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Structural characterization of a new steroidal saponin from *Agave angustifolia* var. *Marginata* and a preliminary investigation of its *in vivo* antiulcerogenic activity and *in vitro* membrane permeability property

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ABSTRACT

A new furostane steroidal saponin was isolated from the leaves of *Agave angustifolia* var. *marginata*. On the basis of chemical conversions and spectroscopic analyses, its structure was established as 3-[*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*β*-D-glucopyranosyl-(1→3)-*O*]-[*O*-6-deoxy-*α*-L-mannopyranosyl-(1→4)-*β*-D-xylopyranosyl-(1→2)-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranosyl)oxy]-(3*β*,5*α*,22*α*,25*R*)-26-(*β*-D-glucopyranosyloxy)-22-methoxy-furostane (**1**). Results of preliminary biological investigations indicated that compound **1** showed significant protective effects against induced gastric ulcers using *in vivo* experimental models and demonstrated negligible toxicity on membrane integrity in the *in vitro* assays.

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Naturally occurring steroidal saponins isolated from medicinal plants are attracting considerable attention and have been reported to possess important biological activities, such as modulation of metabolic processes.[1] Additionally, these compounds have been described to possess therapeutic potential for immune system modulation through different mechanisms, regulating the humoral and cellular immune responses.[2,3] Plants of the genus *Agave* are cultivated worldwide for their potential as nutraceuticals and medicinal properties, many of which are used in the alternative medicine for the treatment of metabolic disorders.[1] Important physiological benefits are described for these compounds, which are demonstrated to reduce cholesterol, normalize triglyceride content, and regulate blood sugar levels, improving gastrointestinal functions.[4]

Agave angustifolia var marginata hort. ex Gentry (Agavaceae) is a horticultural variety, widely cultivated and naturalized through tropical regions around the world with ornamental purposes.[5] Nonetheless, no chemical investigations or biological evaluations were carried out on the constituents of this subspecies. As part of the ongoing efforts in discovering new naturally occurring bioactive compounds, the present work is concerned with structural characterization of a new steroidal saponin from *Agave angustifolia* var. *marginata* along with a preliminary investigation of its antiulcerogenic activity using *in vivo* experimental models and an evaluation of its toxicological potential against membrane integrity in the *in vitro* assays.

The leaves of *A. angustifolia* var. *marginata* were harvested at the Campus of the Federal University of Rio de Janeiro in September 2012. Fresh leaves (5.0 kg), previously cut into small pieces, were extracted with MeOH for 7 days at room temperature. The methanolic solution was concentrated under reduced pressure to remove most of the MeOH and the resulting aqueous solution (500 mL) was extracted with *n*-BuOH (500 mL). The resulting organic phase was evaporated *in vacuo* to give a crude material (14.20 g). Using chromatographic techniques compound **1** was isolated.[6]

Compound **1**, $[\alpha]_D^{25}$ – 43.9° (*c* 0.1, MeOH), was obtained as an amorphous white powder and gave a positive Liebermann-Burchard reaction for a steroidal saponin. It revealed a quasi-molecular weight ion peak at *m/z* 1559,7066 $[M+Na-H]^+$ (calcd 1560, 6617 in the HR-ESI-MS. In the ^{13}C NMR spectrum, of the sixty-nine carbon signals observed, there are six methyl groups (one of which was oxygenated), seventeen methylene groups (seven of which were oxygenated), forty-three methine groups (thirty-six of which were oxygenated) and three quaternary carbon atoms (one of which was oxygenated). The number of hydrogens attached to each individual carbon atom was determined by the APT spectrum. On the basis of the above mentioned MS and NMR spectral data (Table 1),

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Table 1
^{1}H (500 MHz) and ^{13}C (125 MHz) NMR chemical shift values of compound 1 in pyridine-d_5 (δ in ppm, J values in parentheses).[a,b]

