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Leishmanicidal and trypanocidal activity of *Sapindus saponaria*

[Actividad leishmanicida y tripanocida de *Sapindus saponaria*]

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Abstract: Leishmaniasis and trypanosomiasis are parasitic diseases with a high infection rate, being a serious public health issue in the new world. Unfortunately, there are few available commercial drugs, poorly efficient and with increasing parasite resistance. Under these conditions, there is a need for new molecules to develop new and better drugs. One approach to carry out this search is using traditional medicine as information source to obtain new molecules or extracts to control these parasite diseases. *Sapindus saponaria* (Sapindaceae) fruit resin is used in Colombia to treat ulcers caused by Leishmaniasis. In a bioguided study, we have analyzed the *in vitro* effect of fruit resin, chromatographical fractions from fruit resin and also pure compounds against Leishmania species (*L. panamensis*, *L. braziliensis*, *L. amazonensis* and *L. donovani*) and *Trypanosoma cruzi*. The *in vivo* antileishmanial effect was established under the hamster model for cutaneous leishmaniasis by *L. panamensis*; refined extract of *S. saponaria* and pure saponins displayed high *in vitro* and *in vivo* activity as leishmanicides. In addition, extracts caused low viability on *T. cruzi* amastigotes. The use of the crude extract can be a good alternative against cutaneous leishmaniasis, due to its activity, reduced hemolytic effect, and easy production procedures

Keywords: Leishmaniasis, trypanosomiasis, *Sapindus saponaria*, triterpene saponins.

Resumen: La Leishmaniasis y la tripanosomiasis son enfermedades parasitarias con una alta incidencia, siendo un serio asunto de salud pública en el nuevo mundo. Desafortunadamente, hay pocas drogas comerciales disponibles, con pobre eficiencia y con una creciente resistencia parasitaria. Bajo esas condiciones, se necesitan nuevas moléculas para desarrollar nuevas y mejores drogas. Una aproximación para llevar a cabo esa búsqueda es usar la medicina tradicional como fuente de información para obtener nuevas moléculas o extractos para controlar esas enfermedades parasitarias. La resina de *Sapindus saponaria* (Sapindaceae) se usa en Colombia para tratar úlceras causadas por la Leishmaniasis. En un estudio bioguiado, se analizó el efecto *in vitro* de varios extractos de la resina, sus fracciones cromatográficas y algunos compuestos puros, contra varias especies de Leishmania (*L. panamensis*, *L. braziliensis*, *L. amazonensis* y *L. donovani*) panamensis y *Trypanosoma cruzi*. El efecto lesihmanicida *in vivo* fue establecido usando el modelo en hamster de leishmaniasis cutánea producida por *L. panamensis*; los extractos refinados de *S. saponaria* y las saponinas puras mostraron alta actividad *in vitro* e *in vivo* como leishmanicidas. Además, los extractos causaron una baja viabilidad en amastigotes de *T. cruzi*. El uso de extractos refinados en vez de saponinas puras podría ser una buena alternativa contra leishmaniasis cutánea debido a su actividad, poco efecto hemolítico y procedimientos de producción mucho más fáciles.

Palabras clave: Leishmaniasis, tripanosomiasis, *Sapindus saponaria*, saponinas triterpenicas

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INTRODUCTION

Parasitic diseases caused by protozoan parasites such as *Leishmania* spp and *Trypanosma cruzi* are affecting thousands of people around the world. Leishmaniasis is a prevalent disease in 88 countries worldwide affecting 12 million people with 1.500.000 new cases each year. It also accounts for 500.000 cases of visceral leishmaniasis and 1.500.000 of cutaneous leishmaniasis, and 310.000.000 people at risk of infection (WHO. 2014a). On the other hand, about 7.000.000-8.000.000 million people are infected with *T. cruzi* in 21 Latin-American countries, with approximately 41.000 new cases per year (WHO. 2014b).

The massive displacement of population, weather change, growing resistance to drugs treatment, lack of therapeutic alternatives and long treatments are creating a significative rise in these diseases (Wilkinson & Kelly, 2009; Yasinzai *et al.,* 2013; Alsford *et al.,* 2013) with resulting increases in public health problems mainly regarding to poor.

These diseases are almost neglected due to the low interest by big pharmaceutical companies to search and develop antiparasite compounds in this field, motivated by the small economic landscape that they represent as diseases with prevalence basically in developing countries. However, research on specific treatments against the parasite is a priority. Therefore, is necessary, to search for new drugs from a different perspective to the pharmaceutical industry. Traditional medicine is an important source of information of natural products and research based on this approach can provide both new molecules from promissory secondary metabolites and validation of ethnomedical uses of crude extracts or partially fractionated through chemical methods. Natural product chemistry can offer new more molecules to develop drugs against these disease (Wink, 2012; Schmidt *et al.,* 2012a; Schmidt *et al.,* 2012b; Singh *et al.,* 2014).

