Evaluation of Antinociceptive and Anti-Inflammatory Activities of a New Triterpene Saponin from *Bauhinia variegata* Leaves

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A new triterpene saponin, named as 23-hydroxy-3α-[*O*-α-L-^{1}C$_4$-rhamnopyranosyl-(1”→4’)-*O*-α-L-^{4}C$_1$-arabinopyranosyl-oxy]olean-12-en-28-oic acid *O*-α-L-^{1}C$_4$-rhamnopyranosyl-(1””’→4””)-*O*-β-D-^{4}C$_1$-glucopyranosyl-(1””→6”’)-*O*-β-D-^{4}C$_1$-glucopyranosyl ester (**9**), was isolated from the leaves of *Bauhinia variegata* Linn. In addition, six flavonoid compounds along with two cinnamic acid derivatives were isolated and identified based on their chromatographic properties, and chemical and spectral data (ESI-high resolution-MS^n, ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC, and HMBC). Compound **9** was found to be nontoxic (LD_{50}) and to have significant anti-inflammatory and antinociceptive activities. It also showed a slight antischistosomal activity.

Key words: Bauhinia variegata, Triterpene Saponin, Anti-Inflammatory Activity

Introduction

The genus *Bauhinia* includes 250 species, inclusive shrubs, lianas, and small trees, and is distributed mainly in Africa, Asia, and Latin America (Ildis and Chcd, 1992). *Bauhinia variegata* is a flowering plant species in the family Fabaceae. Its powdered bark is traditionally used as tonic, astringent and for treating ulcers and skin diseases. The roots are used as antidote to snake poison (Reddy and Yadava, 2003). Many plants of the genus are used in traditional medicine because of their interesting biological activities such as analgesic (El-Khatib and Kaleel, 1995), astringent, diuretic (Braca *et al*., 2001) anti-inflammatory (Pokhrel *et al*., 2002; El-Khatib *et al*., 1995; Braca *et al*., 2001; Sosa *et al*., 2002), antimicrobial (Reddy *et al*., 2003; Kittakoop *et al*., 2004), antimalarial (Kittakoop *et al*., 2000), antipyretic (El-Khatib *et al*., 1995), antioxidant (Braca *et al*., 2001; Kumar *et al*., 2005; De Sousa *et al*., 2004), hypoglycemic (De Sousa *et al*., 2004), cytotoxic (Rajkapoor *et al*., 2003, 2006), and antidiabetic (Braca *et al*., 2001; Hussain *et al*., 2004). Several *Bauhinia* species have already been chemically studied and found to contain mainly flavonoids (Braca *et al.*, 2001; Reddy *et al*., 2003; Yadava and Reddy, 2001; Salatino *et al*., 1999; Yadava and Tripathi, 2000; Jain *et al*., 2004; Wollenweber *et al*., 2000) besides cinnamic acids (Braca *et al*., 2001), phenanthraquinone (Zhao *et al*., 2005), sterols (Iribarren and Pomilio, 1987), and triterpenoidal saponins (Athikomkulchai *et al*., 2003).

The present article deals with the isolation and structural determination of a new triterpenoidal saponin, six flavonoids and two cinnamic acid derivatives from the leaves of *Bauhinia variegata.* In addition, we report herein the anti-inflammatory, antinociceptive, and antischistosomal activities of the saponin compound.

Material and Methods

Equipment

The NMR spectra were recorded at 300 or 500 (^{1}H) and 75 or 125 (^{13}C) MHz on a Varian Mercury 300 and a JEOL GX-500 NMR spectrometer, and δ values are reported in ppm relative to TMS in DMSO-d_6 or pyridine-d_5. ESI-high resolution-MS analyses were run on an LTQ-FT-MS spectrometer (Thermo Fisher Scientific, Bremen, Germany). UV spectra of pure samples were recorded as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 spectrophotometer.

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Chromatographic material

For chromatographic analyses, Sephadex LH-20 (Pharmacia, Uppsala, Sweden), microcrystalline cellulose S and silica gel G 60 powder (Merck, Germany) were used for column chromatography, and Whatman No. 1 paper sheets for 2D- and comparative paper chromatography (PC) (Whatman, England). The homogeneity of the saponin fractions was tested on thin layer chromatography (TLC) F254 plates, and the spots were visualized by spraying with sulfuric acid reagent (10% in MeOH) followed by heating at 100 °C for 3 min.

Plant material

Leaves of *Bauhinia variegata* were collected at El-Orman Botanical Garden, Giza, Egypt. The plant was authenticated by Dr. Amaal Haggag, Ornamental Research Institute, Zamalek, Cairo, Egypt. Voucher specimens (Reg. No. B-1) are kept in the herbarium of the Medicinal Chemistry Department, Theodor Bilharz Research Institute, Giza, Egypt.