Position	δ_H (J in Hz)	δ_C	APT	Position	δ_H (J in Hz)	δ_C	APT
Aglycon				*β*-D-Glc*p* I			
1*α*	1.52	37.1	CH_2	1	5.12 d (8.1)	104.6	CH
1*β*	0.78			2	4.27	79.8	CH
2*α*	1.62	29.8	CH_2	3	4.15	87.6	CH
2*β*	2.04			4	3.79	70.3	CH
3	3.80	77.4	CH	5	3.79	77.4	CH
4*α*	1.35	35.6	CH_2	6	3.96; 4.38	62.8	CH_2
4*β*	1.79						
5	0.88	44.5	CH	*β*-D-Glc*p* II			
6*α*, 6*β*	1.16	28.8	CH_2	1	5.58 d (7.8)	104.4	CH
7*α*	0.80	32.3	CH_2	2	4.08	74.4	CH
7*β*	1.53	3	4.08	86.7	CH		
8	1.92	34.1	CH	4	4.08	69.0	CH
9	0.51	54.2	CH	5	3.76	77.6	CH
10	–	35.6	C	6	4.02; 4.20	61.0	CH_2
11*α*	1.20	21.1	CH_2				
11*β*	1.40			*β*-D-Glc*p* III			
12*α*	1.01	39.9	CH_2	1	5.10 d (7.6)	105.8	CH
12*β*	1.64	2	4.00	75.8	CH		
13	–	40.9	C	3	4.15	78.1	CH
14	1.00	56.2	CH	4	4.13	71.6	CH
15*α*	1.76	30.7	CH_2	5	3.92	78.4	CH
15*β*	2.01	6	4.25; 4.48	62.0	CH_2		
16	4.59	79.6	CH				
17	1.74	64.2	CH	*β*-D-Xyl*p*			
18	0.80 s	16.4	CH_3	1	5.28 d (7.6)	104.7	CH
19	0.65 s	12.2	CH_3	2	3.92	75.5	CH
20	2.22	40.4	CH	3	4.06	75.1	CH
21	1.20 d (7.0)	16.3	CH_3	4	4.15	75.8	CH
22	–	112.5	C	5	3.48; 4.21	63.8	CH_2
23*α*	2.03	32.3	CH_2				
23*β*	1.41			*α*-L-Rha*p*			
24*α*, 24*β*	1.16	28.8	CH_2	1	5.45 br s	99.3	CH
25	1.90	34.1	CH	2	4.63	72.4	CH
26*α*	3.61 dd (9.6; 6.5)	75.3	CH_2	3	4.53	72.4	CH
26*β*	3.95	4	4.32	73.8	CH		
27	0.98 d (6.5)	17.2	CH_3	5	4.92	69.8	CH
OCH_3	3.29 s	47.2	CH_3	6	1.70 d (6.2)	18.5	CH_3
3-*O*-Sugars				26-*O*-Sugars			
β-D-Gal*p*				*β*-D-Glc*p* IV			
1	4.88 d (7.8)	102.4	CH	1	4.84 d (7.7)	104.6	CH
2	4.40	73.0	CH	2	4.01	75.2	CH
3	4.08	74.9	CH	3	4.23	78.5	CH
4	4.46	81.3	CH	4	4.23	71.6	CH
5	3.96	75.8	CH	5	3.93	78.4	CH
6	4.23; 4.65	60.6	CH_2	6	4.40; 4.59	62.8	CH_2

[a] Overlapped proton signals are reported without designated multiplicity.
[b] Assignments were based on HSQC, HMBC, COSY and APT experiments.

compound **1** was assumed to be a saponin with the molecular formula $C_{69}H_{116}O_{37}$, bearing seven monosaccharide moieties.

Upon the hydrolysis with 1 M H_2SO_4, **1** gave tigogenin, which was identified by co-TLC [$CHCl_3$/MeOH (97:3, v/v)] with an authentic sample. Compound **1** also gave rhamnose (Rha), xylose (Xyl), glucose (Glc) and Galactose (Gal), which were identified by co-TLC [*n*-BuOH/acetone/H_2O (4:5:1)] with authentic samples. $CeSO_4$-H_2SO_4 and orcinol-H_2SO_4 were the spray reagents used for tigogenin and monosaccharides, respectively. The molar carbohydrate composition of **1** indicated the presence of one rhamnose, one xylose, one galactose and four glucoses.[7] The absolute configuration of each sugar residue was determined to be D for Clc, Xyl and Gal, and L for Rha based on GC analysis of their trimethylsilylated (−)-2-butyl-glycosides.[8]