Sapindus resin and other type of saponin exhibit several biological activities, such as antihelmintic (Lal *et al.,* 1976), molluscicide (Upadhyay & Singh, 2011), also possess antiprotozoal activity (Diaz *et al.,* 1993). In Colombia, the resin is applied on the wound caused by leishmania (R. Fonnegra, Universidad de Antioquia, Botanical Garden, personal communication). Based on these facts, we set out to look for antiparasite substances from Colombian flora under bioguided studies, to determine the *in vitro* effect of the crude extract, several chromatographical fractions and pure compounds of this plant resin against several *Leishmania* species *(L. panamensis, L. braziliensis, L. amazonensis* and *L. donovani)* and *Trypanosoma cruzi*. In addition, to carry out *in vivo* assays for therapeutical response using the experimental model of cutaneous leishmaniasis in hamster experimentally infected with *L. panamensis*.

MATERIALS AND METHODS

General procedures

NMR spectra were carried out on a Bruker AMX at 300 MHz using Pyridin *d*-5 as solvent, and TMS as internal standard. Chemical shifts (δ) are reported in ppm and the coupling constants (*J*) in Hz. The high resolution mass spectra were obtained in a Waters Micro mass Q-TOF. Infrared spectra were acquired in a FTS 6000 (BioRad), the samples were prepared as KBr tablets. The melting points were taken in open capillaries using a Melt-Temp® (Electrothermal) device and are uncorrected. Column chromatography was carried out on silica gel 60 (200-300 mesh, Merck®) and Sephadex LH-20 (Sigma®), for thin layer chromatography silica gel 60 F_{254} impregnated aluminum sheets (0.25 mm, Merck®) were used. Compounds were detected under UV light (254, 360 nm), spraying with H_2SO_4 (10%) and heating at 110º C.

Plant material and extraction

Sapindus saponaria fruit were collected in Medellín Colombia); a voucher specimen was deposited in the Herbarium at the University of Antioquia (HUA) under the identification code 156643. Dry fruits (3 kg) were extracted with ethanol (96%), then filtered and the solvent evaporated under reduced pressure to dryness.

Fractionation and purification of compounds

The previously prepared crude extract was dissolved in a mixture of n-hexane-CH_2Cl_2-MeOH (2:1:1, v/v) and filtrated. The resulting solution was fractioned on Sephadex LH-20 using the same solvent mixture described above. The fractions were collected and combined according to their polarity profiles by tlc CH_2Cl_2-MeOH (9:1) as follow: non polar compounds (S1), medium polarity (2S, 3S, 4S) and polar compounds (5S). The medium polarity fractions (2S, 3S, 4S) were separated on a chromatographic column packed with silica gel 60 using CH_2Cl_2-MeOH mixture as eluent, 4.5:0.5 v/v, until 1:1. From this step, five new fractions were collected (3S-B, 3S-C, 3S-D, 3S-E, 3S-F); fractions 3S-B and 3S-C were mixed

and separated with repeated chromatographic columns packed with silica gel 60 using CH_2Cl_2-MeOH (9:1) as eluent to give the pure compounds 1A (1.0 g), 2A (0.6 g) and 3A (0.8 g) as white solids. The composition profile of each fraction was checked by tlc, eluting with CH_2Cl_2-MeOH (9:1) and H_2SO_4 spraying with heating at 110 ºC.

Acid hydrolysis of saponin mixture

A mixture of saponins (10 g) was dissolved in 150 mL of methanol and then 100 mL of distilled water were added. Four mL of HCl 0.1 N were added to the mixture and refluxed five hours at 90º C, after this time the methanol was removed and the resulting suspension was neutralized with K_2CO_3. Then extracted with ethyl acetate, the organic layer was dried over anhydrous Na_2SO_4 and the solvent evaporated to dryness, yielding 800 mg of a white solid highly soluble in pyridine, and identified as hederagenin by comparison of the spectroscopical data with those previously reported in the literature (Joshi *et al.*, 1999; Kanchanapoom *et al.*, 2001).

***In vitro* Leishmanicidal activity**

The antileishmanial activity for the first five fractions (1S-5S) and sub-fractions from 3S (3SB-3SF) were tested on axenic and intracellular *L. panamensis* amastigotes, strain (MHOM/87/UA/UA140) transfected with the green fluorescent protein gene (GFP) in agreement with methods previously reported (Varela *et al.*, 2009)

The capacity of these chromatographical fractions in comparison to the meglumine antimoniate (Glucantime®, Sanofi-Aventis de Colombia) to kill axenic amastigotes of *L. panamensis* was determined based on the viability of the parasites evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method as described previously (Taylor *et al.*, 2010). Briefly, parasites were cultivated in Schneider's medium pH 5.4 supplemented with 20% heat inactivated FBS for 3 days at 32º C. Afterwards they were harvested, washed and resuspended at 2 x 10^6 axenic amastigotes/mL in fresh medium. Each well of a 96-well plate was seeded with 100 µL of parasite suspension (in duplicate) and 100 µL of each concentration of the test compound were added. Plates were incubated at 32 ºC. After 72 hours of incubation, the effect of drugs was determined by measuring the activity of the mitochondrial dehydrogenase by adding 10 µL/well of MTT (Sigma) solution (0.5 mg/mL) and incubating at 32 °C for 3 hours. The reaction was stopped by adding a 50% isopropanol solution with 10% sodium dodecyl sulfate for 30 min. Cell viability was determined based on the quantity of formazan, which was measured with a Bio-Rad ELISA reader set at 570 nm. Parasites cultivated in the absence of the compound but maintained under the same conditions were used as controls for growth and viability. The results are expressed as Effective Concentration 50 (EC_{50}) by using a logarithmic regression with the probit method (Finney, 1978). The selectivity index (SI) was calculated by the quotient between citotoxicity on U937 cells and PM and leishmanicidal activity on axenic amastigotes ($SI = LC_{50} / EC_{50}$).

