# A New Bioactive Steroidal Saponin from *Agave brittoniana*

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A new bisdesmosidic furostanol saponin was isolated from leaves of *Agave brittoniana* Trel. Its structure was established as 3-[*O*-6-deoxy-$\alpha$-L-mannopyranosyl-(1→4)-*O*-$\beta$-D-glucopyranosyl-(1→3)-*O*]-[*O*-$\beta$-D-glucopyranosyl-(1→3)-$\beta$-D-glucopyranosyl-(1→2)-*O*-$\beta$-D-glucopyranosyl-(1→4)-$\beta$-D-galactopyranosyl)oxy]-(3$\beta$,5$\alpha$,6$\alpha$,22$\alpha$,2*R*)-26-($\beta$-D-glucopyranosyloxy)-6,22-dihydroxy-furost-12-one. Its structural identification was performed using detailed analyses of $^1$H and $^{13}$C NMR spectra including 2D NMR spectroscopic techniques (DEPT, COSY, HETCOR and COLOC) and chemical conversions. The steroidal saponin showed no haemolytic effects in the *in vitro* assays and demonstrated antiinflammatory activity using *in vivo* models.

*Key words: Agave brittoniana*, Agavaceae, Steroidal Saponin, Antiinflammatory Activity

## Introduction

*Agave brittoniana* Trel. (Agavaceae) is native to Brazil and is used with ornamental purposes. In the traditional medicine, the aerial parts of this plant are used against infection and inflammation of the urinary system. As part of our programme on the chemical investigation of bioactive steroidal saponins, we have examined the leaves of *A. brittoniana*. In our preceding paper [1], we reported the structure of a steroidal saponin and its effects on the $Na^+$-ATPase and ($Na^+$+$K^+$)-ATPase activities. Further studies have led to the isolation of a new bisdesmosidic furostanol saponin, whose structure was elucidated by spectral data and chemical conversions. The haemolytic property and the antiinflammatory activity of the isolated saponin were investigated.

## Experimental Section

### *Plant material*

Fresh leaves of *Agave brittoniana* Trel. were obtained from the ornamental plant garden of the Federal University of Rio de Janeiro, Rio de Janeiro, in February 2003, and a voucher specimen is maintained in the Laboratory of Chemistry of Medicinal Plants at this University.

### *General procedures*

Melting points were determined by an Electrothermal 9200 micro-melting point apparatus and are uncorrected. The optical rotations were measured on a Perkin Elmer 243B polarimeter. IR spectra were measured on a Perkin Elmer 599B spectrometer. The MALDI-TOFMS was conducted using a Perseptive Voyager RP mass spectrometer. GC-MS analyses were performed with a Shimadzu GCMS-QP5050A gas chromatograph mass spectrometer using an ionization voltage of 70 eV and an ionization current of 60 $\mu$A for EI. GC was carried out with FID using a DB-1 glass capillary column (0.25 × 25 m, 0.25 micron; J. & W. Scientific Incorporated, Folsom, CA, USA). NMR spectra were measured in [$D_5$]pyridine (100 mg of **1** in 0.5 mL) at 25 °C with a Varian Gemini 200 NMR spectrometer, with tetramethylsilane ($\delta$ = 0.0) used as internal standard. $^1$H NMR spectra were recorded at 200 MHz and $^{13}$C NMR spectra at 50 MHz. Silica gel columns (230 – 400 mesh ASTM, Merck) and Sephadex LH-20 (Pharmacia) were used for CC. TLC was performed on silica gel plates (Kieselgel $60F_{254}$, Merck) using the following solvent systems: (A) $CHCl_3$/MeOH/$H_2O$ (65 : 35 : 10, v/v/v, lower phase) for steroidal saponin **1**, (B) $CHCl_3$/MeOH (95 : 5, v/v) for pseudosapogenin and (C) *n*-BuOH/$Me_2CO$/MeOH (4 : 5:1, v/v/v) for monosaccharides. Spray reagents were orcinol/$H_2SO_4$ for steroidal saponin **1** and $Ce(SO_4)_2$ for pseudosapogenin.