The IR spectrum of compound **1** showed meaningful absorptions at 3420, 2928 and 1072 cm^{-1} attributed to O—H, C—H and C—O stretching vibrations, respectively. The absorptions at 918 and 893 cm^{-1} (intensity 918 < 893 cm^{-1}) indicated a 25*R*-furostan steroidal structure.[9] The 25*R* stereochemistry of compound **1** was deduced based on difference among chemical shift between the geminal protons of glycosyloxy methylene (H_2-26) having unsubstituted 23-, 24- and 25-positions.[10,11] The value $\Delta H\text{-}26_{ab} = 0.34$ (δ 3.61 for H-26_a and 3.95 for H-26_b) provided evidence for the 25*R* configuration. Besides, the ^{1}H NMR spectrum of compound **1** displays a doublet signal at δ 0.98 typical of a 25*R*-furostanol saponin. The signal at δ 112.5 was assigned to the acetalic quaternary carbon which is characteristic for a furostane skeleton possessing a OMe group at C-22 on the 26-*O*-glycosidic form.[12] The A/B trans-ring fusion was inferred by the signals at δ 44.5 (CH, C-5), 32.3 (CH_2, C-7), 54.2 (CH, C-9) and 12.2 (CH_3, C-19) indicating that the aglycon of **1** is a 5*α*H steroidal sapogenin.[13]

The ^{1}H NMR spectrum (Table 1) of compound **1** displayed seven anomeric hydrogen atoms at δ_H 4.84 (d, J = 7.7 Hz), 4.88 (d, J = 7.8 Hz), 5.10 (d, J = 7.6 Hz), 5.12 (d, J = 8.1 Hz), 5.28 (d, J = 7.6 Hz), 5.45 (br s) and 5.58 (d, J = 7.8 Hz) which gave correlations in the HSQC spectrum with ^{13}C NMR signals (Table 1) at δ_C 104.6, 102.4, 105.8, 104.6, 104.7, 99.3 and 104.4, respectively. Evaluation of chemical shifts and spin-spin couplings allowed the identification of one *β*-galactopyranosyl unit (*β*-Gal*p*), one *α*-rhamnopyranosyl unit (*α*-Rha*p*), one *β*-xylopyranosyl unit

Table 2
The key correlations in HMBC experiment of **1**.

δ_H (^{1}H)	δ_C (^{13}C)
4.88 (β-D-Gal*p* H-1)	77.4 (Aglycon C-3)
5.12 (β-D-Glc*p* I H-1)	81.3 (β-D-Gal*p* C-4)
5.58 (β-D-Glc*p* II H-1)	87.6 (β-D-Glc*p* I C-3)
5.10 (β-D-Glc*p* III H-1)	86.7 (β-D-Glc*p* II C-3)
5.28 (β-D-Xyl*p* H-1)	79.8 (β-D-Glc*p* I C-2)
5.45 (α-L-Rha*p* H-1)	75.8 (β-D-Xyl*p* C-4)
4.84 (β-D-Glc*p* IV H-1)	75.3 (Aglycon C-26)
3.29 (Aglycon H_3-22)	112.5 (Aglycon C-22)
1.20 (Aglycon H-21)	112.5 (Aglycon C-22)
0.98 (Aglycon H-27)	75.3 (Aglycon C-26)

(β-Xyl*p*) and four β-glucopyranosyl units (β-Glc*p* I, β-Glc*p* II, β-Glc*p* III and β-Glc*p* IV). The HMBC experiment (Table 2) of compound **1** displayed long range couplings between H-1 of β-D-Gal*p* at δ_H 4.88 and C-3 of the aglycon at δ_C 77.4 and between H-1 of β-D-Glc*p* IV at δ_H 4.84 and C-26 of the aglycon at δ_C 75.3, indicating that β-D-Gal*p* and β-D-Glc*p* IV are linked to the C-3 and C-26, respectively. The linkage modes for compound **1** (aglycon and seven monosaccharides) were established by HMBC (see Table 2 and Fig. 1) and methylation analysis.[14] The ^{1}H and ^{13}C NMR spectral data (Table 1) of compound **1** were assigned based on the HSQC, HMBC, COSY and APT experiments and by comparison of its ^{1}H and ^{13}C NMR signals with those reported in the literature.[15,16]