Animals

Male Swiss albino CD-1 mice (6–8 weeks old) and male and female Albino rats (150–180 g) were obtained from Schistosome Biology Supply Center (SBSC), Theodor Bilharz Research Institute, Giza, Egypt. Mice were used for determination of LD_{50} values and after infection with a *Schistosoma mansoni* strain [(80 ± 10) cercariae/mouse] using the body immersion technique (Liang *et al.*, 1987). Mice were used for studying the antischistosomal and part of the anti-inflammatory activates, while rats were used for the anti-inflammatory test using a carrageenan solution.

Drugs and chemicals

Praziquantel (PZQ) (Distocide, E.P.I.Co. Pharmaceuticals, Cairo, Egypt), Cremophore-El (Sigma Chemical Co., St. Louis, , MO, USA), indomethacine (Kahira Pharmaceuticals, Cairo, Egypt).

Extraction and isolation

The air-dried powdered leaves of *B. variegata* (500 g) were extracted under reflux with hot 70% MeOH (3 × 4 l). After evaporation of the solvent, the obtained dry residue was defatted with $CHCl_3$ under reflux (3 × 1 l). The resulting residue (35 g) was suspended in water (500 ml) and extracted with ethyl acetate (3 × 500 ml), followed by *n*-butanol (3 × 300 ml). 2D-PC analysis proved that the chloroform extract is free from polyphenols and flavonoids. These compounds were found in the ethyl acetate fraction, whereas saponins were concentrated in the *n*-butanol extract. The ethyl acetate extract (12 g) was fractionated on a silica gel column (Ø 5.0 × 75 cm). Elution was started with chloroform, followed by ethyl acetate whose polarity was gradually increased by methanol portions. Six collective fractions were obtained (A–F). Fraction A (1.68 g) was found to be an oily, dark brown material of no phenolic character. Compound **1** crystallized from Fraction B (1.10 g) and was purified by recrystallization from MeOH. Two major dark purple spots were detected in fraction C (2.80 g). Further purification of this fraction by repeated column chromatography on Sephadex LH-20 with EtOH afforded pure samples of **2** (35 g) and **3** (38 g). Fraction D (2.60 g) was rechromatographed on a Sephadex LH-20 column using 95% EtOH as an eluent to afford pure samples of compounds **4** (45 mg) and **5** (48 mg). Column chromatography of fraction E (2.50 g) on cellulose eluted with EtOH/H_2O gave subfraction (I) (1.40 g), eluted with 60% EtOH, and (II) (0.95 g), eluted with 80% EtOH. Further purification of subfraction (I) on Sephadex with MeOH as an eluent gave a pure sample of compound **6** (58 mg), while purification of subfraction (II) gave **7** (67 mg). Compound **8** (65 mg) was obtained by repeated chromatographic fractionation of fraction F on Sephadex LH-20 using 70% EtOH as a mobile phase. All separation processes were followed up by comparative PC using Whatman No. 1 paper with *n*-BuOH/HOAc/H_2O (4:1:5, upper layer, S_1) and 15% aqueous HOAc (S_2) as solvent systems and specific spray reagents (*e.g.* Naturstoff, $FeCl_3$, and NH_3). The *n*-butanol extract (17 g) was subjected to column chromatography over silica gel with $CHCl_3$/MeOH [1:0 (1 l), 1:1 (3 l), and 0:1(2 l)]. Three fractions (G–I) were obtained according to the differences in composition indicated by TLC analysis. Fraction H was subjected to repeated fractionation on silica gel columns using $CHCl_3$/ethyl acetate/MeOH/H_2O (32:28:35:2) as eluent to afford a pure sample of **9** (185 mg).

23-Hydroxy-3α-[O-α-L-$^1C_4$-rhamnopyranosyl-(1”→4’)-O-α-L-$^4C_1$-arabinopyranosyl-oxy] olean-12-en-28-oic acid O-α-L-$^1C_4$-rhamnopyranosyl-(1’’’’’→4’’’’)-O-β-D-$^4C_1$-glucopyranosyl-

(1''''→6''')-O-β-D-$^4C_1$*-glucopyranosyl ester* (**9**): Off-white amorphous powder. – R_f 0.53 (S_3). – Negative ESI-high resolution-MS: *m/z* = 1219.6117 $[M–H]^-$ (calcd. 1219.6132 for $C_{59}H_{96}O_{26}$), 749.3170 [M–H–deoxyrhamnosyl-2hexosyl]$^-$, 603.2053 [M–H–deoxyrhamnosyldihexosyl-deoxyrhamnosyl]$^-$, 585.4926 [M–H–deoxyrhamnosyldihexosyl-deoxyrhamnosyl–H_2O]$^-$, 471.4434 [M–H–deoxyrhamnosyl-dihexosyl-deoxyrhamnosylpentoside]$^-$ = [aglycone–H]$^-$. – ^{1}H- and ^{13}C NMR: see Table I.

