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Full Length Research Paper

Inhibition of angiotensin converting enzyme by *Rhazya stricta*, *Moringa peregrina* and *Achillea fragrantissima*, used in traditional system of medicine in Arabian Peninsula: Implication in the management of hypertension

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In recent years, there has been a marked increase in the popularity of medicinal plants derived from the traditional sources of knowledge. This is in congruence to the associated benefits of ease of accessibility and low cost of disease management. Additionally, most of the potent drugs being used currently for chronic diseases are derivatives of natural products. In the current study, the potential of hydro-alcoholic extracts of *Rhazya stricta, Moringa peregrina* and *Achillea fragrantissima* to inhibit angiotensin converting enzyme (ACE) which is involved in the etiology of hypertension and cardiovascular disorders, were examined. The herbal derivatives of these plants have been used for a long time now, in traditional systems of various ethno-pharmacological cultures in Arabian Peninsula and worldwide. The study also evaluated the anti-oxidative properties of these extracts which are reflected by their ability to inhibit DPPH radicals. The phytochemical analysis which focused on the tannin contents of the three extracts demonstrated a direct proportionality between the tannin content and the ACE inhibition activity of the tested extracts in the order: *R. stricta* > *M. peregrina* > *A. fragrantissima.* The results partially support the use of these plants as nutraceuticals and warrant the need for further studies on isolation and characterization of the bioactive constituents of these plants that are responsible for the associated pharmacological property of inhibiting ACE.

Key words: Medicinal plants, hypertension, angiotensin converting enzyme, oxidative stress.

INTRODUCTION

Elevated blood pressure is currently one of the leading risk factors for disease and disability worldwide (Rahimi et al., 2015). Angiotensin converting enzyme (ACE) is a key component in the renin angiotensin aldosterone

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system which is involved in the regulation of blood pressure by cleaving angiotensin I to angiotensin II which is a potent vasoconstrictor (Balasuriya and Rupasinghe, 2011). The enzyme also restricts the vasodilatory and natriuretic properties of bradykinin by catalyzing its degradation to inactive components, thereby further contributing in the physiological manifestation of vascular dynamics of blood flow. Since, the activating cascade of this system is associated with vascular hypertension, ACE inhibition has become a major target for management of hypertension (Hansen et al., 1995). ACE inhibitors prevent the formation of angiotensin II by ACE and thereby reduce peripheral vascular resistance and blood pressure. Although, ACE inhibitors currently available such as captopril are generally effective in reducing blood pressure, their efficacy appear to vary between different ethnic groups and age levels (Brown and Vaughan, 1998).

Moreover, it is being increasingly considered safer to apply nutritional approach that relies on dietary habits and traditional herbal medicines to treat chronic diseases like diabetes and hypertension (Liu et al., 2003; Ullah et al., 2015). It has been reported that more than 50% of drugs used in modern medicine are isolated from herbs or derived from modification of phytochemicals (De Smet, 2002). Recent studies have stated that 75-90% (developing world) and 80% (less developed/developing countries) of the current world population relies on the use of herbal medicines for their primary health care (Robinson and Zhang, 2011; Mehta et al., 2015). Bioactive components of these herbal derivatives such as terpenoids and polyphenolic compounds including flavonoids, hydrolysable tannins, xanthones, procyanidins, caffeolyquinic acid derivatives have been found to be effective as natural ACE inhibitors (Kang et al., 2003; Loizzo et al., 2007).

In the light of the developing interests in the pharmacology of natural products such as ACE inhibitors, the present study has examined three plants with ethno-medicinal significance in Arabian Peninsula for the presence of ACE inhibitory and anti-oxidative properties. The study also demonstrates the presence of tannins as an important molecule which might be responsible for the observed pharmacological properties. Plants such as *Rhazya stricta, Moringa peregrina* and *Achillea fragrantissima* are used in folk medicine for the treatment of various ailments, including chronic and pathogenic diseases like diabetes (Ullah et al., 2015), inflammatory conditions (Ali et al., 1998) and microbial infections (Hammad et al., 2014).

MATERIALS AND METHODS

Materials

Fresh leaves of *R. stricta, A. fragrantissima* and *M. Peregrina* were procured from the local market supplying plants popular for traditional and medicinal value (Tabuk, KSA). These were authenticated by a plant taxonomist from the Department of Biology, Faculty of Science, University of Tabuk. Methanol and ultrapure water were purchased from Thermo Fisher Scientific (Fremont, CA, USA). Tannic acid and 2,2-diphenyl-1-pycrylhydrazyl (DPPH) was delivered by Fluka (USA). Angiotensin converting enzyme from porcine kidney, its substrate N-Hippuryl-L-histidyl-L-leucine hydrate, standard hippuric acid and PBS were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Captopril was purchased from Calbiochem, Germany. All other chemicals and reagents were of analytical grade.

Extraction procedure

The extraction procedure was followed as described by Suarez et al. (2010), with slight modifications. The leaves were rinsed with cold water and dried under shade. Dried plant material was crushed into small pieces in wooden mortar and pestle and further ground to fine dry powder with a blender. A quantity of 300 g dry leaves powder was then soaked in 2 L of 80% methanol in a conical flask placed in a water bath at 40°C for 24 h with constant shaking. The extracted mixture was then filtered through a double layered clean cheese cloth and subsequently through double layered Whatman paper. The filtrate was concentrated under reduced pressure at 35°C using a Bucchi rotavapor R-210 (Switzerland). Finally, the concentrated extract was further placed under vacuum at -30°C for 3-4 days to yield solid/thick pastes. The residual material of *R. stricta, A. fragrantissima* and *M. Peregrina* was weighed 54, 38 and 45 g, providing total yields of 18, 12.6 and 15% (w/w), respectively.

Angiotensin converting enzyme inhibition assay

ACE inhibition assay was carried out as per Hooper et al. (1987) with modifications. In brief, a pre-incubation mixture contained 100 mM Tris-HCl buffer with 300 mM NaCl and 10 μM $ZnCl_2$, pH 8.3/positive control/test sample of various concentrations and 2 mU of ACE enzyme. The reaction mixture was mixed and pre-incubated at 37°C for 10 min. Following pre-incubation, substrate (N-Hippuryl-L-histidine-L–leucine tetrahydrate) was added to a final concentration of 5 mM. The reaction mixture was mixed and incubated at 37°C for 30 min. The reaction was arrested by boiling in water bath for 4 min. A control reaction was also carried out without the test samples. The reaction mixture was centrifuged at 15,000 rpm for 10 min at 25°C. The supernatant was transferred to HPLC vials and subjected to HPLC analysis. The product (hippuric acid) and other components were separated by reverse phase HPLC method using a C18 column. HPLC was performed using Shimadzu Model LC-2010AHT (Shimadzu Corporation, Tokyo, Japan). Briefly, the stationary phase used was an octadecylsilane column (Phenomenex Luna® HPLC column, C18, 5 µm, 250 x 4.6 mm). The mobile phase consisted of gradient mixture of HPLC buffer (A) and acetonitrile (B), which were separately filtered through filter (0.45µm, Pall India Pvt Ltd., India) and degassed by sonication for 3 min. The HPLC column was allowed to equilibrate for 60 min before the start of analysis. Separation was carried out with 10 min isocratic elution (70% A and 30% B). The flow rate of mobile phase was maintained at 1.5 ml/min throughout the analysis and detector wavelength was kept at 230 nm for detection of hippuric acid.