The effect of all fractions and drugs against intracellular amastigotes of *L. panamensis* was evaluated by flow cytometry examination of infected PMA-induced U937 cells incubated in the presence or absence of each fraction and drug (Varela *et al.*, 2009; Pulido *et al.*, 2012). After 72 hours growth PMA-induced U937 cells were exposed to promastigotes of GFP transfectant *L. panamensis* at a parasite:cell ratio of 35:1. The cells with the parasites were incubated for 2 hours 34 ºC, 5% CO_2. Free parasites were removed by washing twice with PBS and fresh medium was added to the infected cells. Twenty-four hours later medium was replaced with fresh medium containing the test compound and infected cells were exposed to the drug for 72 hours. The parasite load in presence and absence of the compounds was calculated from the fluorescence intensities of CD33 and GFP positive infected macrophages. Tests were performed in two separate experiments and each concentration by triplicate. The results are expressed as Effective Concentration 50 (EC_{50}), by using a logarithmic regression with the probit method (Finney, 1978).

The SI was calculated by the quotient between citotoxicity on U937 cells and PM and leishmanicidal activity on intracellular amastigotes ($SI = LC_{50} / EC_{50}$).

Antileishmanial activity of compounds 1A, 2A, 3A and the crude extract of *S. saponaria,* was also evaluated on the promastigote stage of *L. amazonensis* (IFLA/BR/75/PH8), *L. braziliensis* (MHOM/BR/75/M2903) and *L. donovani* (MHOM/74/*PP75*) reference strains (Instituto Boliviano de Biología de Altura) was determined according to the inhibition of parasite growth following the methodology described by Flores *et al.* (2008). The activity was calculated as viability percentage of the

treated parasites against the control. Results are expressed as Inhibitory Concentration 50 (IC_{50}) derived from a typical dose response curve.

In vitro cultures of the promastigote stage of *L. amazonensis, L. braziliensis,* and *L. donovani* strains (Instituto Boliviano de Biología de Altura) were used to test compounds 1A, 2A, 3A and the crude extract of *S. saponaria,* following the methodology described by Flores *et al.,* (2008). Results are expressed as inhibitory concentration 50 (IC_{50}) derived from a typical dose response curve.

In vivo* test using the experimental model of cutaneous leishmaniasis Hamster/*L. panamensis

Therapeutic response and toxicity of compounds were evaluated in the experimental model of cutaneous leishmaniasis (hamster/*L. panamensis*) and their effects were compared to Glucantime®. Outbred adult (8-10- week-old, 140-160 g) Golden hamsters (*Mesocricetus auratus*) of both genders were used in all of the experiments (n = 5 per group). The animals were maintained under standard caging conditions and were provided with commercial rodent food and water *ad libitum.* The experiments were approved by the Ethic Committee for the Animal Experimentation of the University of Antioquia.

Under general anesthesia with Ketamine and Xylazine hydrochloride, the animals were inoculated intradermically in the snout with 100 mL of PBS alone or containing $1x10^6$ promastigotes of *L. panamensis* (MHOM/CO/87UA140) obtained from a 5 days culture. Initiation of treatment schedules took place seven - eight weeks after the inoculation when infection was well established, and lesions were conspicuous (Osorio *et al.,* 2003; Robledo *et al.,* 2005; Flores *et al.,* 2008; Varela *et al.,* 2009). Compounds were solubilized in carboxymethylcellulose and administered to hamsters at 40 mg/kg/d for 10 days orally. A control group received daily carboxymethylcellulose by oral administration. A second control group received and Glucantime® by intramuscular injections at a dose of 40 mg/kg/d for 10 days.

Lesion size was measured prior to treatment and at days 15 and 30 after finished the treatment by measuring the diameter of the inoculated nose with a digital calibrator and expressed in millimeters. Their parasitological stage was determined by culture of material obtained from the lesions in NNN medium. At the end of the trial animals were also bled to quantify serum level of BUN, creatinine and bilirrubin to detect any toxicity associated with the treatment. Animals were monitored during the study to detect any change in behavior or weight. At the end of the experiment hamsters were sacrified using a CO_2 chamber (as recommended by Ethical Committee for Animal Care) and the parasite burden was estimated by a semi-quantitative limiting dilution culture assay. The cervical lymph nodes and snouts were aseptically removed, weighed, and tritured in PBS supplemented with penicillin (100 U/mL), streptomycin (10 mg/mL) and 10% of HIBFS. The tissue samples were adjusted to a concentration of 0.1 mg/mL of Schneider's medium, and 100 µL per well were distributed in flat-bottom microplates containing a layer of NNN medium overlaid with a final volume of 100 µL Schneider's medium. Samples were serially four-fold diluted up to $16,7 \times 10^6$ and incubated for four weeks at 26 °C. Parasite burden was expressed as the maximum dilution in which parasites were detected by examination under an inverted microscope.