Acid hydrolysis of ***9***

20 mg of **9** were hydrolyzed with 4 M HCl in EtOH on a boiling water bath for 4 h. The solvent was then evaporated until most of HCl was eliminated. The residue was diluted with 15 ml H_2O and neutralized with $NaHCO_3$. The suspension was extracted with 100 ml $CHCl_3$. The sapogenin was identified in the $CHCl_3$ layer by TLC (MeOH/$CHCl_3$, 1.5:9.5). The H_2O phase was then concentrated and subjected to PC (ethyl acetate/

Table I. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) spectral data of compounds **9** and **9a** (in pyridine-d_5). δ in ppm and *J* values in Hz are given in parentheses. All carbon and proton resonances were assigned on the basis of 2D ^{1}H-^{1}H COSY, HSQC and HMBC.

No.	**9**		**9a**		No.	**9**		**9a**	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
1	38.69		38.78		1'	104.00	5.11 d (6.5)	104.06	5.11 d (6)
2	25.88		25.85		2'	69.36	4.71 dd (9, 6.5)	69.33	4.70 dd (9.6)
3	80.67	4.27 t-like (3.1)	80.67	4.28 t-like (3)	3'	74.38	4.19 dd (8.5, 3.5)	74.42	4.10 dd (8.5, 3.5)
4	43.16		43.15		4'	75.44	4.60 m	73.78	4.58 m
5	46.77		46.27		5'a	65.34	3.73*	65.38	3.69 brd (10.5.)
6	17.22		17.78		5'b		4.15 brd (11)		4.25 brd (10.5)
7	32.65		32.84		1''	102.40	5.87 br s	101.32	6.19 brs
8	39.55		39.38		2''	72.00	4.73 *	72.03	4.73 brd
9	47.84		47.97		3''	72.20	4.66 *	72.19	4.65 dd (9.5, 3.5)
10	36.53		36.52		4''	73.66	4.31 t-like (10.5)	73.78	4.29 t-like (10.5)
11	23.47		23.47		5''	69.01	4.33 m	69.03	4.40 m
12	122.78	5.39 brs	122.22	5.46 brs	6''	18.19	1.63 d (6.5)	18.19	1.63 d (6.5)
13	143.15		144.41		1'''	95.29	6.24 d (8)		
14	41.78		41.77		2'''	74.99	3.97 dd (8, 9.5)		
15	27.97		27.98		3'''	76.82	3.81 t-like (9.5)		
16	23.10		23.30		4'''	70.53	4.29 *		
17	47.36		47.36		5'''	77.71	4.01 m		
18	41.30	3.18 dd (13.5, 3.5)	41.59	3.28 dd (13.5, 3.5)	6''' a,b	69.96	4.95, 4.68*		
19	46.67		46.02		1''''	104.52	4.99 d (7.8)		
20	30.38		30.57		2''''	73.79	4.06*		
21	33.85		33.84		3''''	76.16	4.11*		
22	32.41		32.49		4''''	78.30	4.43 t-like (9.5)		
23	63.50	4.13 d (10.5) 3.75 d (10.5)	63.50	4.26 d (10.5) 3.74 d (10.5)	5''''	77.90	4.10 m		
24	13.62	1.08 s	13.63	1.02 s	6'''' a,b	60.94	4.08, 4.21*		
25	15.84	0.97 s	15.70	0.99 s	1'''''	101.32	6.26 br s		
26	17.22	1.12 s	17.07	1.07 s	2'''''	72.23	4.66 *		
27	25.68	1.16 s	25.79	1.22 s	3'''''	72.42	4.61 *		
28	176.15		179.79		4'''''	73.15	4.17*		
29	32.74	0.85 s	32.88	0.92 s	5'''''	68.88	4.21 m		
30	23.33	0.87 s	23.34	0.93 s	6'''''	18.19	1.70 d (6.5)		

* Unresolved proton resonances.

C_5H_5N/H_2O, 12:5:4) against authentic sugar samples, whereby D-glucose, L-rhamnose, and L-arabinose were identified.

Alkaline hydrolysis of ***9***

About 10 mg of **9** were refluxed with 10 ml 1 M NaOH for 3 h. The hydrolysate mixture was neutralized and the prosapogenin was extracted with *n*-butanol to give **9a**. The aqueous phase was then concentrated and subjected to PC (ethyl acetate/C_5H_5N/H_2O, 12:5:4) against authentic sugar samples, whereby D-glucose and L-rhamnose were identified.