Anti-oxidant activity by DPPH radical scavenging assay

Antioxidants reduce DPPH free radical to 2,2-diphenyl-1-picryl hydrazine, a colorless compound. DPPH assay was carried out as per the method of Vani et al. (1997). In brief, the total reaction mixture contained methanol/positive control/various increasing

Table 1. IC_{50} values of the tested hydro-methanolic extracts for ACE inhibition and DPPH radical scavenging activity.

Extract	ACE Inhibition {IC_{50} (µg/ml)}	DPPH radical Inhibition {IC_{50} (µg/ml)}
R. stricta	3015.83±1.24	417.24±1.63
M. peregrina	3705.78±0.98	67.99±0.85
A. fragrantissima	5254.95±2.78	95.91±0.52

Analysis was done in three parallel determinations. The IC_{50}values were determined by logarithmic regression and are presented together with their respective 95% confidence limits.

concentrations of standard compound/test extract (as shown in the figures) and DPPH to a final concentration of 0.132 mM. The reaction mixture was incubated at 25°C for 20 min and absorbance was read at 510 nm using UV-spectrophotometer. The control reaction was carried out without test extracts which were replaced by appropriate volumes of the vehicle. Ascorbic acid was used as a standard compound (results not shown). Results were presented as % inhibition of radicals (compared to control without any test agent).

Analysis of tannin content

The extract was subjected to analysis for the presence of tannins. Quantitative determination of tannin was done using tannic acid as a reference compound (Makkar, 2000).

Statistical analysis

All experiments were performed in three different sets, with each set in triplicate. The data are expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed for analysis of variance (ANOVA) followed by F-test using SPSS version 11.5 (SPSS, Inc., Chicago, IL). Values of P, which were ≤0.05, were considered significant.

RESULTS AND DISCUSSION

Tested extracts of R*. stricta, M. Peregrina* and *A. fragrantissima* showing progressive inhibition of mammalian angiotensin converting enzyme

As presented in Figure 1B, C and D, the hydro-methanolic extract of the three tested extracts of *R. stricta*, *M. Peregrina* and *A. fragrantissima* showed a dose-response with progressive inhibition of ACE activity proportionate to the increase in the extract concentration. The standard anti-hypertensive drug captopril was used as a reference for the standardization of the assay as shown in Figure 1A. The anti-ACE activity of the three extracts were demonstrated by different inhibition potential as shown in Table 1, which provides the IC_{50} values reflecting the efficiency of the extracts in the following order: *R. stricta* > *M. peregrina* > *A. fragrantissima*. Hypertension is the most common cardiovascular disease and a major health issue in both developed and developing countries. There have been a number of treatment modalities for hypertension that include the use of diuretics, β-blockers, calcium channel blockers and angiotensin II receptor blockers. However, the most common of these are angiotensin converting enzyme inhibitors. ACE inhibitors have wide therapeutic potential in the treatment of heart failure and high blood pressure. Randomized, placebo-controlled trials have shown that ACE inhibitors are effective in lowering blood pressure and in the treatment of cardiovascular dysfunction (Pitt et al., 2000). Recent studies have shown the emergence of a number of plant-derived extracts with potential to provide lead molecules for the synthesis and development of new anti-hypertensive agents (Chaudhary et al., 2015; Gasparotto et al., 2011).

DPPH radical inhibition profile of the extracts showing progressive dose-response curve

Substantive data indicate that reactive oxygen species (ROS) and oxidative stress are important elements of cardiovascular diseases including atherosclerosis, hypertension and congestive heart failure (Sugamura and Keaney, 2011). Low density lipoprotein complexes (LDL) contain lipid species that are subjected to oxidation in the presence of several ROS known to exist in vascular wall (Sorescu et al., 2001). Oxidative modification of LDL is known to be a feature of the atherosclerotic process (Witztum and Steinberg, 2001). In hypertensive patients, angiotensin II increases chronically and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is activated, which causes a rise in ROS (Sharifi et al., 2013). In consideration of these observations, a multi-dimensional approach requires a herbal candidate which has pleiotropic action mechanisms that could target various factors involved in the etiology of cardiovascular disorders. In the experiment presented in Figure 2A, B and C, the antioxidant capacity of the three extracts was evaluated using DPPH radical scavenging assay. As shown in the figure, these extracts led to the progressive scavenging of DPPH radical in a dose-dependent manner. However, it is the *M. peregrina* extract which seems to be idealistic in addition to its ACE inhibition activity, it also possess the most potent anti-oxidative properties as compared to the other two extracts (Table 1).

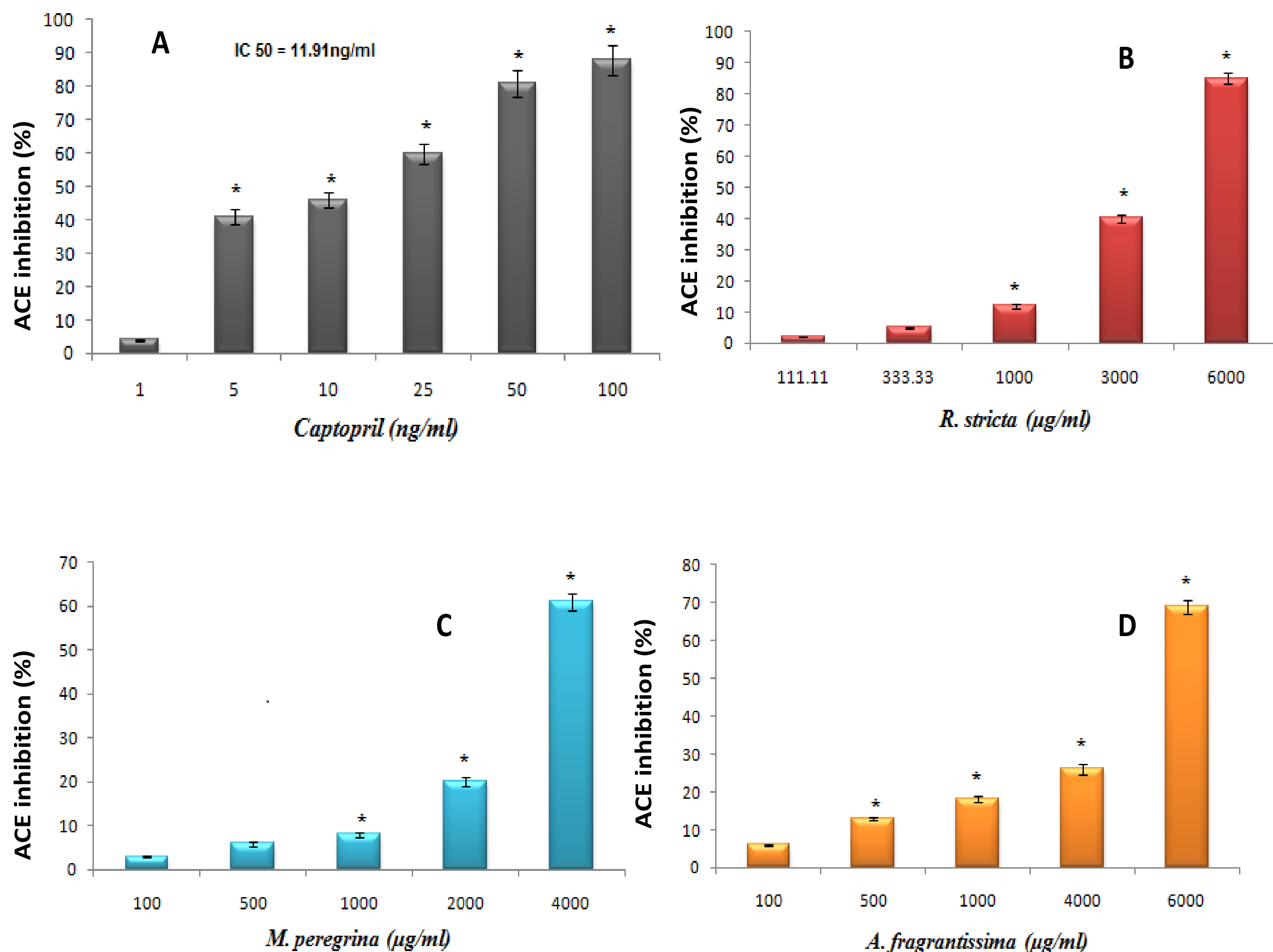

Figure 1. Inhibition profile of mammalian angiotensin inhibiting enzyme by (A) Standard drug captopril and the hydro-methanolic extracts of (B) *R. stricta*, (C) *M. peregrina* and (D) *A. fragrantissima.* Values reported are ± SEM of three independent experiments. **P* ≤ 0.05: significant when compared with the control (in the absence of tested concentrations of extract).

