peaks at *m/z* 434 $[M-2\times HOAc]^+$ (4%), 419 (20%), 248 (100%), 203 (66%), 189 (23%), and 133 (44%). These data suggested that a C-12 double bond and a C-28 carboxyl group were present in the molecule. Signals at δ 143.8 (C-13) and 122.2 (C-12) in the ^{13}C-nmr spectrum supported the presence of Δ^{12} double bond. The spectrum also indicated the presence of two additional olefinic carbons at δ 138.2 (C-5) and 125.5 (C-6). Singlets at δ 184.0, 171.0, and 170.8 confirmed the presence of a carboxyl group and two acetoxyl groups.

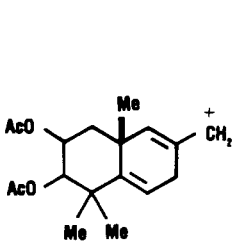
Hydrolysis of compound **3** with ethanolic KOH afforded a crystalline material, mp 245–246° (dec) [**4**]. Compound **4** did not form an acetonide, indicating that the hydroxyl groups at C-2 and C-3 were diaxial.

Methylation of **4** with CH_2N_2 yielded a crystalline compound, mp 205–206°, and the eims showed the molecular ion peak at *m/z* 484 (1%).

The spectral data of compound **3** and its derivatives indicate that compound **3** is 2β,3α-diacetoxyoleana-5,12-dien-28-oic acid. To our knowledge compound **3** has not been reported previously.

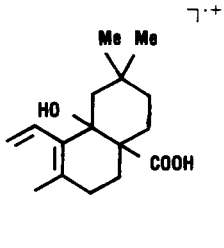
Compound **5** showed absorption at 225 nm in the uv spectrum. The ^{1}H-nmr spec-

trum exhibited signals at δ 5.41 (m, 2H, vinylic protons), 5.13 (dd, 1H, J = 10.0, 4.0 Hz, H-2β), 4.76 (d, 1H, J = 10.0 Hz, H-3α), 2.06 (3H, s, OAc), and 1.98 (3H, s, OAc). These values are in close agreement with known 2α,3β-diacetoxy triterpenoids (9). A one-proton singlet at δ 2.55 may be due to a hydroxyl group, the presence of which was indicated in the ir spectrum. The hydroxyl group appears to be tertiary as it could not be acetylated. The spectrum showed seven C-Me singlets at δ 1.24, 1.21, 1.06, 0.99, 0.96, 0.93, and 0.73 consistent with an oleanane skeleton. The eims showed the molecular ion at *m/z* 570 (1%). Other peaks observed at *m/z* 305 (1%) and 264 (2%) were attributed to fragments **a** and **b**. These data revealed the presence of a C-



a

m/z 305



b

m/z 264

12 double bond, a C-28 carboxyl group, and a C-18 hydroxyl group in the molecule. Thus, compound **5** has been identified as 2α,3β-diacetoxy-18-hydroxyoleana-5,12-dien-28-oic acid. This is the first reported isolation of **5** from natural sources.

The $CHCl_3$ extract and triterpenes **2** and **4** (hydrolyzed products of **3**) obtained from the seeds of *V. negundo* exhibited anti-inflammatory activity as shown in Table 1.

TABLE 1. Percent Inhibition of Edema (after 3.5 h) Exhibited by *Vitex negundo* $CHCl_3$ Extract and Triterpenes **2** and **4**.

Group	Dose mg/kg, p.o.	Mean increase in paw volume ml ± SD	% inhibition of edema	Significance
Control	—	0.66 ± 0.091	—	—
Standard (ibuprofen)	50	0.24 ± 0.045	63.2	$P<0.001$
$CHCl_3$ extract	500	0.31 ± 0.043	34.8	$P<0.05$
Compound **2**	50	0.54 ± 0.087	18.7	$P<0.01$
Compound **4**	50	0.43 ± 0.071	34.3	$P<0.001$

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points reported are uncorrected. The uv and ir spectra were recorded on Perkin-Elmer Hitachi 200 and Perkin-Elmer 1430 ratio recording infrared spectrophotometers, respectively. The ^{1}H-nmr spectra were determined on a Varian EM 390 spectrometer. The ^{13}C-nmr spectra were recorded on a Bruker WP 400 NMR spectrometer operating at 100 MHz and on a Jeol FX-90Q spectrometer operating at 22.49 MHz. The eims were obtained with a Jeol D-300 mass spectrometer attached to a JMA-200 system. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Gc was obtained on a 6 ft × ⅛ in. column of 3% SE-30 on 100-200 HP Chrom G, and the Liebermann-Burchard test was used to detect the presence of triterpenes. *V. negundo* seeds were procured from the local market and their authenticity confirmed by comparison with a reference sample. A voucher specimen is deposited in the herbarium of the Department of Pharmaceutical Sciences, Panjab University, Chandigarh.