Trypanocidal activity

Compounds 1A, 2A, 3A and nifurtimox were dissolved in DMSO and added to a suspension of *T. cruzi* epimastigotes ($3x10^6$/mL, strain MF). Parasite growth was monitored by nephelometry for a time period of 10 days. No toxic effect was observed for DMSO. 100% of viable parasites correspond to absorbance values measured from 570 to 690 nm per milligram of protein in the MTT assay without treated trypomastigotes (Faundez *et al.,* 2005). The activity was calculated as viability percentage of the treated parasites against the control.

***In vitro* Cytotoxic assay on mammal cells**

Cytotoxicity was evaluated for fractions 1S - 5S and 3SB-3SF on the human promonocitic cell line U937 (ATCC CRL-1593.2™) and peritoneal macrophages (PM) from hamsters using a 96 well plate for cell culture as described previously (Taylor *et al.,* 2011). Results are expressed as Lethal Concentration 50 (LC_{50}) calculated by the Probit method and corresponds to the concentration at which 50% of the cell population died (Finney, 1978).

Erythrocyte Hemolysis

O+ human erythrocytes previously collected with CPD (anticoagulant solution) were centrifugated and washed twice with isotonic phosphate buffer (10 mM, pH 7.4) at 500 *g* x 5 min. The pellet was resuspended to 40% v/v with the same washing buffer. For

each compound, 200 µL of each concentration were added to the vial and then 4 µL of cells suspension (40%) were added, the final hematocrit was 0.8%. An isotonic solution (NaCl 150 mM) was used as basal hemolysis control and for maximal hemolysis 1% tween 20 was used. The suspension was incubated at 37º C for 20 minutes. After this time, the samples were centrifugated at 500 g for 5 minutes and the supernatant transferred to a low affinity 96 well plate. The amount of free hemoglobin was determined reading spectrophotometrically at 540 nm (Microplate reader, BIORAD 610). Hemolysis percentage was calculated with the equation % hemolysis = [(Sample Abs - NaCl Abs) / (Tween Abs - NaCl Abs)] * 100. Testing concentrations were 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1.25 µg/mL and 0.625 µg/mL in DMSO.

RESULTS

Compounds structure

The crude extract of *S. saponaria* by thin layer chromatography exhibited a triterpene profile, in addition to presence of high amounts of sugars. Compounds 1A, 2A and 3A (Figure 1) possess the same triterpene moiety. The isolated saponin mixture subjected to acid hydrolysis allowed identification of hederagenin as aglycone, by comparing their spectroscopic data with those previously reported (Joshi *et al.*, 1999; Kanchanapoom *et al.*, 2001).

All compounds were identified through ^{1}H NMR, ^{13}C NMR and experiments COSY ^{1}H-^{1}H, JMOD, HMQC and HMBC. Compound 1A is, therefore, the hederagenin-3-O-(3,4-O-diacetyl-ß-D-xylopyranosyl)-(1-->3)-α-L-rhamnopyranosyl-(1-->2)-α-L-arabinopyranoside previously reported (Murgu & Rodrigues-Filho, 2006); in addition, compounds 1A, 2A and 3A were also isolated from *Gliricidia sepium* (Kojima *et al.*, 1998).

Table 1
Activity on amastigotes of *L. panamensis* of chromatographic fractions from *S. saponaria* crude extract

Treatment	Fraction	LC_{50} (µg/mL) U937 cells	Inhibition % at 50µg/mL
Crude extract fractionation on sephadex LH-20.	Crude extract	47.2	57.0
	2-S	19.7	96.8
	3-S	10.3	76.0
	4-S	67.5	64.3
	5-S	462.2	47.1
Fraction 3S separation on Silica gel 60	3S-B	13.9	97.5
	3S-C	<15.6	92.1
	3S-D	8.2	92.1
	3S-E	152.9	82.1
	3S-F	355.2	58.5
Glucantime®		400	50

The difference in the structure of the compounds is mainly due to the kind of sugar components and the number of acetoxyl groups in the glycosidic chain (Figure 1). For compound 1A, sugar residues follow the sequence Ara-Rha-Xyl (the last diacetylated), saponin 2A has the sequence Ara-Rha-Ara as can be deduced from the multiplicity of the proton at C-4, which appear as a broad singlet instead of a double triplet as it is the case for xylose. In the last sugar component of the sequence of compound 2A, diacetoxylation at C-3 and C-4 is found, according to cross peaks between the protons at these positions and the acetoxyl carbonyls signals. For compound 3A, the sequence and sugar type is the same as in compound 1A, but the difference is the acetylation only at C-4 of the xylose unit.

Hederagenin-3-O-(3,4-O-diacetyl-ß-D-xylopiranosyl-(1-->3)-α-L-rhamnopyranosyl-(1-->2)-α-L-arabinopyranoside (1A)

White solid. mp. 190-192ºC. IR (KBr cm^{-1}): 3448 (broad), 2944, 1741, 1384, 1250, 1250. HR ESI-TOF MS, *m/z* 967.5067 [M + H], calcd for $C_{50}H_{78}O_{18}$ 967.5222 [M + H].