### *Extraction and isolation*

Fresh leaves of *A. brittoniana* (4.0 kg) were extracted with MeOH (9.0 L) for 72 h at r. t. The extract was concentrated under reduced pressure to remove most of the MeOH and the resulting aqueous phase was shaken with *n*-BuOH [water/*n*-BuOH (1 : 1, v/v)]. This procedure was repeated and

Table 1. $^{13}$C and $^{1}$H NMR spectral data for compound **1**[a].

[a] $^{13}$C (50 MHz) and $^{1}$H NMR (200 MHz) in [D$_5$]pyridine; $\delta$ in ppm, $J$ in Hz. Assignments based upon $^{1}$H-$^{1}$H COSY, HETCOR and COLOC experiments. The following convention is used: Agl = aglycone, Gal = $\beta$-D-galactopyranosyl, Glc = $\beta$-D-glucopyranosyl, Rha = $\alpha$-L-rhamnopyranosyl.

| Position | | $^{13}$C, $\delta$ | $^{1}$H, $\delta$ ($J$) | DEPT | Position | | $^{13}$C, $\delta$ | $^{1}$H, $\delta$ ($J$) | DEPT |
|---|---|---|---|---|---|---|---|---|---|
| Agl | 1 | 35.84 | | $CH_2$ | Glc | 1 | 104.40 | 5.07 d (7.8) | CH |
| | 2 | 29.54 | | $CH_2$ | | 2 | 80.31 | | CH |
| | 3 | 77.70 | 3.84 m | CH | | 3 | 87.45 | | CH |
| | 4 | 34.20 | | $CH_2$ | | 4 | 70.13 | | CH |
| | 5 | 53.84 | | CH | | 5 | 76.88 | | CH |
| | 6 | 71.29 | 3.54 br d (8.8) | CH | | 6 | 62.43 | | $CH_2$ |
| | 7 | 41.29 | | $CH_2$ | Glc | 1′ | 103.59 | 5.49 d (7.5) | CH |
| | 8 | 32.92 | | CH | | 2′ | 74.23 | | CH |
| | 9 | 54.94 | | CH | | 3′ | 87.45 | | CH |
| | 10 | 35.84 | | C | | 4′ | 68.94 | | CH |
| | 11 | 37.59 | | $CH_2$ | | 5′ | 77.47 | | CH |
| | 12 | 212.53 | | C=O | | 6′ | 61.65 | | $CH_2$ |
| | 13 | 54.94 | | C | Glc | 1″ | 104.92 | 5.04 d (7.5) | CH |
| | 14 | 55.51 | | CH | | 2″ | 75.04 | | CH |
| | 15 | 31.00 | | $CH_2$ | | 3″ | 77.47 | | CH |
| | 16 | 79.27 | | CH | | 4″ | 71.09 | | CH |
| | 17 | 55.13 | | CH | | 5″ | 77.89 | | CH |
| | 18 | 16.15 | 1.17 s | C*Me* | | 6″ | 62.03 | | $CH_2$ |
| | 19 | 13.09 | 0.75 s | C*Me* | Glc | 1‴ | 104.12 | 5.14 d (8.0) | CH |
| | 20 | 42.59 | | CH | | 2‴ | 75.04 | | CH |
| | 21 | 13.91 | 1.36 d (6.9) | CH*Me* | | 3‴ | 76.01 | | CH |
| | 22 | 110.56 | | C | | 4‴ | 77.47 | | CH |
| | 23 | 30.21 | | $CH_2$ | | 5‴ | 76.67 | | CH |
| | 24 | 27.74 | | $CH_2$ | | 6‴ | 60.74 | | $CH_2$ |
| | 25 | 33.75 | | CH | Glc | 1⁗ | 105.01 | 4.83 d (7.8) | CH |
| | 26 | 74.74 | | $CH_2$ | | 2⁗ | 75.22 | | CH |
| | 27 | 16.77 | 0.98 d (6.6) | CH*Me* | | 3⁗ | 78.61 | | CH |
| Gal | 1 | 102.09 | 4.83 d (7.8) | CH | | 4⁗ | 71.69 | | CH |
| | 2 | 72.60 | | CH | | 5⁗ | 78.51 | | CH |
| | 3 | 75.04 | | CH | | 6⁗ | 63.01 | | $CH_2$ |
| | 4 | 79.60 | | CH | Rha | 1 | 103.42 | 5.74 br s | CH |
| | 5 | 74.82 | | CH | | 2 | 71.95 | | CH |
| | 6 | 60.28 | | $CH_2$ | | 3 | 72.14 | | CH |
| | | | | | | 4 | 73.38 | | CH |
| | | | | | | 5 | 69.90 | | CH |
| | | | | | | 6 | 18.01 | 1.71 d (6.2) | CH*Me* |