The tannin contents of the extracts correlate with their ACE inhibition activity

Previous studies have reported a positive association of the tannin contents of various plant derivatives and their anti-ACE properties with tannin molecules acting as non-specific inhibitors of the enzyme (Liu et al., 2003). As shown in Figure 3, the tannin content of the three plant extracts were compared and the result demonstrated the highest to lowest trend as: *R. stricta* > *M. peregrina* > *A. fragrantissima.* As mentioned above, a similar trend has been obtained in the ability of the extracts to inhibit ACE, thereby indicating a degree of correlation in the tannin content with the ACE inhibition potential. ACE is a zinc-containing peptidyl dipeptide hydrolase. It is known that the active site of ACE consist of three parts: a carboxylate binding functionality such as the guanidinium group of Arg, a pocket that accommodates a hydrophobic side chain of C-terminal amino acid residues, and a zinc ion. It has been suggested that the anti-ACE activity of polyphenolic compounds might involve the formation of chelate complexes with the zinc atom within the active centre of zinc-dependent metallopeptidases, thereby rendering it ineffective in catalysis (Ojeda et al., 2010).

Conclusion

In the early years of the new millennium, about 26.4% of the world's population suffered hypertension, whereas it has been predicted that this statistic would increase by 60% in 2025 (Kearney et al., 2005). Since the proportion of hypertensive population is expected to increase unprecedentedly, new preventive and therapeutic approaches for management of hypertension are essential. The current

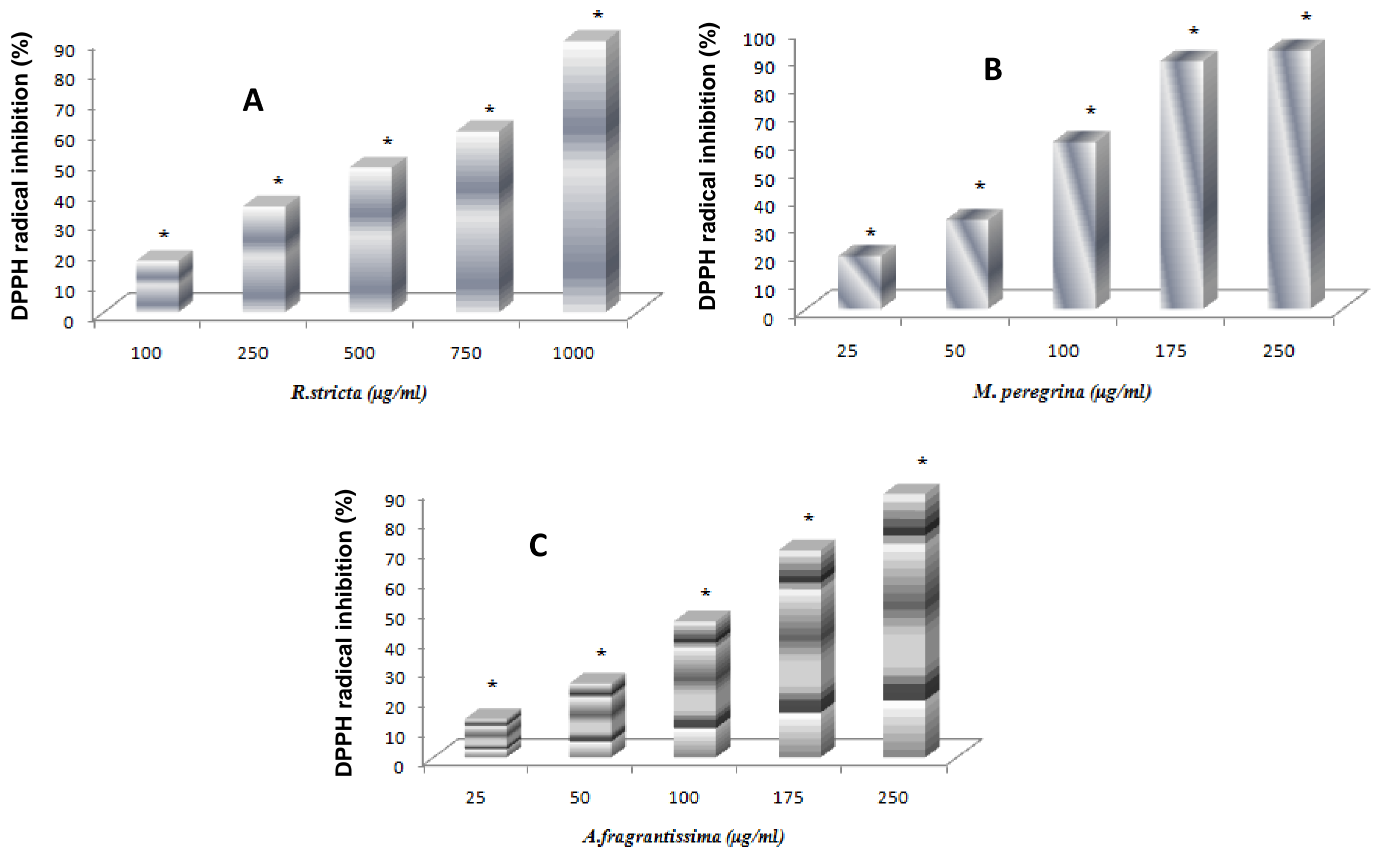

Figure 2. Radical scavenging capacity of the hydro-methanolic extracts of (A) *R. stricta*, (B) *M. peregrina* and (C) *A. fragrantissima*, expressed by its ability to inhibit DPPH radicals. Values reported are ± SEM of three independent experiments. *$P \leq 0.05$: significant when compared with the control (in the absence of tested concentrations of extract).