Hederagenin-3-O-(3,4-O-diacetyl-α-L-arabinopyranosyl-(1-->3)-α-L-rhamnopyranosyl-(1-->2)-α-L-arabinopyranoside (2A)

White solid. mp. 193-195ºC. IR (KBr cm^{-1}): 3447 (broad), 2943, 1733, 1384, 1242, 1053. HR ESI-TOF MS, *m/z* 967.5416 [M + H], calcd for $C_{50}H_{78}O_{18}$ 967.5222 [M + H].

Hederagenin-3-O-(4-O-diacetyl-ß-D-xylopiranosyl-(1-->3)-α-L-rhamnopyranosyl-(1-->2)-α-L-arabinopyranoside (3A)

White solid. mp. 195-197ºC. IR (KBr cm^{-1}): 2942, 1734, 1386, 1253, 1053. HR ESI-TOF MS, *m/z* 925.5466 [M + H], calcd for $C_{48}H_{76}O_{17}$ 925.5116 [M + H].

Hederagenin**.** White solid. mp. 333-335, IR (KBr cm^{-1}): 3451, 2943, 1698, 1386, 1037.

Leishmanicidal activity of fractions and isolated compounds from S. saponaria

Activity on L. panamensis amastigotes

The crude extract from ripe fruit of *S. saponaria* was separated in 5 fractions (1S - 5S) through column chromatography using Sephadex LH-20; these fractions were evaluated at 50 µg/mL on *L. panamensis* amastigotes. The crude extract has a significant inhibitory activity (57% of inhibition) and low toxicity, 47.2 µg/mL, (Table 1). Fraction 2S showed a high percentage of inhibition (96.8%) and a LC_{50} of 19.7 µg/mL, fraction 3S has high inhibitory activity (76%) and a LC_{50} of (10.3 µg/mL), whereas 4S has 64.3% of inhibition but is less cytotoxic (LC_{50} 67.5 µg/mL). Fractions 1 and 5 were practically inactive in amastigotes of *L panamensis*.

According to these results and the similarities observed among the fractions by thin layer chromatography, fractions 2S, 3S and 4S were combined. From this mixture five new fractions were obtained (3S-B, 3S-C, 3S-D, 3S-E, 3S-F), almost all of which were over 80% active on amastigotes, being 3S-E the least cytotoxic (152.9 µg/mL, Table 1). However, yields from 3S-E fraction were the lowest and its composition was rich in high amounts of sugars; though, it still contained the same compounds of the other three fractions.

Fractions 3S-B and 3S-C were mixed and after several chromatographical procedures three compounds were purified (1A, 2A, 3A) and their structures assigned by spectroscopical methods. Similarly, fractions 3S-E and 3S-F were also mixed.

Compounds 1A, 2A and 3A, their aglycone hederagenin, and the crude extract were evaluated on axenic and intracellular amastigotes. It was observed that intracellular parasites were more sensitive than axenic amastigotes to the tested compounds (Table 2). At low concentrations, all pure compounds and the crude extracts were highly activity against intracellular amastigotes, but less active against axenic amastigotes; however, cytotoxicity in U937 cells was higher. The small differences in the structure of compounds 1A, 2A and 3A do not change their potency significantly (Table 2), and glycosilation level does not account for an important change in the activity as can be seen when comparing the results with those for hederagenin. Nevertheless, glycosilation must be important in the *in vivo* experiments possibly by increased in absorption.

Activity on several species of Leishmania promastigotes

With the aim to determine the biological effect on several species of *Leishmania* and metabolic stages of the parasites, compounds 1A, 2A and 3A were evaluated (Table 3). *L. donovani* was the less sensitive to the effects of the tested compounds with an IC_{50} value of 68 µg/mL for all pure compounds, but it was slightly more sensitive to the crude extract with IC_{50} 35.3 µg/mL. In the case of *L. braziliensis* and *L. amazonensis* promastigotes, a higher sensitivity to all compounds was detected; though an inexplicable resistance to compound 2 was noticed (Table 3). Apparently, there is no need to get pure compounds, and that a semi-processed extract with less amounts of sugar could be a promissory material to start other experiments related to the *in vivo* effects, and to develop a formulation against leishmaniasis. This is an advantage since complex, expensive and time consuming purification technologies will not be required to get active components.

Table 2
Cytotoxicity and leishmanicidal activity of compounds 1A, 2A, 3A, hederagenin and the crude extract on peritoneal macrophages, and human promonocitic cells U937. Results are expressed as mean ± SD. n = 2

Substance	LC_{50} (µg/mL)		EC_{50} (µg/mL)		IS[a] U937, PM	
	U937	PM[b]	AA[c]	IA	AA	IA
1A	1.3 ± 0.3	15.3 ± 2.2	8.6 ± 2.3	2.5 ± 0.3	0.2, 1.8	0.5, 6.12
2A	2.2 ± 0.4	10.1 ± 1.8	16.6 ± 0.1	2.7 ± 0.6	0.1, 0.6	0.8, 3.7
3A	3.7 ± 0.4	11.1 ± 0.1	10.8 ± 1.1	2.1 ± 0.2	0.3, 1.0	1.8, 5.3
Hederagenin	13.5 ± 3.0	29.6 ± 4.0	18.1 ± 1.5	10.7 ± 1.8	0.7, 1.6	1.2, 2.1
C Ex[e]	26.3 ± 1.7	34.7 ± 5.1	57.7 ± 0.6	14.5 ± 0.8	0.5, 0.6	1.8, 2.4