LD_{50}

Aqueous solutions of **9** at doses of 25, 50, 100, 200, 400, 1000, and 2000 mg/kg body weight (b.w.), respectively, were given for 1 d. Mortality and morbidity of animals were recorded 24 h later. Results were analyzed statistically, and LD_{50} values were calculated according to Litchfield and Wilcoxon (1949).

Study of the antischistosomal activity

Male mice (18–20 g) were infected with the Egyptian strain of *S. mansoni* cercariae [(80 ± 10) cercariae/mouse]. They were classified into 5 groups (each group of 6 mice) according to the following treatment regimen: infected control group; mice which received the vehicle of the drugs; second and third groups treated with compound **9** in doses of 100 and 200 mg/kg b.w. 7 weeks post infection (WPI), respectively; a fourth group was treated with PZQ orally in a dose of 500 mg/kg b.w. for 2 consecutive days 6 WPI (Gönnert and Andrews, 1977); a fifth group was treated with indomethacine 7 WPI in a dose of 20 mg/kg b.w. All animals were sacrificed 9 WPI and perfused with cold saline using a Master Flex pump (Cole-Parmer Instrument Company, Barringtom, ME, USA) to recover worms either from hepatic or portomesenteric tissue (Duvall and DeWitt, 1967). The antischistosomal activity of the drug was assessed parasitologically by studying the worm load and distribution, oogram pattern (% egg developmental stages) in the small intestine of mice (Pellegrino and Bernner, 1956), and tissue egg load (Klöetzel, 1967) in both liver and intestine.

Test of the anti-inflammatory activity in mice infected with S. mansoni: Estimation of prostaglandin E_2 (PGE_2) using titer zyme ELISA

PGE_2 in serum

Serum was separated from blood by centrifugation at 3000 rpm for 10 min. PGE_2 was calculated from the standard curve as (in ng/ml) according to the method of Lands and Smith (1982).

PGE_2 in liver homogenate

1 g of liver from each mouse was homogenized using a homogenizer (B. Braun Melsungen AG, Germany). The protein concentration was measured according to Lowry *et al.* (1951). The level of PGE_2 in liver homogenate and isolated granulomas was calculated from the standard curve (in ng/g tissue).

PGE_2 in isolated granuloma

Granulomas were isolated (Pellegrino and Bernner, 1956) from livers of animals sacrificed 9 WPI with *S. mansoni* cercariae. After isolation, granulomas were washed three times with Hanks balanced salt solution (HBSS), homogenized in HBSS, and centrifuged at 3000 rpm to remove all supernatant, and frozen at –70 °C.

Measurement of granuloma diameter

Measurements were done only for granulomas containing a single egg in their centres. The mean diameter of each granuloma was obtained in microns by measuring two diameters of the lesions at right angles to each other with the help of an occular micrometer according to the following equation (Mahmoud and Warren, 1974):

mean diameter
= sum of the two diameters/2.

100 lesions from each group of at least 7 animals were measured.

Test of the anti-inflammatory activity in experimentally induced synchronous pulmonary granulomas

Induction of synchronous pulmonary granulomas

Isolation of schistosome eggs was conducted using a modification of the method of Coker and Von Lichenberg (1956). Schistosome eggs were isolated from livers of mice infected 8 weeks previously with 200 mg/kg b.w. cercariae of *S. mansoni* eggs and suspended in phosphate-buffered

saline at a concentration of 8000 eggs/ml. 4000 eggs were injected into the tail vein of 8-week-old normal mice, using a tuberculin syringe with a 21-gauge needle (Domingo and Warren, 1968). These eggs were embolized in the lungs, inducing focal granulomas.

Experimental design

Mice were divided into 3 groups. 16 d after ova induction, the first group received the vehicle of the drug (infected control), the second group was orally treated with compound **9** in a dose of 200 mg/kg for 7 d, and the third group received indomethacine orally in a dose of 20 mg/kg for 7 d. All animals were sacrificed 32 or 48 d after ova induction, and granulomas were isolated from lungs as previously described (Pellegrino and Bernner, 1956) for determination of PGE_2. Parts of lungs were fixed in 10% formalin for measuring the lung granuloma diameters (Mahmoud and Warren, 1974).

Study of the anti-inflammatory activity using carrageenan solution

An acute anti-inflammatory effect of **9** was tested according to the method described by Winter *et al.* (1962). The compound was given orally in aqueous solution to male and female albino rats. 1 h later, 0.1 ml of an 1% carrageenan solution in saline was injected into the pad of a right hind paw, and 0.5 ml saline into the left paw. The volume of the injected paws was measured 4 h after administration. Animals were sacrificed and both hind paws were excised and weighed separately. 20 mg/kg indomethacine was used as positive control and the percentage oedema was calculated according to the equation

$$\% \text{ oedema} = [(\text{mass of right paw} - \text{mass of left paw})/\text{mass of left paw}] \cdot 100.$$

Study of the antinociceptive activities

The visceral nociceptive (acetic acid-induced writhing test) and central nociceptive (hot plate test) tests were used.