The sequence of the sugar chain of **1** was confirmed by methylation analysis,[14] which furnished 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl rhamnitol, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl xylitol, 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 galactitol and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methyl glucitol. These results indicated that the sugar-aglycon linkages and the sequence of the sugar chain of compound **1** were as shown in Fig. 1. Based on the above findings, the structure of compound **1** was established as 3-[*O*-β-D-glucopyranosyl-(1→3)-*O*-β-D-glucopyranosyl-(1→3)-*O*]-[*O*-6-deoxy-α-L-mannopyranosyl-(1→4)-β-D-xylopyranosyl-(1→2)-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranosyl)oxy]-(3β,5α,22α,25*R*)-26-(β-D-glucopyranosyloxy)-22-methoxy-furostane (Fig. 1).

According to the literature, steroidal saponins are shown to possess several physiological properties depending on their chemical structures, such as the capacity for alteration of membrane permeability.[1] Additionally, these compounds isolated from medicinal plants have been reported to have important biological activities, including modulation of metabolic processes, protective activity against induced gastric mucosal lesions and significant effects against liver injury in experimental models.[17,18] Several commercial preparations are made from some species of this genus, which are largely consumed as nutritional supplements, and important beneficial effects on the gastrointestinal physiology are reported for these nutraceuticals, such as the reduction of cholesterol and regulation of triglyceride contents.[19,20] However, the active principles responsible for these physiological benefits are sometimes unknown. In order to explore the utilization of this species in the alternative medicine and with the aim of evaluating the biological activity of compound **1**, we investigated its antiulcerogenic potential, with a preliminary evaluation of its protective effects against induced gastric ulcers using *in vivo* experimental models[21], along with an evaluation of its toxicological potential against membrane integrity in the *in vitro* assays.[22]

The antiulcerogenic activity was evaluated by measuring the inhibition of acute gastric lesions induced by acidified ethanol,[23,24] which tends to dissolve the components of the mucous membrane of the stomach, bringing gastric blood flow to a standstill that contributes to the development of the hemorrhage and necrotic aspects of tissue injury.[25] In an attempt to establish a correlation between spirostane and furostane type structures, the furostanol saponin **1** (AAM-1) isolated from *Agave angustifolia* var. *marginata* was compared to a spirostanol saponin (ABS-2), previously isolated in our laboratory.[26] By macroscopic observations, in the control animals that received only water before acidified ethanol administration, intense and widespread gastric hyperemia and thickened lesions were evident. In contrast, the stomachs of the animals which received compound **1** showed an aspect with significant reduction in gastric hyperemia and in number and severity of lesions. The intensity of gastric ulcers was quantified by the percentage of the injury area in relation to the control group (Fig. 2). This protective action is significantly enhanced in comparison to the reference compound sucralfate, a cytoprotective medication. This result suggests that compound **1** probably interfere with the ulcerogenic mechanism, showing a cytoprotective property. In addition, the steroidal spirostane saponin ABS-2 showed an antiulcerogenic potential slightly higher than compound **1**, in spite of its powerful hemolytic property (Fig. 3).

The mechanisms which protect the gastric mucosa against acute attack by necrotic agents involve a variety of events. Among these, a crucial role is played by mucus production, which is an important protective factor for the gastric mucosa and consists of a viscous, elastic and adherent barrier formed by water and glycoproteins that covers the entire gastrointestinal mucosa. The protective properties of the mucus barrier depend not only on its