the resulting organic phase was evaporated *in vacuo* to give a crude material (18.3 g), which was dissolved in MeOH (300 mL) and roughly chromatographed (305 mg/5 mL each time) on Sephadex LH-20 (3.8 × 65 cm) with MeOH. The fractions were combined based on the TLC profiles to give the saponin mixture (3.2 g). Further purification by chromatography on a silica gel column (2.8 × 90 cm) eluted with $CHCl_3$/MeOH/$H_2O$ (7 : 3 : 1, lower phase) provided a TLC homogeneous compound **1** (123 mg, $R_f$ = 0.30) which gave a dark blue color with orcinol/$H_2SO_4$.

*Compound **1***

Colorless needles. – M. p. > 280 °C. – $[\alpha]_D^{25} = -30$ ($c$ = 0.1, MeOH). – IR (KBr): $\nu$ = 3411 (O–H), 2929 (C–H), 1705 (C=O), 1652, 1455, 1424, 1378, 1317, 1262, 1202, 1159, 1074, 1038, 914, 897, 590 cm$^{-1}$ [(25*R*)-furostanol, intensity 914 < 897 cm$^{-1}$]. – $^{1}$H and $^{13}$C NMR data given in Table 1. – MALDI-TOFMS: *m/z* = 1606.6387 (calcd. 1606.6403 for $C_{69}H_{114}O_{40}Na$, $[M+Na]^+$).

*Acid hydrolysis of **1***

A solution of saponin **1** (100 mg) in 1 M HCl/1,4-dioxane (1 : 1, v/v; 10 mL) was heated in a sealed tube for 1 h at 100 °C. After cooling, the reaction mixture was neutralized with 3 % KOH in MeOH and evaporated to dryness. The salts that deposited on addition of MeOH were filtered off and the filtrate was passed through a Sephadex LH-20 column with MeOH to give the hydrolysate (89 mg) which was column chromatographed over silica gel using a discontinuous gradient of $CHCl_3$/MeOH (19 : 1 to 1 : 1, v/v) to yield the pseudosapogenin (17.3 mg) and a sugar fraction (63.4 mg). The identity of the aglycone was established by comparison with spectral data through IR, $^{1}$H and $^{13}$C NMR and EIMS [2]. The sugar fraction (1 mg) was dissolved in pyridine (100 $\mu$L) and analyzed by silica gel-TLC in the above described solvent system (C). After spraying with orcinol/$H_2SO_4$, rhamnose gave a green spot at $R_f$ = 0.75, glucose gave a blue spot at $R_f$ = 0.70 and galactose gave a purple spot at $R_f$ = 0.66.

*Molar carbohydrate composition and D,L configurations*

The molar carbohydrate composition of compound **1** was determined by GC-MS analysis of its monosaccharides as their trimethylsilylated methylglycosides obtained after methanolysis (0.5 M HCl in MeOH, 24 h, 80 °C) and trimethylsilylation [3]. The configurations of the glycosides were established by capillary GC of their trimethylsilylated (–)-2-butylglycosides [4].