Writhing test: The acetic acid abdominal constriction test was performed as described by Collier *et al.* (1968). Vehicle, aspirin (100 mg/kg), and the test compound **9** were orally administered to mice 30 min before the experiment. Then 0.1 ml/g of 0.7% acetic acid-saline was injected intraperitoneally 10 min after the injection, and the frequency of writhings in mice was counted for the next 10 min.

Hot plate test: The hot plate test was used to measure the response latencies according to the method described previously by Eddy and Leimbach (1953) with minor modifications. In this experiment, the hot plate (Ugo Basile, Model-DS37) was maintained at (55 ± 0.2) ºC. The reaction time was noted by observing either the licking of the hind paws or the jumping movements before and after drug administration. The cut-off time was 20 s and 10 mg/kg morphine sulfate (El-Nasr Pharmaceutical Co.) was administered intraperitoneally as reference drug.

Statistical analysis

Results were given as means ± SE. Data were analyzed using one-way ANOVA, followed by Duncan's test. The statistical significance of difference was taken as $P < 0.05$.

Results

General

The phytochemical investigation of *B. variegata* leaves proved that the ethyl acetate extract was free from saponin. Repeated column chromatography of the collective six fractions (A–F) obtained using different eluents resulted in the isolation of six flavonoids, **1**–**3** and **6**–**8**, and two cinnamic acid derivatives, **4** and **5**, while chromatographic separation of the *n*-butanol extract resulted in the isolation of the bidesmosidic triterpenoidal saponin **9**. On the basis of their chromatographic properties, acid hydrolysis products, and spectroscopic analyses (UV, ESI-high resolution-MS^n, 1D and 2D NMR), the structures of the isolated compounds were identified as quercetin (**1**), 3,3'-dimethoxy quercetin (**2**), 3,3',6-trimethoxy quercetin (**3**) (Harborne, 1982), caffeic acid (**4**), ferulic acid (**5**) (Lu and Ly *et al.*, 2002), quercetin 3-*O*-β-D-$^4C_1$-glucopyranoside (**6**), quercetin 3-*O*-β-D-$^4C_1$-galacturonopyranoside (**7**), quercetin 3-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1'''→2'')-*O*-β-D-$^4C_1$-glucopyranoside (**8**) (Harborne, 1982), and 23-hydroxy-3α-[*O*-α-L-$^1C_4$-rhamnopyranosyl-(1''→4')-*O*-α-L-$^4C_1$-arabinopyranosyl-oxy]olean-12-en-28-oic acid *O*-α-L-$^1C_4$-rhamnopyranosyl-(1''''''→4'''')-*O*-β-D-$^4C_1$-glucopyranosyl-(1''''→6''')-*O*-β-D-$^4C_1$-glucopyranosyl ester (**9**) (Fig. 1).

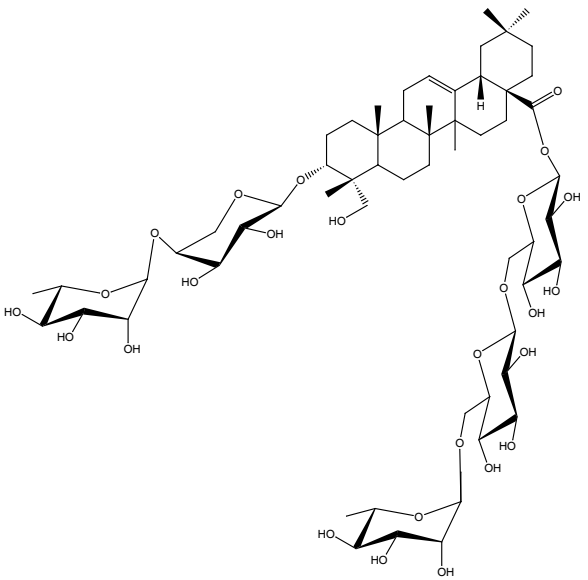

Fig. 1. Chemical structure of **9** isolated from *B. variegata* leaves.