[a] IS: Index of selectivity = CL50/EC50; [b] PM: peritoneal macrophages; [c] AA: Axenic Amastiogotes: [d] IA: Intracellular Amastigotes; [e] CEx: Crude Extract

Table 3
Leishmanicidal activity on promastigotes of different species of isolated saponins from *Sapindus saponaria*

Substance	IC_{50} (µg/mL)		
Specie/strain	*L. amazonensis* PH8	*L. brazilensis* M2903	*L. donovani* PP75
Crude extract	23.4 ± 0.007	32.0 ± 0.14	35.35 ± 3.88
1A	23.0 ± 0.56	32.0 ± 0.21	68.30 ± 0.49
2A	68.4 ± 0.35	32.4 ± 0.28	68.60 ± 0.14
3A	23.2 ± 0.28	28.1 ± 0.42	66.30 ± 1.34

Amphotericin IC_{50}: 0.2 µg/mL Pentamidine IC_{50}: 10 µg/mL

Table 4
Effect of *S. Saponaria* fractions on the lesion size (snout) and parasite load in the experimental model of cutaneus leishmaniasis (Hamster/*L. panamensis*).

Treatment	Lesion size[a] (mm^2 ± SD)			% reduction lesion [b]	% reduction parasite load
	Preinfection	Pre treatment	30 days post-treatment		
Crude extract (3S)	16.9 ± 0.9	18.7 ± 1.5	20.9 ± 2.9	0	0
3S-E,F	13.6 ± 0.5	16.4 ± 1.8	18.7 ± 3.1	0	14.3
3S-D	14.3 ± 0.8	20.0 ± 4.9	20.0 ± 1.6	0	31.0
CMC[c]	16.2 ± 1.6	21.2 ± 3,4	18.1 ± 5.8	14.6	0
Glucantime®	15.4 ± 0.8	18.6 ± 0.3	15.5 ± 0.4	16.6	100
Control (uninfected/ untreated)	12.5 ± 0.4	13.5 ± 0.3	14.1 ± 0.3	0	NA[d]

a. Lesion size: Area of induration
b. %reduction lesion size = 100 – [(Lesion size 30 days post-treatment/lesion size pre-treatment) x 100]
c. CMC: carboxymethylcellulose
d. Not apply.

Antileishmanial effect in the animal model

The main mixtures of saponins, i.e. chromatogramphical fraction 3S-D, the crude extract and fractions 3S-E-F, were tested in an *in vivo* assay against *L. panamensis*, considering the previously described *in vitro* results and using the experimental model of cutaneous leishmaniasis in the hamster model, according to modifications on the size of the ulcer and the parasite load in the snout.

The *in vivo* results showed that the chromatographical fraction 3S-E-F and the crude extract did not influence the wound evolution caused by parasites; however, a decrease in parasitaemia of 14.3% was observed for 3SE-3SF. Fraction 3S-D delayed the wound evolution (Table 4) and parasitaemia was reduced in 31.0% in comparison to the control. Glucantime® (40 mg/Kg, IM) showed absence of parasites 15 days postreatment and 100% decreased in parasitaemia.

The biochemical assays before and after treatment with compounds indicated the absence of toxic effects with the application of these extracts, indicating probably a resistant strain (Table 5). Compounds 1A and 2A displayed the lowest percentages of viability; compound 1A was the most effective with values of 5.85% (1.44 mg/mL) and 6.21% (0.208 mg/mL) with and without the addition of nifurtimox, respectively. The results were similar for the lowest concentration tested on compound 1A. The activity related to the crude extract was 30.63 and 29.67% (plus nifurtimox) of viability. These results suggest lack of synergism among compounds tested and nifurtimox, because their viability is very similar and we could only see a very small increase in activity when the compounds were assayed together.

Trypanocidal activity of crude extract and saponins 1A and 2A from S. saponaria

Compounds 1A, 2A and the crude extract were tested on *Trypanosoma cruzi* epimastigotes to evaluate their activity. This assay was carried out in two ways; first, using the compounds or crude extract alone at 1.44, and 0.288 mg/mL for compound 1A, 0.208 mg/mL for compound 2A and 2.8 mg/mL for crude extract, and second adding nifurtimox, one of the drugs of choice for the treatment of trypanosomiasis, at the same concentrations of the previously described compounds. When the effect of nifurtimox was evaluated against the parasites, the viability was 78.02%,

Table 5
Effect of crude extract and saponins 1A and 2A on *Trypanosoma cruzi* viability with and without adding Nifurtimox

Compound	% of viability[a]	Concentration (mg/mL)
Control	100,00 ± 0,17	
Nx[b]	78,00 ± 0,06	10 µM
Crude extract	30,63 ± 0,07	2,8
Crude extract + Nx	29,67 ± 0,08	2,8
1A	6,21 ± 0,01	1,44
1A + Nx	5,85 ± 0,01	1,44
2A	9,86 ± 0,01	0,208
2A + Nx	6,92 ± 0,01	0,208
1A	8,13 ± 0,02	0,288
1A + Nx	11,30 ± 0,01	0,288

[a]Mean value + Standard deviation; [b]Nifurtimox.