EXTRACTION AND FRACTIONATION OF COMPOUNDS **1–3** AND **5**.—The defatted seed powder (11 kg) was extracted exhaustively with $CHCl_3$ in a Soxhlet apparatus, and the resulting residue (40.6 g) was chromatographed over a Si gel (Acme, 100–200 mesh, 800 g) column. Elution with $CHCl_3$-MeOH (99:1) (2 × 500 ml) afforded 0.9 g of residue. MeOH was added, and the MeOH-insoluble portion was crystallized from EtOAc, mp 82° (0.03 g, aliphatic acids). Further elution with the same solvent (3 × 500 ml) gave 8.0 g of residue which was acetylated with Ac_2O and pyridine. The acetate thus obtained, after chromatographic purification and crystallization from MeOH, gave a crystalline product **1**, mp 195–196° (0.025 g). Further eluates of the column using $CHCl_3$-MeOH (98:2) (10 × 500 ml) afforded a dark green mass (3.2 g) that was found to be a mixture of two compounds. Resolution of the mixture on Si gel gave two entities: vanillic acid, crystallized from C_6H_6/Me_2CO, mp 204–205° (0.035 g); and compound **2**, crystallized from MeOH, mp 222–224° (0.35 g). Subsequent elutions with the same solvent (15 × 500 ml) yielded a dark brown residue (7.0 g). This residue was washed with Me_2CO, and the Me_2CO-insoluble part (4.0 g) was treated with Ac_2O (15 ml) and pyridine (30 ml) at room temperature. The resulting acetates were purified by Si gel tlc and yielded compound **3**, crystallized from $CHCl_3$/petroleum ether, mp 182–183° (0.91 g), and compound **5**, crystallized from $CHCl_3$/MeOH, mp 205° (0.025 g). The eluates obtained with $CHCl_3$-MeOH (95:5) (15 × 500 ml) gave a dark brown residue (7.5 g) which on rechromatography and crystallization from $CHCl_3$/MeOH afforded β-sitosterol-D-glucoside, mp 283° (0.42 g).

Compound **1**.—Compound **1** gave a positive test for triterpenes. $[\alpha]^{20}D$ +48.6° ($CHCl_3$, c = 0.0109); ir ν max (KBr) cm^{-1} 2932, 2850, 1735, 1697, 1382, 1368, 1245; ^1H nmr ($CDCl_3$) δ 5.19 (t, 1H, J = 2.2 Hz, H-12), 4.46 (t, 1H, J = 7.5 Hz, H-3α), 1.98 (3H, s, OAc), C-Me signals at δ 1.02 (3H, s), 0.91 (6H, s), 0.81 (6H, s), 0.72 (3H, s), 0.59 (3H, s); ^{13}C nmr ($CDCl_3$) δ 36.7 (C-1), 28.0 (C-2), 80.9 (C-3), 39.6 (C-4), 55.4 (C-5), 18.2 (C-6), 32.9 (C-7), 37.0 (C-8), 52.6 (C-9), 36.8 (C-10), 23.3 (C-11), 125.7 (C-12), 138.0 (C-13), 48.0 (C-14), 22.7 (C-15), 24.1 (C-16), 32.6 (C-17), 47.5 (C-18), 45.9 (C-19), 30.6 (C-20), 33.8 (C-21), 38.3 (C-22), 28.1 (C-23), 21.2 (C-24), 15.5 (C-25), 17.1 (C-26), 183.5 (C-27), 25.9 (C-28), 32.9 (C-29), 23.6 (C-30), 170.9 (OAc), 21.9 (Me); eims *m/z* (rel. int.) $[M]^+$ 498 (1), 438 (3), 248 (100), 203 (45), 189 (28).

Compound **2**.—This compound gave positive test for triterpenes. $[\alpha]^{26}D$ +61.9° (MeOH, c = 0.0021); uv (MeOH) λ max (log ε) 224 (2.61); ir ν max (KBr) cm^{-1} 3200, 1700, 1390, 1375, 1040; ^1H nmr ($CDCl_3$/DMSO-d_6) δ 5.27 (m, 2H, H-6 and H-12), 3.87 (m, 1H, H-2β), 3.34 (d, 1H, J = 3.0 Hz, H-3β), signals for C-Me groups at 1.14 (3H), 1.09 (3H, 0.98 (3H), 0.96 (3H), 0.92 (3H), 0.84 (3H), 0.81 (3H); eims (rel. int.) $[M]^+$ 470 (1), 425 (2), 248 (100), 203 (65), 189 (20), 133 (39).

The acetate of compound **2** was prepared by published methods (2): ^1H nmr ($CDCl_3$) δ 5.28 (2H), 4.98 (1H, 2-H), 4.70 (1H, 3-H), 2.11 (3H, s, OAc), 1.96 (3H, s, OAc), C-Me signals at 1.25 (3H), 1.18 (3H), 1.12 (3H), 1.04 (3H), 0.97 (3H), 0.87 (3H), 0.76 (3H).