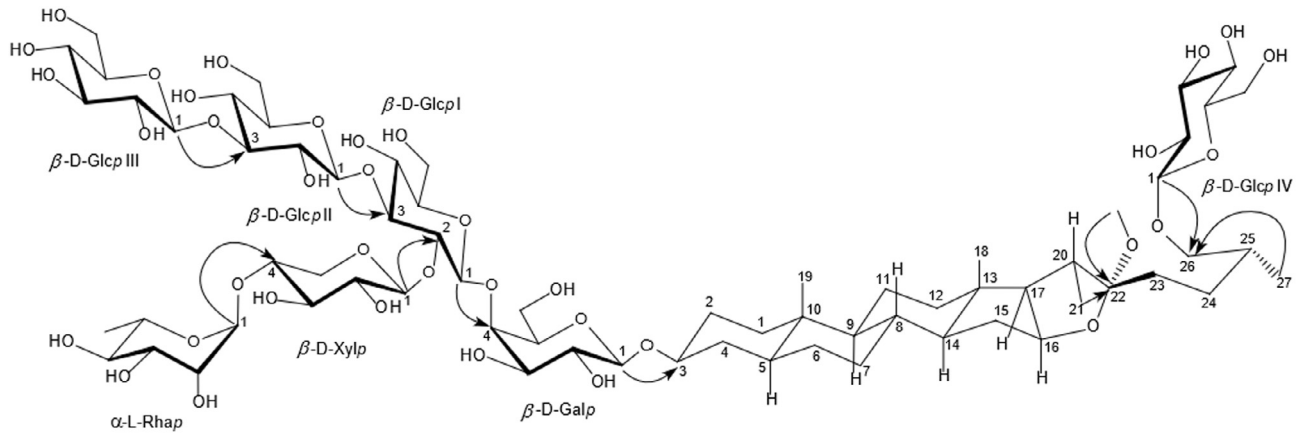

Fig. 1. The structure of compound **1** isolated from *Agave angustifolia* var. *marginata* and its main HMBC (→) correlations.

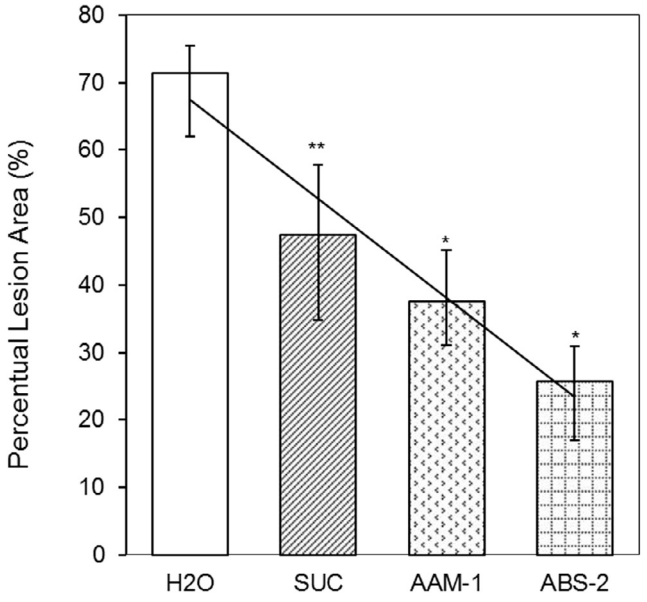

Fig. 2. Antiulcerogenic activity of compound **1** (AAM-1, 100 mg/kg, po) and the reference compounds (ABS-2, 100 mg/kg, po) and sucralfate (SUC, 100 mg/kg, po) against acidified ethanol induced gastric lesions. Results are mean ± S.E.M. (n = 5); $^{*}p < 0.01$, $^{**}p < 0.05$, significantly different from the control group.

structure but also on the amount or thickness of the layer covering the mucosal surface.[27] Since literature reports indicated that steroidal saponins possess the ability to increase the mucosal defensive factors, inducing the turnover of glycoproteins in the mucosal cells, thus increasing the quantity of cellular mucus, probably this can be the mode of action of compound **1**, preventing the penetration of the necrotizing agent or interacting with the macromolecules of the gastric mucosa.[28]

Since antiulcerogenic activity generally is correlated to cytoprotective properties, in order to investigate the effects of compound **1** on membrane integrity, an *in vitro* hemolysis assay was used to evaluate its toxicity, because erythrocytes represent a simplified model system useful in the evaluation of metabolic alterations and oxidative damages.[29] Compound **1** (AAM-1) was evaluated for hemolytic activity and compared with adjuvants commonly used in animal and human experimental models,[30] showing an attenuated hemolytic potential (HD_{50} 275 μg/mL) similar to the commercial adjuvants. In contrast, the spirostane steroidal saponin ABS-2 (HD_{50} 25 μg/mL), previously isolated in our laboratory and used as positive control,[26] demonstrated a highly hemolytic potential (Fig. 4).