Hemolysis

With the aim to establish the hemolytic capability of these substances due to their triterpene moiety, the crude extract and compounds 1A, 2A and 3A were evaluated at five concentrations (Figure 2). All compounds caused 100% hemolytic effect at 10 µg/mL, but at the same concentration the crude extract showed a low hemolytic action (4.64%). Compound 1A produced 89% hemolysis at 5.0 µg/mL. This effect dropped when the concentration was decreased to 2.5 µg/mL; at lower concentrations no significant changes in the hemolytic effect were observed, remaining under 4%. This effect could be due to low concentration of these triterpenes in some chromatographical fractions or crude extracts and the presence of high amounts of sugars.

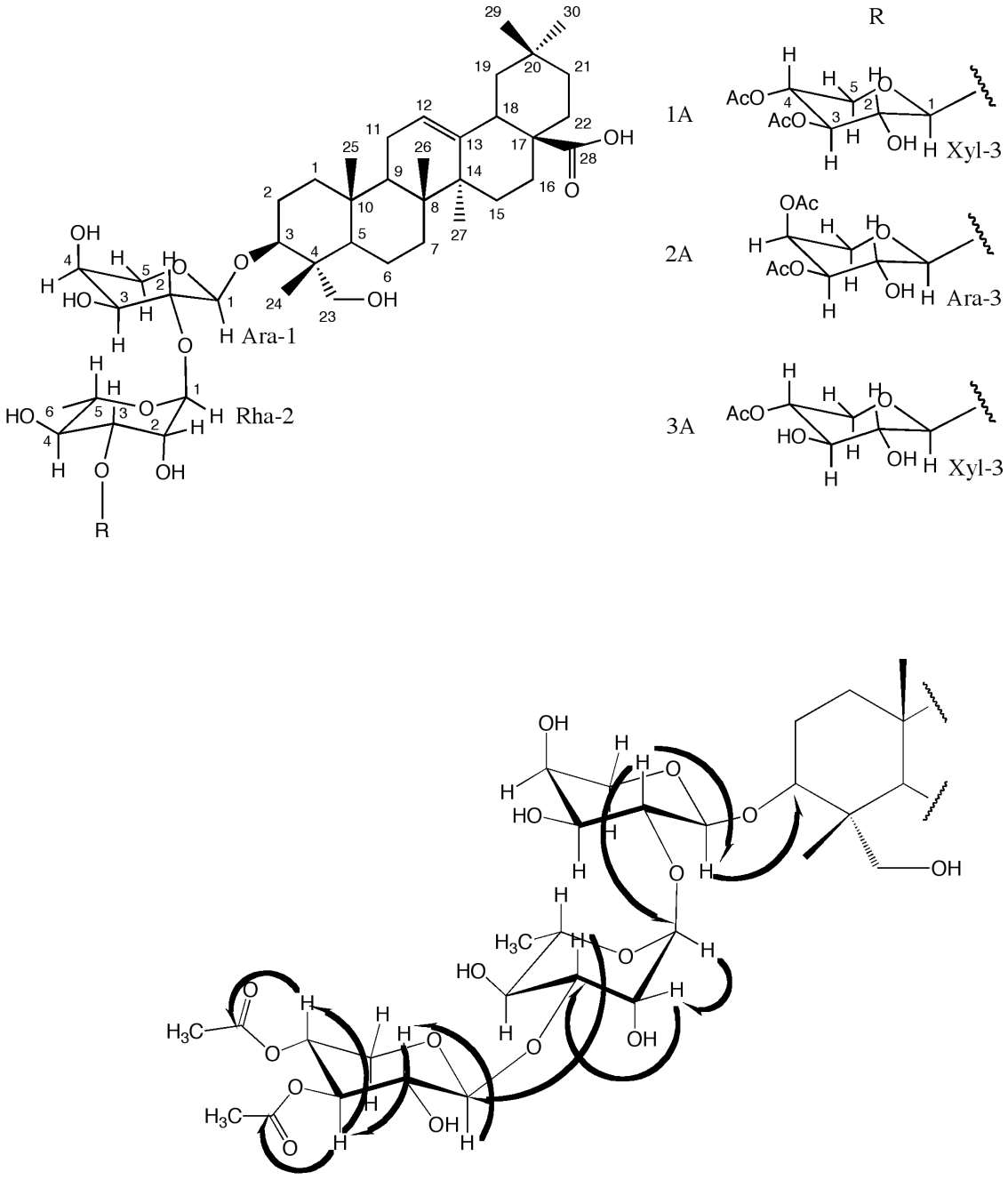
DISCUSSION

Research on natural products is an important source of new molecules with therapeutic potential in the treatment of a broad number of diseases, among them parasitic diseases such as leishmaniasis and trypanosomiasis, which have been neglected (Davis *et al.*, 2004; The Lancet, 2014).

Sapindus saponaria has been reported to be used in stomachal ulcer treatment skin lesions and inflammation (Meyer *et al.*, 2002; Guirado & Orlando, 2005). In Colombia, the resin is applied on the wound caused by leishmania; this biological activity is in agree to this results, since antiparasite activity in crude extracts, fractions and pure compounds has been detected, both *in vitro* and *in vivo* experiments. In addition, from the crude extract of S*apindus saponaria*, three pure triterpene saponins were isolated and their structure was elucidated by spectroscopic methods. These type of molecules are characterized by the presence of several sugar units.