*Methylation analysis*

Compound **1** was methylated with dimethyl sulfoxide/lithium methylsulfinyl carbanion/methyl iodide [5]. The methyl ethers were obtained after hydrolysis (4 N trifluoroacetic acid, 2 h, 100 °C) and analyzed as partial alditol acetates by GC-MS [6].

*Animals*

Swiss albino male mice, weighing 15 – 20 g, were used. The animals were housed under standard environmental conditions and fed with standard rodent diet and water *ad libitum*.

*Statistical analysis*

The experimental data were tested for statistical differences using the Student's *t* test.

*Haemolytic activity*

A normal human red blood cell suspension (0.6 mL of 0.5 % ) was mixed with 0.6 mL diluent containing 5, 10, 20, 30, 40, 50, 100, 250, and 500 $\mu$g mL$^{-1}$ of compound **1**, aluminum hydroxide, and 5 – 500 $\mu$L mL$^{-1}$ of Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) in saline solution. Mixtures were incubated for 30 min at 37 °C and centrifuged at $70 \times g$ for 10 min. Saline and distilled water were included as minimal and maximal haemolytic controls. The haemolytic percents developed by the saline control were subtracted from all groups. The adjuvant concentration inducing 50 % of the maximum haemolysis was considered the $HD_{50}$ (graphical interpolation). Experiments included triplicates at each concentration [7].

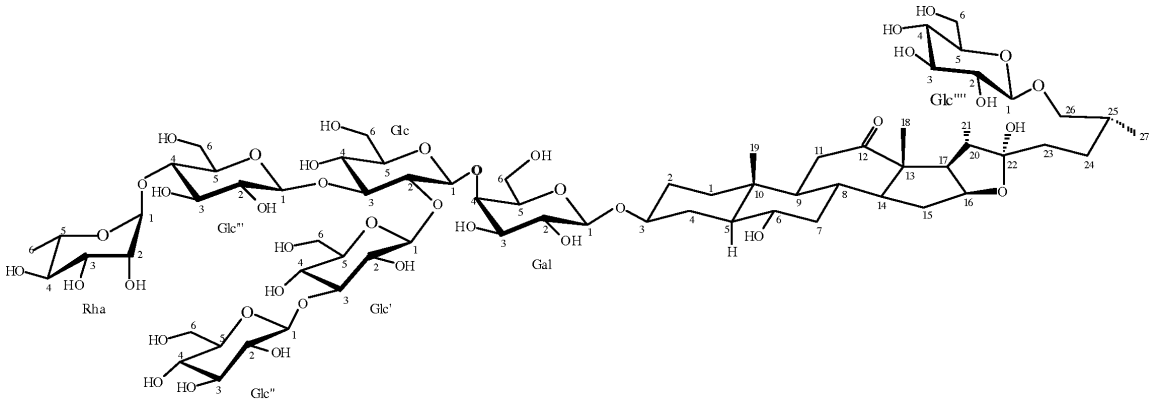
*Antiinflammatory activity*

The effects of compounds **1** and **1a** on acute inflammation were evaluated by measuring the topical edema induced by croton oil in the mouse ear skin [8]. Adult albino male mice (15 – 20 g) were used for the experiment. The animals were maintained on a standard laboratory diet with free access to water. Edema was induced by topical application of the croton oil solution (40 $\mu$g in 10 $\mu$L of acetone per dose) to the inner surface of the right ear of each mouse. Compounds **1** and **1a**, dissolved in 50 % aqueous ethanol, were applied topically (500 $\mu$g per ear) immediately after the inflammatory agent. The standard drugs, indomethacin (250 $\mu$g per ear) and dexamethasone (100 $\mu$g per ear) were administered dissolved in acetone. The thickness of each ear was measured using a micrometer (Mitutoyo Series 293). The edema inhibition was expressed as the reduction in thickness with respect to the control group.