Morphological alterations were analyzed by optical microscopy,[31] and the results indicated that treatment of erythrocytes with compound **1** (AAM-1) showed a diminishing membrane disruption potential, demonstrating an attenuated toxicity on membrane integrity even at the highest concentration evaluated

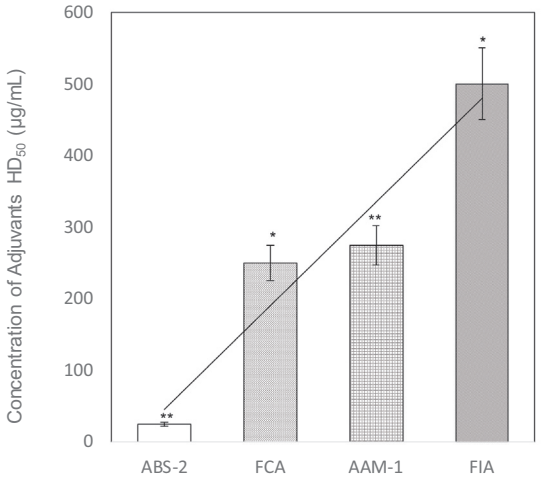

Fig. 4. Hemolytic activity (μg/mL) of compound **1** and commercial adjuvants commonly used in animal and human experimental models. The adjuvant concentration inducing 50% of the maximal hemolysis was considered the median hemolytic dose (HD_{50}; graphical interpolation). Each experiment included triplicates at each concentration. Results are mean ± S.E.M. (n = 5); $^{*}p < 0.01$, $^{**}p < 0.05$ significantly different from the control. Abbreviations: FCA: Freund's Complete Adjuvant; FIA: Freund's Incomplete Adjuvant; ABS-2: *Agave brittoniana* saponin control; AAM-1: *Agave angustifolia* var. *marginata* saponin (**1**).

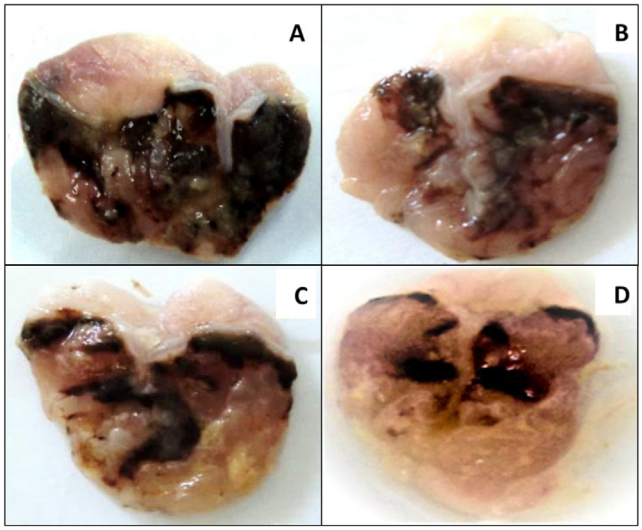

Fig. 3. Effect of oral administrations showing gastric ulcer areas. Images representing macroscopic photographs of the control group (A), sucralfate, 100 mg/kg (B), AAM-1, 100 mg/kg (C) and ABS-2, 100 mg/kg (D). The results are expressed as mean ± S.E.M. (n = 5) and statistical comparison was performed using analysis of variance (ANOVA).