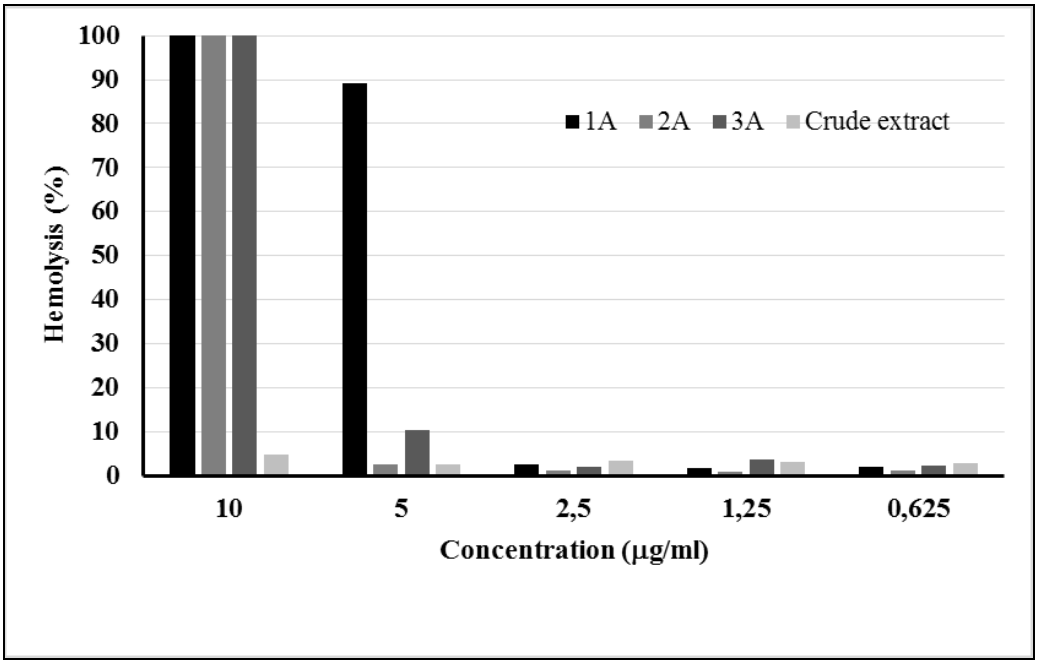
The crude extract, chromatographical fractions and pure compounds have different levels of activity against *L. panamensis* and *T. cruzi*. In preliminary bioassays the crude extract and the chromatographical fractions were found to exhibit good leishmanicidal activity and lower cytotoxicity than the pure compounds. After that, compounds were purified and then assayed against *L panamensis*; all compounds displayed high and similar leishmanicidal activity to those results established with refined extracts. But in fact, these chromatographical fractions are saponins rich mixtures with different ratios and they are susceptible to standardization in two important aspects, their chemical composition and biological activity. The process to purify and obtain only pure compounds is slow and expensive, in contrast to the extracts, but the level of bioactivity of the refined extract is almost the same as that of the pure molecules.

Figure 1
Structure of saponins isolated from *Sapindus saponaria* and HMBC connectivities in carbohydrate moiety



The assays carried out under the animal model gave promissory results, considering that the structure of the compounds has not been optimized neither their concentrations, frequency and administration route. From this study, it is clear that in the time course of the experiments and at the concentration used, no associated toxicity appeared. The crude extract practically has not hemolytic action. Thus, the use of hemolytic saponins could be implemented in the treatment of cutaneous leishmaniasis, using optimized topical formulations, and as such hemolysis is not a pharmacological problem anymore. Other leishmanicidal saponins have been already studied up to very advanced levels, but their studies did not go any further because observed cytotoxicity (Germonprez *et al.*, 2005).

Figure 2
Hemolytic activity of crude extract and compounds 1A, 2A and 3A from *S. saponaria*



On the other hands, significative reduction of *T. cruzi* viability was obtained with application of low concentration of refined extracts from *S. saponaria*. Lower values like 6.21% reduction are caused with application of extract 1A, but did not reduced in a significant way this value when was applied together nifurtimox, a control positive drug. Similar profile of activity was detected with the application of extract 2A. These results were causes despite the presence of a high resistant strain of *T cruzi*, because in this experiment, nifurtimox at 10 mM, only caused a slight reduction in the viability.

Finally, a highlight of this research was the possibility of obtaining refined extracts more than pure compounds, to apply in some diseases, since, as is well known, possibilities like ADME properties and synergism, could be expressed. In addition, chemical purification methods involve a lot of time and budget, to obtain a few milligrams of the active molecule. Moreover, sensitivity and accepted or ruled concentrations

levels to consider a material as promissory are relative to each lab or to each experiment (including culture media and lab conditions), since the behavior parasite strains is sometimes variable (Pink *et al.,* 2006). Besides, the availability of raw material to produce more refined extract or pure compounds is guaranteed, because a tree can produce up to 500 kg of fruit per year, and lately is cultivated in several reforestation programs.

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