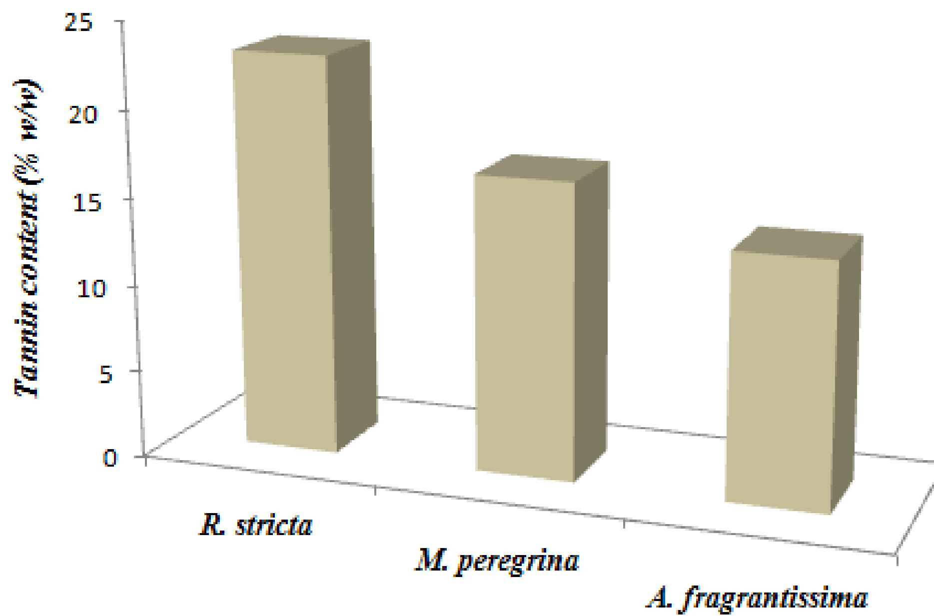

Figure 3. Comparative analysis of the tannin contents of the extracts of *R. stricta*, *M. peregrina* and *A. fragrantissima*.

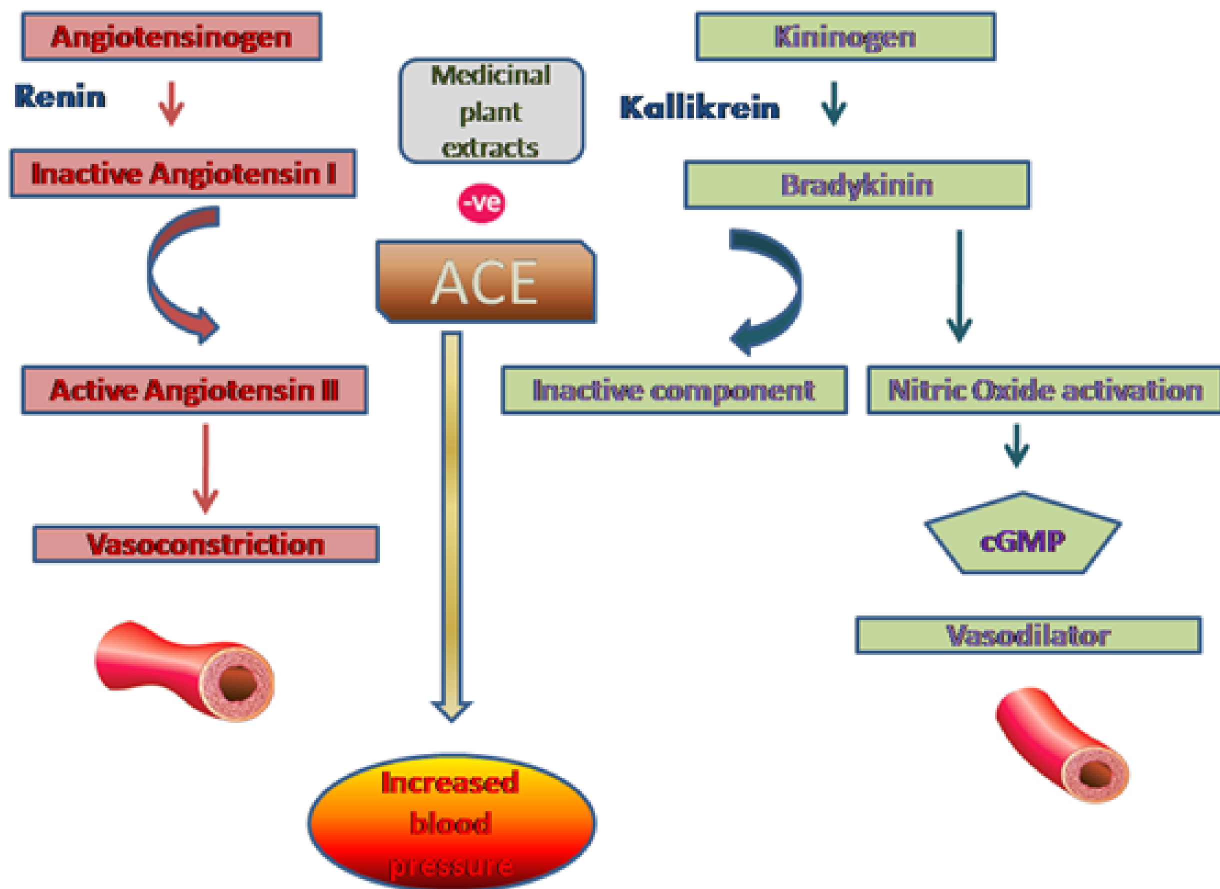

Figure 4. Schematic mechanism of the putative potential of the extracts of the tested medicinal plants in the management of hypertension.

study provides three novel sources of plant-derived ACE inhibitors (Figure 4) which could be further investigated for the isolation and characterization of bioactive molecules responsible for the associated pharmacological properties and which might serve as lead molecules in the development of novel anti-hypertensive drugs.

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Conflict of interest

The authors confirm that this article content has no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Full Length Research Paper

Antiprotozoal activity and cytotoxicity of extracts from *Solanum arboreum* and *S. ovalifolium* (Solanaceae)

Fernanda Londoño[1], Wilson Cardona[1]*, Fernando Alzate[3], Felipe Cardona[4], Ivan D. Vélez[2], Yulieth Upegui[2], Victoria Ospina[2], July Andrea Muñoz[2] and Sara M. Robledo[2]*

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Leishmaniasis, Chagas and Malaria are a major health problem in Latin America. Medicinal plants constitute viable alternatives to conventional medicine in many developing countries. Several *Solanum* species have shown antimicrobial activity, specifically, *Solanum nudum* has shown *in vitro* antimalarial activity. Based on the aforementioned, we determined the antiprotozoal activity and cytotoxicity of alcoholic and non-alcoholic extracts from *Solanum arboreum* and *Solanum ovalifolium.* Extracts were obtained by percolation with solvents of different polarity: hexane, dichloromethane, ethyl acetate and ethanol. All extracts were evaluated *in vitro* for antiprotozoal activity against *Leishmania panamensis*, *Trypanosome cruzi* and *Plasmodium falciparum*. Cytotoxicity was also evaluated. *In vitro* screening showed that dichloromethane (D2) and ethanol (Et2) extracts of *S. arboreum* were the most active against all three protozoa tested here (EC_{50} = 1.2 and 4.6 µg/ml against *L. panamensis,* 13.3 and 2.7 µg/ml against *T. cruzi* and 3.0 and 2.9 µg/ml against *P. falciparum,* respectively). These extract were cytotoxic against human U-937 macrophages (6.1 and 4.4 µg/ml, respectively). Additional studies on toxicity using other cell lines are needed. The activity of the Et2 extract is probably due to the presence of polar compounds such as saponins, flavonoids and coumarins. The activity of D2 extract is probably due to the presence of steroids such as diosgenone. These extracts have potential as a source of compounds for the development of new antiprotozoal therapeutical alternatives and therefore further studies are needed to evaluate and validate the use of the extracts as phytotherapeutics.

Key words: Antiprotozoal activity, antileismanial activity, trypanocidal activity, antiplasmodial activity, *Solanum arboreum, Solanum ovalifolium,* Solanaceae.