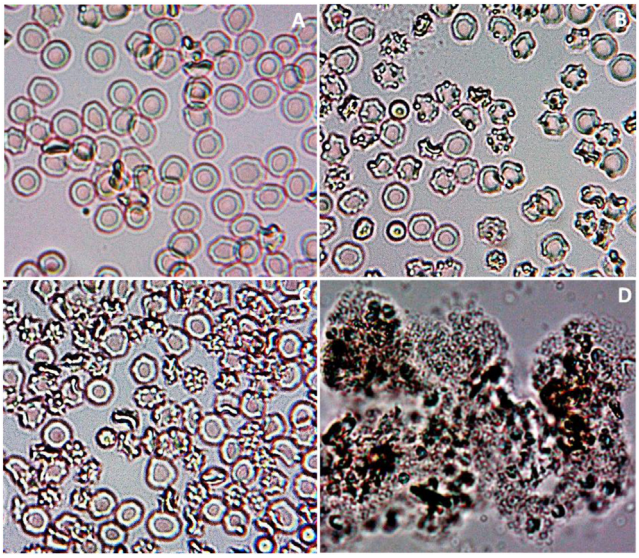

Fig. 5. Effects of the furostanol saponin **1** from *Agave angustifolia* var. *marginata* (AAM-1) and a spirostanol saponin from *Agave brittoniana* (ABS-2) on erythrocyte morphology at the lowest and highest representative concentrations. Optical microscopy analysis, original magnification (A–D) 40x: (A) AAM-1, 10 μg/mL, (B) ABS-2, 10 μg/mL, (C) AAM-1, 1000 μg/mL and (D) ABS-2, 1000 μg/mL.

(1000 μg/mL). In contrast, the spirostane steroidal saponin (ABS-2) showed marked morphological alterations even at lower concentrations (10 μg/mL), since the erythrocytes exhibited many small thorny projections and a shrunken cytoplasm. In addition, several echinocytes were observed. At the highest concentration (1000 μg/mL), it induced powerful lysis, indicating a severe damage and total disruption of the erythrocyte membrane, forming an aggregate of intracellular components, and consequently a cytotoxic potential (Fig. 5). Generally, steroidal saponins possess powerful hemolytic activity because steroids have higher affinities for cholesterol on erythrocyte membranes, as a consequence of its amphipathic structure containing a hydrophobic steroidal nucleus and a hydrophilic carbohydrate moiety. Nonetheless, this is not the case for compound **1**, which demonstrated an attenuated membranolytic activity. This particular behavior can be explained by the assumption that the steroidal saponin **1** possesses sugar units distributed at opposite sides of the aglycon moiety, which considerably reduces its hydrophobicity, resulting in the loss of the amphipathic features.[32]

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21. Laboratory Animals. Male Swiss mice weighing 25–35 g were purchased from Health Sciences Centre, Federal University of Rio de Janeiro, Brazil and acclimatized for 7 days before. Rodent laboratory chow and tap water were provided ad libitum, and maintained under controlled conditions: temp. 24 ± 1o, humidity 50 ± 10%, 12-h light/12-h dark cycle. The protocols were approved by the Animal Research Ethics Committee of the Federal University of Rio de Janeiro. All the procedures were in accordance with the "Principles of Laboratory Animal Care" (National Institute of Health Publication 85–23, revised 1985).
22. Hemolytic Activity. Red blood cells suspension (0.5 mL of 0.5%) was mixed with 0.5 mL of diluent containing 1, 5, 10, 20, 30, 40, 50, 100, 250, 500 and 1000 μg/mL of compound 1 (AAM-1), Agave brittoniana saponin (ABS-2), and 5–500 μg/mL 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 70g for 10 min. The free hemoglobin in the supernatant was measured by absorbance at 412 nm. Saline and distilled water were included as minimal and maximal hemolytic controls, respectively. The hemolytic percents developed by the saline control were subtracted from all groups. The adjuvant concentration inducing 50% of the maximal hemolysis was considered the median hemolytic dose (HD50; graphical interpolation). Each experiment included triplicates at each concentration.
23. Antiulcerogenic Activity. The antiulcerogenic activity was evaluated by measuring acute gastric lesions induced by acidified ethanol. Male Swiss mice in groups of five were fasted for 24 h before the experiment and administered orally with 1 mL of pure water as the negative control or compound 1 (AAM-1, 100 mg/kg), or the Agave brittoniana saponin (ABS-2, 100 mg/kg), or the reference compound sucralfate (100 mg/kg), dissolved in vehicle as positive control. One hour after the treatments, all animals received orally 200 μL of acidified ethanol solution (0.3 M HCl/EtOH) to induce gastric lesions. The animals were killed 1 h after treatment with the ulcerogenic agent and the stomachs removed, opened along the greater curvature and rinsed with physiological saline to determine the lesion damage. The degree of gastric mucosal damage was evaluated from digital pictures using a computerized image analysis system. The percentage of the total lesion area (hemorrhagic lesions) to the total surface area of the stomach was defined as the ulcer index.
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