Biological results

The LD_{50} value of compound **9** was found to be 3000 mg/kg b.w. **9** exhibited slight antischistosomal activity when administered for 7 days in doses of 100 and 200 mg/kg b.w. 7 weeks after infection in comparison with PZQ (Table II). Concerning the anti-inflammatory activity, compound **9** showed reduction in the PGE_2 level in serum (19.4% and 34.7%), liver homogenate (15.4% and 27.8%), and granuloma homogenate (17.0% and 23.2%) in mice infected with *S. mansoni* when compared to indomethacine which exhibited 49.7%, 43.4% and 39.6% reduction in the level of PGE_2 in serum, liver and granuloma homogenate, respectively (Table III). Reduction in the hepatic granuloma diameter was observed after treatment with 100 mg/kg b.w. and 200 mg/kg b.w. of **9** (Table III).

A maximum pulmonary granuloma diameter of (138 ± 4) μm was recorded 16 days after ova injection which gradually diminished to (130 ± 4)

Table II. Effect of compound **9** and praziquantel (PZQ) on parasitological parameters 9 weeks post infection with *S. mansoni* cercariae.

Animal group	Worm reduction (%)	Reduction in total number of ova (%)	Reduction in total number of couples (%)	Hepatic shift (%)
Infected control	–	–	–	19.0
PZQ (2 × 500 mg/kg)	94.5*	93.1*	98.8*	75.1*
9 (100 mg/kg)	7.2	5.2	8.8	23.0
9 (200 mg/kg)	10.3	7.4	8.8	25.0

* Significant different from infected control group at $P < 0.05$.

Table III. Effect of compound **9** (100 and 200 mg/kg) and indomethacine (20 mg/kg) on PGE_2 level in serum, liver and granuloma homogenate and on granuloma diameter.

Animal group	Prostaglandin E_2 (PGE_2)			Granuloma diameter [μm]
	Serum [ng/ml]	Liver homogenate [ng/g]	Granuloma homogenate [ng/g]	
Normal control	0.49 ± 0.10	0.51 ± 0.09	–	–
Infected control	20.10 ± 2.52#	24.01 ± 2.11#	31.80 ± 4.11	222 ± 4
9 (100 mg/kg)	16.21 ± 3.43 (19.4)	20.32 ± 3.22 (15.4)	26.39 ± 3.15 (17.0)	193 ± 6 (12.8) *
9 (200 mg/kg)	13.13 ± 2.91* (34.7)	17.33 ± 3.14* (27.8)	24.41 ± 3.91* (23.2)	181 ± 6* (18)
Indomethacine (20 mg/kg)	10.11 ± 1.12* (49.7)	13.6 ± 1.51* (43.4)	19.21 ± 2.52* (39.6)	165 ± 6* (26)

\# Significant different from normal control group at $P < 0.05$.
* Significant different from infected control group at $P < 0.05$.

and (123 ± 3) μm 32 and 48 days after ova injection. Reduction in the granuloma diameter was observed after treatment with compound **9** by 17.6% and 20.9% and indomethacine by 23.8 and 23.9% 32 and 48 days after ova injection, respectively, when compared with control animals. The PGE_2 level in granulomas was also reduced after treatment with compound **9** by 44.4% and 32.4%, while indomethacine produced 36.1% and 22.1% reduction 32 and 48 days after ova injection, respectively (Table IV).

In the model of mice injected with carrageenan, oral treatment with gradual doses of 40, 80, 100 and 200 mg/kg solution showed reduction in oedema by 21.3%, 26.2%, 39.6%, and 28.3% when compared to the control group. These concentrations of compound **9** showed potency of 38%, 47%, 51%, and 71% when compared to indomethacine (at a dose of 20 mg/kg) (Table V).

Concerning the antinociceptive effects in both mouse models, the visceral and central nociceptive, compound **9** showed 54% inhibition of writhing numbers of the control group compared to 76% produced by aspirin (Table VI). This leads to high potency of 71%. Compound **9** also exhibited 41% increase in the reaction time in the hot plate test compared to 88% increase by morphine; so the potency of this compound was 47% (Table VII).

Table IV. Effect of oral administration of compound **9** compared to indomethacine on lung granuloma diameter and PGE_2 level in lung granuloma homogenate.

Animal group	Granuloma diameter [μm]		PGE_2 in lung granuloma homogenate	
	After 32 d	After 48 d	After 32 d	After 48 d
Control	130.2 ± 4.2	123.3 ± 3.2	30.2 ± 5.4	27.2 ± 4.5
9 (200 mg/kg)	107.3 ± 5.3* (17.6%)	97.5 ± 3.5 (20.9%)	16.8 ± 1.3 (44.4%)	18.4 ± 2.3 (32.4%)
Indomethacine (20 mg/kg)	99.2 ± 3.3* (23.8%)	93.8 ± 2.8 (23.9)	19.3 ± 2.5 (36.1%)	21.2 ± 3.3 (22.1%)

* Significant different from control group at $P < 0.05$.

Table V. Anti-inflammatory activity of compound **9** compared to indomethacine.