INTRODUCTION

Protozoal diseases are a cause of mortality in various developing countries of tropical and subtropical regions. These diseases are significant health problems in endemic countries, this situation is aggravated by increasing treatment failures with available drugs (Bhutta et al., 2014). Leishmaniasis involves a wide spectrum of clinical manifestations, ranging from small cutaneous nodules, plaques or ulcers (cutaneous leishmaniasis) to severe mucosal tissue destruction (mucosal leishmaniasis) or disfunction of vital organs and tissues such as liver, spleen and bone marrow (visceral leishmaniasis). This disease affects more than 12 million

people worldwide and is caused by various species of the *Leishmania* genus, a protozoan parasite that is transmitted to humans through the bite of phlebotominae sandflies of the *Lutzomyia* genus (Alvar et al., 2012). Chagas disease (also named American trypanosomiasis) affects about 10 million people mainly in Latin America. The disease is produced by the protozoan parasite *Trypanosoma cruzi* that is transmitted to the mammalian host through the bite of triatomine bugs belonging to *Triatoma, Rhodnius* and *Panstrongylus* genus (Nouvellet et al., 2015). On the other hand, malaria is a protozoal disease which affects more than 106 countries, affecting 200 million people and causing about one million deaths annually (Bhutta et al., 2014). Human malaria is caused by at least five species of *Plasmodium*, the most important being *Plasmodium falciparum* and *Plasmodium vivax* (World Health Organization (WHO), 2014).

Current chemotherapies are still based on old drugs; pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) to treat cutaneous leishmaniasis; nitroaromatic compounds (benznidazole and nifurtimox) for treatment of Chagas disease or chloroquine, amodiaquine, sulfadoxine/pyrimethamine to treat *P. falciparum* or *P. vivax* malaria, respectively; more recently new Artemisinin-based combination therapy is recommended for the treatment of *P. falciparum* malaria. Unfortunatelly, all of these drugs have several toxic effects on the patients that are associated with high doses and length of therapeutic schemes. Moreover, they are no longer as effective as before due to the emergence of drug resistance in the parasite, which complicates the problem (Chatelain and Ioset, 2011; Den Boer et al., 2011; Keenan and Chaplin, 2015; Fidock et al., 2004).

Medicinal plant species constitute viable alternatives to conventional medicine in a large number of developing countries, especially poor communities that inhabit rural areas lacking access to health services. *S. arboreum* Dunal is a shrub of middle size, unarmed, with obovate leaves, 13 to 40 cm long, glabrous beneath. Inflorescenses are short congested cymes, flowers 4 to 8 mm long, 5-merous, white. Fruits globose berries, 1 cm diameter, green and brown when mature. This is a common species, occurring in most forest from Mexico to Peru. *S. ovalifolium* is a tree, up to 8 m height and 20 cm diameter at breast height (DBH), armed, leaves ovate to lanceolate, pubescent. Inflorescence paniculate, flowers less than 1.5 cm length, sepals green, petals white to lilac. Fruits are green berries. This is an abundant species growing from Venezuela to Peru (Alzate et al., 2012). Habitants of Tumaco (Nariño), on the Colombian Pacific coast, commonly use *S. nudum* Dunal (Solanaceae) to treat fevers and although leaves extracts of this plant have shown *in vitro* antimalarial activity against asexual blood forms of *P. falciparum* (Cardona, 1997; Saez et al., 1998), there are no reports of antiprotozoal activity of the extracts from leaves of *S. ovalifolium* and *S. arboreum*. Although steroids of *Solanum* species are important for their cytotoxicity, it is also known that this cytotoxicity depends on the cell type.

Based on the antiprotozoal activity showed by *S. nundum* and the importance of evaluating other *Solanum* species, this study was aimed to evaluate the leishmanicidal, tripanocidal, anti-plasmodial and cytotoxic activity of extracts from *S. ovalifolium* and *S. arboreum*, with the purpose of contributing to new therapeutic alternatives that could be used against these protozoal diseases.

METHODS AND MATERIALS

Plants

Specimens of *S. ovalifolium* and *S. arboreum* were collected during August and September, 2013, respectively, in Santa Elena (Medellín) and Amalfi, respectively, two municipalities of Antioquia department, Colombia. Leaves were collected for chemical and biological studies. Specimens were identified by F. Alzate **(**Biology institute, University of Antioquia, Medellin, Colombia), voucher specimens are kept at the University of Antioquia Herbarium (HUA) under inclusion numbers 165079 and 183148.

Extraction

The material was dried in an oven at 35°C for 48 h. Powdered leaves of *S. arboreum* and *S. ovalifolium,* 300 and 250 g, respectively, were extracted successively with hexane, then dichloromethane, ethyl acetate, and finally ethanol, in a percolator at room temperature and concentrated in vacuum. The yield obtained for each extract was as follows: hexane (1.92 g, 0.6% and 4.77 g, 1.9% for *S. arboreum* and *S. ovalifolium*, respectively), Dichloromethane (10.93 g, 3.6% and 4.18 g, 1.7%), ethyl acetate (0.80 g, 0.3% and 6.60 g, 2.6%) and ethanol (5.83 g, 1.9% and 27.31 g, 10.9%).

Phytochemical screening

The phytochemical composition of different extracts from *S. arboreum* and *S. ovalifolium* was undertaken. In order to detect the presence of steroids, triterpenoids, phenolics, flavonoids, alkaloids, saponins, anthraquinones and anthocyanosids the method described by Yusuf was adopted (Yusuf, 2014). Coumarins was detected by applying the methods of Matos (Matos, 1997).

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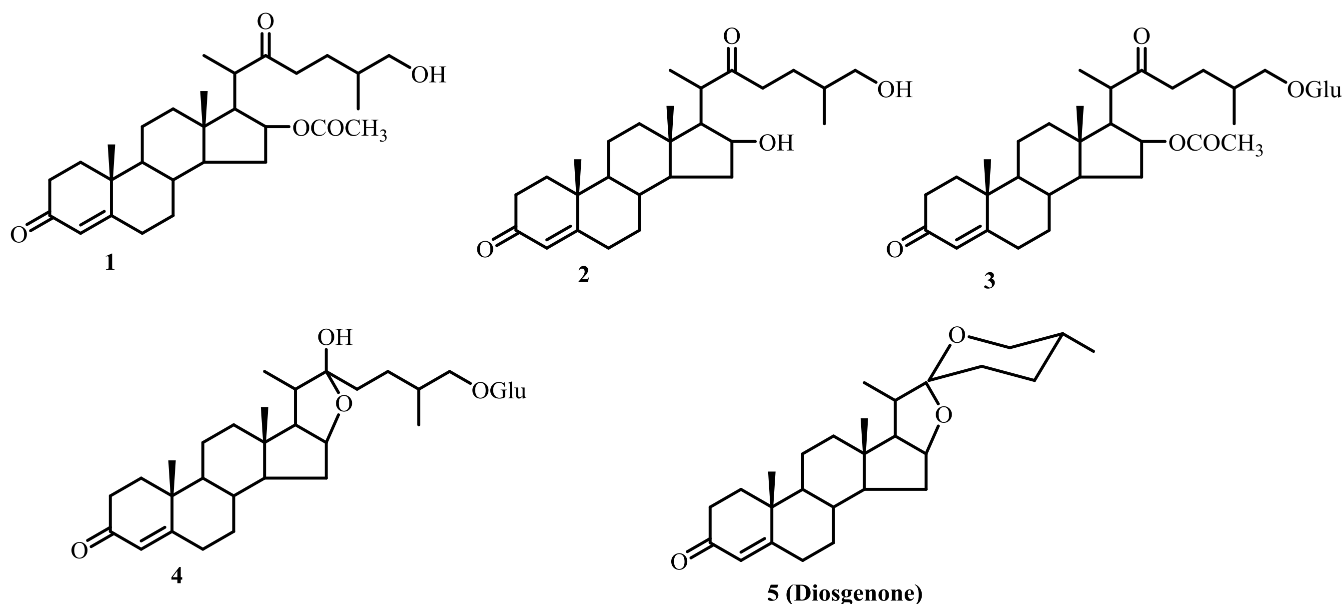

Figure 1. Steroids reported from *Solanum nudum*.