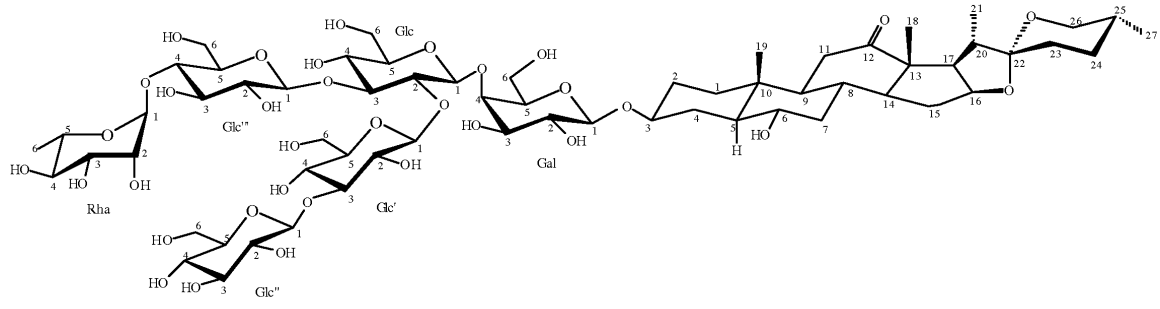
## Result and Discussion

Compound **1** was obtained as colorless needles and gave a positive Liebermann-Burchard test for a steroidal saponin. It revealed a *quasi*-molecular ion peak at *m/z* = 1606.6387, $[M+Na]^+$, in the MALDI-TOFMS. In the $^{13}$C NMR spectrum, the 69 carbon signals observed belong to five methyl groups, fifteen methylene groups (seven of which were oxygenated), forty five methine groups (thirty eight of which were oxygenated) and four quaternary carbon atoms (two of which were oxygenated). On the basis of the above mentioned MS and NMR spectral data, compound **1** was supposed to be a saponin with the molecular formula $C_{69}H_{114}O_{40}$, bearing a chain of seven sugar moieties.

In addition to this, the furostanol glycosidic nature of compound **1** was indicated by the strong absorption bands at 3411 and 1074 cm$^{-1}$ and a 25*R*-furostan steroidal structure (897 and 914 cm$^{-1}$, intensity 914 < 897 cm$^{-1}$) in the IR spectrum [9], confirmed by $^1$H and $^{13}$C NMR spectra [10]. The $^1$H NMR spectra (Table 1) contained signals for two hydrogens at $\delta$ = 3.54 (br. d, $J$ = 8.8 Hz, 1H, 6-H) and 3.84 (m, 1H, 3-H), two secondary methyl hydrogens at $\delta$ = 0.98 (d, $J$ = 6.6 Hz, 3H, 27-H) and 1.36 (d, $J$ = 6.9 Hz, 3H, 21-H) and two angular methyl hydrogens at $\delta$ = 0.75 (s, 3H, 19-H) and 1.17 (s, 3H, 18-H). The above $^1$H NMR spectral data and a comparison of the $^{13}$C NMR signals of the aglycone moiety of **1** with those described in the literature [1, 2, 10] suggested the structure of the aglycone to be 3,6,22,26-tetrahydroxy-(3$\beta$,5$\alpha$,6$\alpha$,22$\alpha$,25*R*)-furostan-12-one. In addition to this, the $^1$H NMR spectrum of **1** showed seven anomeric hydrogens at $\delta$ = 4.83 (d, $J$ = 7.8 Hz, 2H), 5.04 (d, $J$ = 7.5 Hz, 1H), 5.07 (d, $J$ = 7.8 Hz, 1H), 5.14 (d, $J$ = 8.0 Hz, 1H), 5.49 (d, $J$ = 7.5 Hz, 1H) and 5.74 (br s, 1H) corresponding to Gal-H-1 and Glc-H-1$''''$, Glc-H-1$''$, Glc-H-1, Glc-H-1$'''$, Glc-H-1$'$ and Rha-H-1, respectively, indicating the $\beta$-




**1**




**1a**

Fig. 1. Structures of compounds **1** and **1a**.

anomeric configuration for galactose and five glucoses and the $\alpha$-anomeric configuration for rhamnose. The $^{13}$C NMR spectroscopic data (Table 1) for the sugar moieties indicated that all the monosaccharides were in pyranose form.