Animal group	Dose of drug [mg/kg b.w.]	Oedema (%)		
		Mean + SE	Change	Potency
Normal control	Saline	38.3 ± 0.8	–	–
9	40	30.2 ± 1.9	–21.3*	38*
9	80	28.3 ± 2.7*	–26.2*	47*
9	100	27.5 ± 2.4*	–28.3*	51*
9	200	23.1 ± 1.8*	–39.6*	71*
Indomethacine	20	16.9 ± 1.6*	–55.9*	100*

* Significant different from control group at $P < 0.05$.

Table VI. Antinociceptive activity of compound **9** compared with the reference drug using the acetic acid-induced writhing test.

Animal group	Dose of drug	Writhing number [counts/20 min]	Inhibition (%)	Potency (%)
Control (acetic acid/0.7% saline)	0.01 ml/g	67.0 ± 1.7	–	–
9	200 mg/kg	30.8 ± 2.4*	54	71
Aspirin	100 mg/kg	16.2 ± 1.4*	76	100

* Significant different from control group at $P < 0.05$.

Table VII. Antinociceptive activity of compound 9 compared with the reference drug using the hot plate test.

Animal group	Dose of drug	Reaction time [s]	Increase (%)	Potency (%)
Control	Saline	11.0 ± 1.6	–	–
9	200 mg/kg	15.5 ± 0.4*	41	47
Morphine	10 mg/kg	20.7 ± 1.7*	88	100

* Significant different from control group at $P < 0.05$.

Discussion

Compound **9** gave positive Salkoweski and Molisch's reactions indicating its triterpenoid and glycosidic nature. Its negative ESI high-resolution-mass spectrum showed a molecular ion peak at *m/z* 1219.6117 $[M–H]^-$ (calcd. 1219.6132), corresponding to the molecular formula $C_{59}H_{96}O_{26}$. The MS^2 experiment of $[M–H]^-$ ion gave a negative fragment at *m/z* 749.3170 $[M–H–146–2 \times 162]^-$ (loss of a rhamnosyl and two hexosyl units). The MS^3 fragmentation of ion at 749.3170 led to ion at 603.2053 attributed to (loss of extra 146 of a second rhamnosyl), followed by 585.4926 loss of H_2O. The MS^4 of ion fragment at *m/z* 603.2053 gave rise to a product ion at *m/z* 471.4434 $[genin–H]^-$, corresponding to the loss of pentoside from the latter fragment. These data together with the alkaline hydrolysis was compatible with a structure of hydroxyolean 28-rhamnosyl-glucosyl-glucosyl ester with an *O*-rhamnosyl-pentoside, most probably at C-3 (Mshvildadze *et al.*, 2004) (TLC and PC with the authentic samples). The resonances due to six sp^3 methyl carbon atoms at δ_C 13.62, 15.84, 17.22, 23.33, 25.68, 32.74, a primary carbinol at δ_C 63.50 and two sp^2 carbon atoms at δ_C 122.78 and 143.15 in the ^{13}C NMR spectrum of **9** coupled with the corresponding information from the ^1H NMR spectrum [6 tertiary methyl proton singlets, methylene protons linked to oxygen bearing carbon which constituted an AX spin system at 4.13 and 3.74 (d, J = 10.5 Hz, H-23), hydroxymethine proton at δ_H 4.27 for H-3, a proton attributed to H-18 at δ_H 3.18 (dd, J = 13.5, 3.5 Hz) and a broad singlet vinyl proton at δ_H 5.39 of H-12] confirmed the aglycone moiety as 3,23-dihydroxy-olean-12-en skeleton. The relative upfield shift of C-5 at δ_C 46.67 ($\Delta \sim +5$ ppm) was also an evidence for the γ-effect of the carbinol C_{23}-OH. The resonances of C-3 at δ_C 80.67, C-28 at δ_C 176.15 together a with ^1H NMR signal at 6.24 were characteristic of a bis-desmosidic aglycone with 3α-hydroxy group. All assigned ^1H and ^{13}C resonances of the aglycone were confirmed by HSQC and HMBC correlation spectroscopy (Table I). Five anomeric proton signals were assigned at 5.11 (6.5, α-arabinosyl), 5.87 (brs, α-rhamnosyl), 6.24 (8.0, β-gulcosyl ester), 4.99 (7.8, β-glucosyl), 6.26 (brs, α-rhamnosyl) in the ^1H NMR spectrum through their direct one-bond coupling in HSQC with their own anomeric carbon signals at δ_C 104.00, 102.40, 95.29, 104.52, and 101.32, respectively (Table I). The sugar moieties were deduced to adopt α-$^1C_4$-, α-$^4C_1$- or β-$^4C_1$-pyranose stereostructure in case of rhamnosyl, arabinosyl or glucosyl moieties, respectively, on the basis of J values of the anomeric protons and δ values of their ^{13}C resonances (Table I). The interglycosidic and sugar-aglycone linkages were deduced from the long-range three-bond HMBC correlations. The HMBC exhibited correlations between H-1' (5.11) (arabinosyl) and C-3 (80.67) aglycone, H-1" (5.87) rhamnosyl and C-4' (75.44) arabinosyl to establish a diglycoside moiety at C-3 of the aglycone as 3-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1"→4')-*O*-α-L-$^4C_1$-arabinopyranoside. Similarly, correlations between H-1"' (6.24) glucosyl ester and C-28 (176.15) aglycone, H-1"" (4.99) glucosyl and C-6"' (69.96) glucosyl ester, H-1""' (6.26) rhamnosyl and C-4"" (78.30) of the second glucosyl established a triglycosyl ester moiety at C-28 as 28-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1""'→4"")-*O*-β-D-$^4C_1$-glucopyranosyl-(1""→6"')-*O*-β-D-$^4C_1$-glucopyranosyl. All ^1H and ^{13}C resonances were assigned by the aid of HSQC and HMBC correlation peaks and by comparison with the corresponding data of structurally related compounds (Mshvildadze *et al.*, 2004; Shao *et al.*, 1989).