Identification of steroids

All extracts were subjected to analysis by thin layer chromatography using as reference steroids isolated from *S. nudum* [25(R)-spirost-4-en-3-one (1), 16α-acetoxy-26-hydroxycholest-4-ene-3,22-dione (2), 16α,26-dihydroxycholest-4-ene-3,22-dione (3), 16α-acetoxy-26-O-β-D-glucopyranosyloxycholest-4-ene-3,22-dione (4) and 26-O-β-D-glucopyranosyloxy-16α-acetoxycholest-4-ene-3,22-dione (5, diosgenone)] (Figure 1) (Pabón et al., 2009), silica gel as stationary phase, using dichloromethane-methanol (20:1) and hexane-ethyl acetate (8:2) mixtures as mobile phase.

Biological activity assays

The extracts were subjected to *in vitro* evaluation of cytotoxicity on U-937 human cells, antileishmanial and antitrypanosomal activities on intracellular amastigotes of *L. (V) panamensis* and *T. cruzi*, respectively, and antiplasmodial activity on asynchronous cultures of *P. falciparum*.

***In vitro* cytotoxicity**

The cytotoxic activity of the extracts was assessed based on the viability of the human promonocytic cell line U-937 (ATCC CRL-1593.2^{TM}) evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following the methodology described previously (Pulido et al., 2012). Briefly, cells grown in tissue flasks were harvested and washed with phosphate buffered saline (PBS) by centrifuging. Cells were counted and adjusted at 1 × 10^6 cells/ml of RPMI-1640 supplemented with complete 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). One hundred µl were dispensed into each well of a 96-well cell-culture plate and then 100 ml of RPMI-1640 and the corresponding concentrations of the extracts were added, starting at 200 µg/ml in duplicate. Plates were incubated at 37°C, 5% CO_2 during 72 h in the presence of extracts. The effect of extracts was determined by measuring the activity of the mitochondrial dehydrogenase by adding 10 µl/well of MTT solution (0.5 mg/ml) and incubation at 37°C for 3 h. The reaction was stopped by adding 100 µl/well of 50% isopropanol solution with 10% sodium dodecyl sulfate and 30 min incubation. Cell viability was determined based on the quantity of formazan produced according to the intensity of color (absorbance) registered as optical densities (OD) obtained at 570 nm in a spectrophotometer (Varioskan™ Flash Multimode Reader - Thermo Scientific, USA). Cells cultured in the absence of extracts were used as control of viability (negative control), while meglumine antimoniate (Sbv) and amphotericin B (AmB) were used as control for cytotoxicity (non-cytotoxic and cytotoxic drugs, respectively). Assays were performed in two independent runs with three replicates per concentration tested.

***In vitro* hemolytic activity**

The ability to induce hemolysis was evaluated only on those extracts that showed antiplasmodial activity. Briefly, 500 µl of human red blood cells (huRBC), adjusted to 5% hematocrit in RPMI-1640 medium, were placed into each well of a 24-well plate and exposed to four concentrations of each extract (200, 50, 12.5 and 3.125 µg/ml). After 48 h of incubation at 37°C, the concentration of free hemoglobin (as evidence of hemolysis) was determined spectroscopically according to the OD obtained at 542 nm (Varioskan™ Flash Multimode Reader - Thermo Scientific, USA). Non-specific absorbance was corrected by subtracting absorbance of the blank. Determinations were done by triplicate in at least two independent experiments (Conceição et al., 2006).

***In vitro* antileishmanial activity**

The activity of extracts was evaluated on intracellular amastigotes of *L. (V) panamensis* transfected with the green fluorescent protein gene (MHOM/CO/87/UA140pIR-GFP) (Taylor et al., 2011). Effect of each extract was determined according to the inhibition of the infection evidenced by both decrease of the infected cells and decrease of intracellular parasite load. Briefly, U-937 human cells at a concentration of 3 × 10^5 cells/ml in RPMI 1640 and 0.1 µg/ml of

phorbol-12-myristate-13-acetate (PMA) were dispensed into each well of a 24-well cell culture plate and then infected with 5 days-old promastigotes in a 15:1 parasites per cell ratio. Plates were incubated at 34°C, 5% CO_2 during 3 h and cells were washed two times with PBS to eliminate non internalized parasites. One ml of fresh RPMI 1640 supplemented with 10% FBS and 1% antibiotics was added into each well, cells were incubated again to guarantee multiplication of intracellular parasites. After 24 h of infection, culture medium was replaced by fresh culture medium containing each extract at 20 µg/ml or lower (based on the cytotoxicity showed previously by each extract) and plates were incubated at 37°C, 5% CO_2. After 72 h, inhibition of the infection was determined. For this, cells were removed from the bottom plate with a trypsin/EDTA (250 mg) solution; recovered cells were centrifuged at 1100 rpm during 10 min at 4°C, the supernatant was discarded and cells were washed with 1 ml of cold PBS and centrifuged at 1100 rpm during 10 min at 4°C. The supernatant was discarded and cells were suspended in 500 µl of PBS and analyzed by flow cytometry (FC 500MPL, Cytomics, Brea, CA, US). All determinations for each extract and standard drugs were carried out in triplicate, in two independent experiments (Buckner et al., 1996; Pulido et al., 2012). Activity of tested extracts was carried out in parallel with infection progress in culture medium alone and in culture medium with AmB and Sbv as antileishmanial drugs (positive controls). Extracts that showed percentages of inhibition higher than 50% to 20 or fewer µg/ml were then evaluated at four additional concentrations to determine the effective concentration 50 (EC_{50}). Here, infected cells were exposed against each concentration of extracts during 72 h; then, cells were removed and tested by flow cytometry as described before.

In vitro trypanocidal activity

Extracts were tested on intracellular amastigotes of *T. cruzi,* Tulahuen strain transfected with β-galactosidase gene (donated by Dr. F. S. Buckner, University of Washington) (Buckner et al., 1996). The activity was determined according to the ability of the extract to reduce the infection of U-937 cells by *T. cruzi*. Following the procedure previously described, anti-parasite activity was initially screened at a single concentration of 20 mg/ml. In this case, 100 µl of U-937 human cells at a concentration of 2.5×10^5 cells/ml in RPMI-1640, 10% FBS (Fetal Bovine Serum) and 0.1 µg/ml of (Phorbol 12-myristate 13-acetate) were placed in each well of 96-well plates and then infected with phase growth epimastigotes in 5:1 (parasites per cell) ratio and incubated at 34°C, 5% CO_2. After 24 h of incubation, 20 µg/ml of each extract were added to infected cells. After 72 h of incubation, the effect of all extracts on viability of intracellular amastigotes was determined by measuring the β-galactosidase activity by spectrophotometry, adding 100 µM CPRG (Chlorophenol red-β-D-galactopyranoside) and 0.1% nonidet P-40 to each well. After 3 h of incubation, plates were read at 570 nm in a spectrophotometer (Varioskan™ Flash Multimode Reader - Thermo Scientific, USA) and intensity of color (absorbance) was re-gistered as OD. Extracts that showed inhibition percentages higher than 50% were evaluated again at four concentrations selected according to the LC_{50} previously obtained for each extract. Infected cells exposed to benznidazol (BNZ) were used as control for antitrypanosomal activity (positive control) while infected cells in-cubated in culture medium alone were used as control for infection (negative control). Non-specific absorbance was corrected by sub-tracting the OD of the blank. Determinations were done by triplicate in at least two independent experiments (Insuasty et al., 2015).