In the $^{13}$C NMR spectrum of compound **1**, a 1,4-linked inner $\beta$-D-galactopyranosyl unit, a 1,2,3-linked inner $\beta$-D-glucopyranosyl unit, a 1,3-linked inner $\beta$-D-glucopyranosyl unit, a 1,4-linked inner $\beta$-D-glucopyranosyl unit, two terminal $\beta$-D-glucopyranosyl units and a terminal $\alpha$-L-rhamnopyranosyl unit were observed. The $^{1}$H and $^{13}$C NMR chemical shift assignments were made by standard 1D and 2D NMR techniques ($^{1}$H-$^{1}$H COSY, DEPT, HETCOR and COLOC experiments). Its COLOC spectrum displayed long range couplings between galactose-1-H at $\delta$ = 4.83 and aglycone-C-3 at $\delta$ = 77.70, between glucose $-1''''$-H at $\delta$ = 4.83 and aglycone-C-26 at $\delta$ = 74.74, between glucose -1$''$-H at $\delta$ = 5.04 and glucose-C-3$'$ at $\delta$ = 87.45, between glucose-1-H at $\delta$ = 5.07 and galactose-C-4 at $\delta$ = 79.60, between glucose -1$'''$-H at $\delta$ = 5.14 and glucose-C-3 at $\delta$ = 87.45, between glucose-1$'$-H at $\delta$ = 5.49 and glucose-C-2 at $\delta$ = 80.31, and between rhamnose-1-H at $\delta$ = 5.74 and glucose-C-4$'''$ at $\delta$ = 77.47. In addition to this, the methylation analysis of compound **1** furnished 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl glucitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl glucitol. These results indicated that the composition of compound **1** is undoubtedly as shown in Fig. 1.

On acid hydrolysis, compound **1** gave a pseudosapogenin, galactose, glucose and rhamnose. The

pseudosapogenin was identified as 3,6-dihydroxy-(3$\beta$,5$\alpha$,6$\alpha$,25*R*)-spirostan-12-one. Its identity was established by comparison with spectral data through IR, $^{1}$H and $^{13}$C NMR and EIMS experiments [2]. The molar carbohydrate composition of compound **1** indicated the presence of seven neutral monosaccharides: galactose-glucose-rhamnose (1 : 5 : 1) [3]. Their absolute configurations were determined by GC of their trimethylsilylated (–)-2-butylglycosides [4]. D-Galactose, D-glucose and L-rhamnose were identified. Consequently, on the basis of IR, $^{1}$H and $^{13}$C NMR spectroscopy, MALDI-TOFMS and chemical reactions, the structure of compound **1** was established as 3-[(*O*-6-deoxy-$\alpha$-L-mannopyranosyl-(1→4)-*O*-$\beta$-D-glucopyranosyl-(1→3)-*O*]-[*O*-$\beta$-D-glucopyranosyl-(1→3)-$\beta$-D-glucopyranosyl-(1→2)-*O*-$\beta$-D-glucopyranosyl-(1→4)-$\beta$-D-galactopyranosyl)oxy]-(3$\beta$,5$\alpha$,6$\alpha$,22$\alpha$,25*R*)-26-($\beta$-D-glucopyranosyloxy)-6,22-dihydroxy-furost-12-one (**1**).

According to the literature, steroidal saponins possess several physiological properties depending on their chemical structures, such as haemolytic activity and capacity for alteration of membrane permeability [11]. Additionally, these compounds isolated from medicinal plants have been reported to have antiinflammatory activity against several experimental models of inflammation [12]. In order to evaluate the biological properties of the steroidal saponins **1** and **1a** [1] (Fig. 1) isolated from *A. brittoniana*, they were screened for haemolytic activity *in vitro* [7] and antiinflammatory activity *in vivo* [8].