Alkaline hydrolysis of **9** furnished the prosapogenin **9a**, identified as oleanolic acid 23-hydroxy-3α-[*O*-α-L-$^1C_4$-rhamnopyranosyl-(1"→4')-*O*-α-L-$^4C_1$-arabinopyranosyl] from its spectral data (Table I). Therefore, **9** was finally identified as 23-hydroxy-3α-[*O*-α-L-$^1C_4$-rhamnopyranosyl-(1"→4')-*O*-α-L-$^4C_1$-arabinopyranosyl-oxy]olean-12-en-28-oic acid

O-*α*-L-$^1C_4$-rhamnopyranosyl-(1''''→4'''')-*O*-*β*-D-$^4C_1$-glucopyranosyl-(1''''→6''')-*O*-*β*-D-$^4C_1$-glucopyranosyl ester.

Concerning the anti-inflammatory activity, acute inflammation depends on the release of chemical mediators. These mediators bring about oedema formation as results of extravasations of fluid and proteins from the local microvasculature and accumulation of polymorphonuclear leukocytes at the inflammatory site (Dawood *et al*., 2006). Winter *et al*. (1962) presented an inflammatory reaction induced in the hind paw by subplantar injection of carrageenan. This inflammation model is considered the most conventional one for acute inflammation. In the present study, oral treatment with compound **9** (200 mg/kg b.w.; Table V) showed the best reduction in oedema (39.6%) when compared to the control group with high potency (71%) compared to indomethacine (20 mg/kg b.w.). The reduction in oedema was accompanied by significant reduction in the PGE_2 level in serum, liver homogenate and granuloma when compared to the infected control group. A major mechanism of NSAIDs (nonsteroidal anti-inflammatory drugs) like indomethacine is lowering the prostaglandin production through inhibition of cyclooxygenase which has a dual function mediation of inflammation and cytoprotection of the stomach and intestine. Reduction in the hepatic and pulmonary granuloma diameter was observed after treatment with compound **9** which may be due to its anti-inflammatory activity.

Furthermore, compound **9** demonstrated analgesic effects both in the visceral and central nociceptive mouse models. An intraperitoneal injection of acetic acid can produce peritoneal inflammation (acute peritonitis), which causes a response consisting of contraction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body. This writhing response is considered to be a visceral inflammatory pain model, and this method has been associated with increased levels of prostaglandins in the peritoneal fluids (Deraedt *et al.*, 1980). The results here revealed that compound **9** had significantly reduced acetic acid-induced writhing responses, which were more than 70% to those of the reference drug aspirin.

Compound **9** was found to have antinociceptive activity in the hot plate test, which is a specific central antinociceptive test. The antinociceptive effects of compound **9** involve supraspinal modulation as demonstrated by the use of the hot plate. The results suggest that compound **9** has a central antinociceptive effect, as shown by the increase in the reaction time. The present study showed the efficacy of compound **9** in different antinociceptive responses generated by a chemical noxious stimulus produced by acetic acid injections or by a thermal noxious stimulus in the hot plate tests. The anti-inflammatory and antinociceptive activities of compound **9** may be attributed to the oleane-type triterpene (Speroni *et al*., 2007).

With respect to the antinociceptive effects of compound **9** further studies should be carried out to investigate the molecular mechanism of action and participation in the pain inhibitory mechanisms in the central nervous system.

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