In vitro Antiplasmodial Activity

The antiplasmodial activity was evaluated *in vitro* on asynchronic cultures of *P. falciparum* (NF54 strain), maintained in standard culture conditions. The effect of each extract over the growth of the parasites was determined by quantifying the parasite DNA stained with ethidium bromide (EtBr) (Insuasty et al., 2015; Insuasty et al., 2013). Briefly, unsyncronized *P. falciparum* cultures were adjusted to 1.5 to 2% parasitemia and 5% hematocrit in RPMI medium enriched with 10% human serum (complete medium). Then, in each well of a 96-well plate, 500 µl of parasite suspension were dispensed and subsequently exposed against 500 µl of four concentrations of extracts (200, 50, 12.5 and 3.125 µg/ml). Dilutions were prepared from a stock solution of 1.000 µg/ml. Chloroquine (CQ) was used as positive antiplasmodial drug control. Parasites unexposed to any compound were used as control of both growth and viability (negative control). Plates were incubated for 48 h at 37°C in N_2 (90%), CO_2 (5%) and O_2 (5%) atmosphere. After incubation, parasite DNA was extracted and purified by using a lysis solution containing proteinase K. DNA was stained with EtBr and then quantified in a spectrofluorometer (Varioskan, Thermo) reading at 542 nm. The intensity of fluorescence in each experimental condition was registered in arbitrary units of fluorescence (AUF). Non-specific fluorescence was corrected by subtracting fluorescence of unstained DNA. Determinations were done by triplicate in at least two independent experiments.

Data analysis

Cytotoxicity was determined according to cell growth (viability) and mortality percentages obtained for each isolated experiment (extracts, AmB, Sbv and culture medium alone). Results were expressed as 50 lethal concentrations (LC_{50}), corresponding to the concentration necessary to eliminate 50% of cells, calculated by Probit analysis (Finney, 1978). Percentage of viability was calculated by Equation 1, where the optical density (OD) of control corresponds to 100% of viability (cell growth).

$$\% \text{ Viability} = (\text{O.D Exposed cells}) / (\text{O.D Control cells}) \times 100 \qquad (1)$$

In turn, mortality percentage = 100 - % viability of each experimental condition.

Antileishmanial activity was determined according to percentage of infected cells and parasite load obtained for each experimental condition by flow cytometer. The percentage of infected cells was determined as the number of positive events by double fluorescence (green for parasites and red for cells) using dotplot analysis. On the other hand, the parasitic load was determined by analysis of mean fluorescence intensity (MFI) (Pulido et al., 2012). The parasitemia inhibition was calculated by equation 2, where the MFI of control corresponds to 100% of parasitemia. In turn, inhibition percentage corresponds to 100 - % Parasitemia. Results of antileishmanial activity was expressed as 50% effective concentrations (EC_{50}) determined by the Probit method (Finney, 1978):

$$\% \text{ Parasitemia} = (\text{MFI Exposed parasites}) / (\text{MFI Control parasites}) \times 100 \qquad (2)$$

Similarly, trypanocidal activity was determined according to the percentage of infected cells and parasite load obtained for each experimental condition by colorimetry. The parasite inhibition was calculated by equation 3, where the OD of control corresponds to 100% of parasites. In turn, inhibition percentage corresponds to 100 - % of Parasites. Results of trypanocidal activity were also expressed as EC_{50} determined by the Probit method (Finney, 1978):

$$\% \text{ Parasite} = (\text{O.D Exposed parasites}) / (\text{O.D Control parasites}) \times 100 \qquad (3)$$

Table 1. Phytochemical screening of *Solanum arboreum* and *S. ovalifolium* extracts.

Extract/compound	Phenols	Alkaloids	Flavonoids	Saponins	Anthraquinones	Anthocyanosids	Triterpenes	Coumarins
Solanum arboreum								
Hexane	-	-	-	-	-	-	+	-
Diclorometane	-	-	-	-	-	-	+	-
Ethyl acetate	+	-	-	-	-	-	+	+
Ethanol	+	-	+	+	-	+	+	+
Solanum ovalifolium								
Hexane	-	-	-	-	-	-	+	-
Diclorometane	-	-	-	-	-	-	+	-
Ethyl acetate	+	-	-	-	-	-	+	+
Ethanol	+	-	+	+	-	+	+	-

Note: + = presence; - = absence

The anti-*Plasmodium* activity of each extract was evidenced by the reduction of the fluorescence (Flu). Indeed, the viability percentage was calculated by equation 4:

$$(\%)\ \text{Viability} = [(\text{FLU})\ \text{of parasites exposed to extracts} - (\text{FLU})\ \text{culture medium}] / [(\text{FLU})\ \text{of parasites unexposed to extracts} - (\text{FLU})\ \text{culture medium}] \times 100 \quad (4)$$

Then, the inhibition growing percentage was calculated according to equation 5:

$$\text{Inhibition}\ (\%) = 100 - \%\ \text{of Viability} \quad (5)$$

The hemolytic activity of tested extracts was evidenced by the percentage of hemoglobin free content calculated according to equation 6:

$$\%\text{hemolysis} = (\text{O.D Exposed cells}) / (\text{O.D Control cells}) \times 100 \quad (7)$$

The cytotoxicity was graded according to the LC_{50} value as high cytotoxicity: $LC_{50} < 100$ µg/ml, moderate cytotoxicity: $LC_{50} > 100$ to < 200 µg/ml, and potentially non-cytotoxicity: $LC_{50} > 200$ µg/ml. Antiprotozoal activity (leishmanicidal, trypanocidal, or antiplasmodial) was graded according to the EC_{50} or IC_{50} values as high activity: $EC_{50} < 20$ µg/ml, moderate activity: $EC_{50} > 20$ to < 50 µg/ml, potentially non activity: $EC_{50} > 100$ µg/ml. The selectivity index (SI) was calculated by dividing the cytotoxic activity and the leishmanicidal activity using the following formula: $SI = LC_{50} / EC_{50}$ or IC_{50}.

RESULTS AND DISCUSSION

Phytochemical screening

Preliminary phytochemical analysis showed a similar composition of secondary metabolites in both species. This analysis revealed the presence of triterpenes in all extracts, while phenols were only present in ethyl acetate and ethanol extracts. Saponins, flavonoids and anthocyanosids were found in the polar extracts and coumarins were detected in the ethyl acetate extracts, in both species. Coumarins were also observed in the ethanol extract of *S. arboreum*. No anthraquinones nor alkaloids were found in any extract. The results are listed in Table 1.

Identification of steroids

Comparative studies by thin layer chromatography of main secondary metabolites of *Solanum nudum* with *S. ovalifolium* and *S. arboreum* extracts are shown in Table 2. The presence of compounds 1 and 2 is observed in the hexane and dichloromethane extracts from *S. arboreum* (H2 and D2, respectively). Diosgenone 5 was detected in dichloromethane extract from *S. ovalifolium* (D1) and hexane, dichloromethane and ethyl acetate extract (H2, D2 and EA2, respectively) from *S. arboreum.*

Biological activities

The effect of extracts on cell growth (viability) was assessed in human macrophages (U-937 cells) which are the host cells for *L. (V) panamensis* and *T. cruzi* parasites. On the other hand, the antiparasite activity of these extracts was tested on intracellular amastigotes of *L. (V.) panamensis* and *T. cruzi* and total forms of *P. falciparum* according to the ability of extracts to reduce the amount of parasites after exposure.