Generally, steroidal saponins possess powerful haemolytic activity because steroids have higher affinities for cholesterol on erythrocyte membranes [11]. As a consequence of its amphipathic structure containing a hydrophobic steroidal nucleus and a hydrophilic carbohydrate moiety, this seems to be the case for compound **1a**. Nonetheless, this is not the case for compound **1**, demonstrated by the absence of haemolytic effects (Fig. 2). This behavior can be explained by the assumption that the steroidal saponin **1** possesses sugar units distributed at opposite sides of the aglycone moiety, which considerably reduces its hydrophobicity, resulting in the loss of the amphipathic features. In addition to this, compounds **1** and **1a** were evaluated for antiinflammatory activity using an acute inflammation model. The results were measured by inhibition of the edematous response induced by a common irritating agent [8]. A topical application of compound **1a**, having a spirostane skeleton, potently suppressed the edema formation induced by the inflammatory agent, provoking an inhibition similar to the reference compounds indomethacin and dexamethasone. Compound **1**, having a furostane skeleton, showed a moderate activity (Fig. 3). These results suggest that the E and F rings of spirostane steroids play a key role in the mediation of the inflammatory response and the haemolytic activity [13].

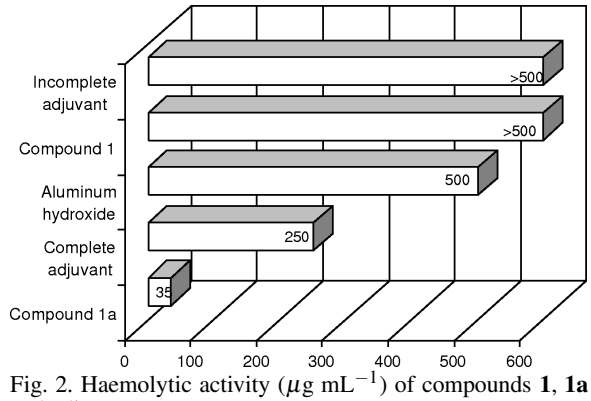



Fig. 2. Haemolytic activity ($\mu$g mL$^{-1}$) of compounds **1**, **1a** and adjuvants.

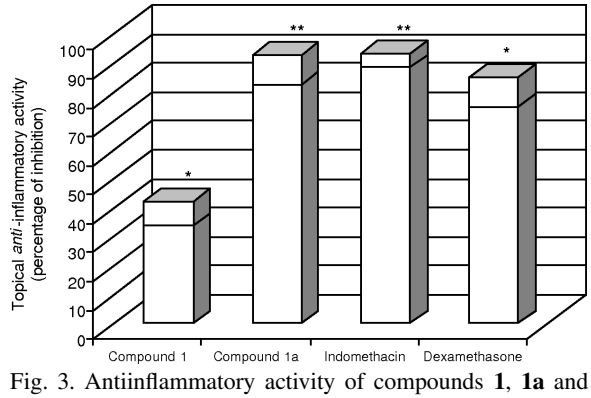



Fig. 3. Antiinflammatory activity of compounds **1**, **1a** and reference compounds against mouse ear edema induced by croton oil. Results are mean ± S.E.M. ($n$ = 5); $^{*}p < 0.05$, $^{**}p < 0.01$, significantly different from the control group.

In comparison with the literature reports, compound **1a** showed antiedematous properties with potency similar to that of bioactive compouds from other medicinal plants used against inflammatory conditions, such as *Bupleurum rotundifolium* [14] and *Abrus precatorius* [15], while compound **1** showed an inhibition of edema which was about the same order of magnitude as that observed for compounds isolated from *Lonicera japonica* [16], using the same experimental induction of topical inflammation. The biological re-

sults obtained suggest that the steroidal saponins **1** and **1a** may be the potential therapeutic agents involved in inflammatory disorders justifying the use of this plant in popular medicine.

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