The methyl ester was prepared by reaction with CH_2N_2 and the usual workup afforded needle-shaped crystals: mp 285–286°; ir ν max (KBr) cm^{-1} 1722, 1238; eims (rel. int.) $[M]^+$ 484 (1), 262 (34), 203 (100), 189 (21).

Compound **2**, on refluxing with dry Me_2CO in the presence of *p*-toluenesulfonic acid, afforded the acetonide as a gum. Tlc showed the acetonide to be a single spot: eims (rel. int.) $[M]^+$ 510 (1), 464 (1), 452 (1), 406 (5), 248 (100), 203 (48).

Compound **3**.—Compound **3** gave a positive test for triterpenes, $[\alpha]^{26}D$ +10.76° (MeOH, c = 0.0065); uv (MeOH) λ max (log ε) 225 (2.62); ir ν max (KBr) cm^{-1} 3355, 1745, 1698, 1250, 1380, 1367, 1042, 1030; ^1H nmr ($CDCl_3$) δ 5.28 (2H, m, H-6 and H-12), 4.72 (br s, 1H, H-2α), 4.64 (br s, 1H, H-3β), 2.05 (3H, s, OAc), 1.99 (3H, s, OAc), C-Me signals at δ 1.26 (3H), 1.07 (6H), 0.90 (9H), 0.76 (3H); ^{13}C nmr ($CDCl_3$) δ 41.5 (C-1), 69.8 (C-2), 80.5 (C-3), 39.0 (C-4), 138.2 (C-5), 125.5 (C-6), 32.6 (C-7), 40.5 (C-8), 47.0 (C-9), 37.6 (C-10), 23.0 (C-11), 122.2 (C-12), 143.8 (C-13), 46.0 (C-14), 28.0 (C-15), 25.4 (C-16), 41.2 (C-17), 43.0 (C-18), 45.5 (C-19), 30.0 (C-20), 32.0 (C-21), 38.7 (C-22), 27.0 (C-23), 16.3 (C-24), 17.0 (C-25), 17.8 (C-26), 25.4 (C-27), 184.0 (C-28), 20.5 (C-29), 23.4 (C-30), 171.0 (OAc at C-2), 170.8 (OAc at C-3), 15.8 (Me); eims (rel. int.) $[M]^+$ 554 (1), 434 (4), 419 (20), 248 (100), 203 (66), 189 (23), 133 (44).

Hydrolysis of compound **3** with KOH in EtOH afforded an amorphous product **4**, mp 245–246° (dec.); ir ν max (KBr) cm^{-1} 3420, 1700, 1460, 1380, 1367, 1052. The hydrolyzed product did not form an acetonide. The hydrolysis product was converted into the methyl ester with CH_2N_2: mp 205–206°; ir ν max (KBr) cm^{-1} 3420, 1730, 1265; eims (rel. int.) $[M]^+$ 484 (1), 203 (100), 189 (24).

Compound **5**.—Compound **5** gave a positive Liebermann-Burchard test. $[\alpha]^{26}D$ −13.33° (MeOH, c = 0.0052); uv (MeOH) λ max (log ε) 225 (2.81); ir ν max (KBr) cm^{-1} 3250, 1760, 1710, 1390, 1380, 1265, 1169, 1058, 1048, 980; ^1H nmr ($CDCl_3$) δ 5.41 (m, 2H, H-6 and H-12), 5.13 (dd, 1H, J = 10.0 Hz, 4.0 Hz, H-2β), 4.76 (d, 1H, J = 10.0 Hz, H-3α), 2.55 (s, 1H, OH), 2.06 (s, 3H, OAc), 1.98 (s, 3H, OAc), C-Me signals at δ 1.24 (3H), 1.21 (3H), 1.06 (3H), 0.99 (3H), 0.96 (3H), 0.90 (3H), 0.73 (3H); eims (rel. int.) $[M]^+$ 570 (1), 552 (1), 305 (1), 264 (2), 246 (6), 219 (8).

ANTI-INFLAMMATORY ACTIVITY.—Anti-inflammatory activity was determined by the method of Winter *et al.* (10) using Sprague-Dawley male rats (weight range 100–160 g). The animals were divided into control, standard, and test groups of five animals each. The crude extract and the pure compounds suspended in normal saline-Tween 20 (95:5) were administered to the test animals at 500 and 50 mg/kg, p.o., respectively. Ibuprofen (50 mg/kg, p.o.) suspended in the same delivery system was given to the animals of the standard group while the control animals received normal saline-Tween 20 (95:5) 10 ml/kg, p.o. Edema was induced by subcutaneous injection of 0.1 ml of 1% solution of carrageenan in normal saline in the subplantar region of the left hind paw. The paw edema was measured plethysmographically before and at intervals of 2, 3, and 3.5 h after injection of carrageenan. The effects observed after 3.5 h were found to be maximum, and the results are expressed as arithmetic means ± S.D. The data were analyzed using Student's *t*-test (11), and the results are given in Table 1.

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