The initial screening identified only extracts D2 and Et2 as having activity against intracellular amastigotes of *L. (V) panamensis* and *T. cruzi* and H2, D2, and Et2 showed activity against total forms of *P. falciparum.* With these extracts the percentage of parasite growth inhibition was higher than 52% (Table 3). Other extracts, H1, D1, Et1, and EA2, showed low activity and therefore were not further evaluated for EC_{50}. The most active extract for intracellular amastigotes of *L. (V) panamensis* was D2 with EC_{50} of 1.2 µg/ml, followed by extract Et2 with EC_{50} value of 4.6 µg/mL (Table 3), while the most active extracts against *T. cruzi* were Et2 with EC_{50} value of 2.7 µg/ml followed by D2 with EC_{50} of 13.3 µg/ml

Table 2. Steroids detection in *Solanum arboreum* and *S. ovalifolium* extracts by thin layer chromatography.

Extract /compound	1	2	3	4	5 (Diosgenone)
H1	-	-	-	-	-
D1	-	-	-	-	+
EA1	-	-	-	-	-
Et1	-	-	-	-	-
H2	+	+	-	-	+
D2	+	+	-	-	+
EA2	-	-	-	-	+
Et2	-	-	-	-	-

Table 3. *In vitro* cytotoxicity and antiprotozoal activity of *Solanum arboreum* and *S. ovalifolium* extracts.

Product	LC_{50} (µg/ml)[a]	INHIBITION (%)[b]			EC_{50} (µg/ml)[c]		
		L.p	T.c	P.f	L.p	T.c	P.f
H1	>200	0.0	42.4 ± 6.5	16.4 ± 2.8	NE[d]	37.2 ± 2.6	NE
D1	89.4 ± 11.1	0.0	45.9 ± 3.3	26.6 ± 1.6	NE	19.1 ± 2.2	NE
EA1	97.7 ± 11.2	19.8 ± 1.5	39.5 ± 1.9	14.4 ± 1.7	NE	NE	NE
Et1	>200	0.0	36.9 ± 2.7	24.3 ± 2.3	NE	NE	NE
H2	35.6 ± 2.1	22.0 ± 0.8	47.5 ± 5.0	52.6 ± 1.8	NE	NE	9.4 ± 0.5
D2	6.1 ± 0.9	67.9 ± 2.4	62.8 ± 4.7[i]	66.9 ± 4.6	1.2 ± 0.3	13.3 ± 2.4	3.0 ± 0.4
EA2	125.0 ± 13.8	15.3 ± 2.6	34.0 ± 5.1	1.9 ± 0.9	NE	NE	NE
Et2	4.4 ± 0.2	52.2 ± 2.9	67.4 ± 9.8[j]	59.4 ± 4.3	4.6 ± 0.8	2.7 ± 0.3	2.9 ± 0.3
SNMet [h]	49.3 ± 7,1	-	-	-	-	-	-
Sb(V)[e]	495.9 ± 55,6	79.4 ± 2.1[f]	NA[m]	NA	6.3 ± 0.9	NA	NA
AmB	42.1 ± 2.0	76.0 ± 3.0[g]	NA	NA	0.04 ± 0.01	NA	NA
BNZ	179.0 ± 4.2	NA	66.3 ± 5.7[k]	NA	NA	1.8 ± 0.2	NA
CQ	0.017 ± 0.002	NA	NA	52.7 ± 8.3[l]	NA	NA	0.02 ± 0.01

Extracts *S. ovalifolium*: H1 (hexane), D1 (dichloromethane), EA1 (ethyl acetate) and Et1 (ethanol); Extracts *S. arboreum*: H2 (hexane), D2 (dichloromethane), EA2 (ethyl acetate) and Et2 (ethanol). Data represent the mean value ± Standard deviation; [a] LC_{50}: Lethal Concentration 50; Cytotoxic extract: LC_{50}<100 µg/ml. No cytotoxic extract: LC_{50} >200 µg/ml. Leishmanicidal active extract: EC_{50} <50 µg/ml. Moderately active extract: EC_{50}<100 µg/ml; No Active extract: EC_{50}>100 µg/ml; [b] % Inhibition at 20 µg/ml; [c] EC_{50}: Effective Concentration 50; [d] NE: Not evaluated because inhibition was below 50%; [e] SbV: pentavalent antimonial meglumine antimoniate. [f] Dose employed: 10 µg/ml; [g] Dose employed: 0.05 µg/ml; [h] *Solanum nudum* methanol extract; [i] Dose employed: 10 µg/ml; [j] Dose employed: 5.0 µg/ml; [k] Dose employed: 10.7 µg/ml; [l] Dose employed: 0.05 µg/ml; [m]Not applicable.

(Table 3). The Et2, D2 and H2 extracts that were active against *P. falciparum* showed IC_{50} of 2.9 µg/ml, 3.0 µg/mL, and 9.4 µg/mL, respectively (Table 3).

Cytotoxicity was observed for H2, D2, Et2 D1, EA1 and EA2 extracts with LC_{50} < 100 µg/ml (Table 3). Lower cytotoxicity was obtained for H1 and Et1 extracts (LC_{50} > 200.0 µg/ml). AmB was cytotoxic (LC_{50} = 42.1 ± 2.0 µg/ml) while Sbv was not cytotoxic (LC_{50} = 495.9 ± 55.6 µg/ml). On the other hand, D2, Et2 and H2 extracts did not produce hemolysis of huRBC at 200 µg/ml (the maximum concentration tested).

When protozoal activity was compared with cytotoxicity, we found that biological activity of D2 was selective for *L. panamensis* (SI 5.1) while D1 and H1 were selective for *T. cruzi* with SI 2.4, and 1.1, respectively (Table 4). H2 was selective for *P. falciparum* (SI 1.7), Et2 was selective for *T. cruzi* and *L. panamensis* (1.6 and 1.0, respectively). The selectivity of H2 was higher than that showed by CQ (1.7 vs 0.34). Although D2 extract showed better activity than SbV, its SI was affected by high cytotoxicity. Similarly, Et2 showed activity comparable to benznidazole, with the SI also being affected by the high cytotoxicity.

Et2 and D2 extracts of *S. arboreum* showed activity against all parasites tested here: *L. panamensis* and *T. cruzi* and *P. falciparum;* the activity of the Et2 extract is probably due to the presence of polar compounds such as saponins, flavonoids and coumarins (Table 1), which have long been recognized to exhibit antiprotozoal activity (Pierson et al., 2010; Maes et al., 2004; Robledo

Table 4. Selectivity of biological activity of *Solanum arboreum* and *S. ovalifolium* extracts.

Extract/control	Selectivity Index (SI)[a]		
	Lp	Tc	Pf
H1	NC[b]	1.1	NC
D1	NC	2.4	NC
H2	NC	NC	1.7
D2	5.1	0.46	0.4
Et2	1.0	1.6	0.3
SNMet[c]	NA[d]	NA	0.9
Sb(V)[e]	78.6	NA	NA
Amphotericin B	1052.5	NA	NA
Benznidazole	NA	99.4	NA
Chloroquine	NA	NA	0.34

[a]SI = LC_{50} / EC_{50} or IC_{50}; NA = NA: Not applicable; [b] NC: Not calculated because EC_{50} was not determined; [c] *Solanum nudum* methanol extract; [d] Not applicable; [e] SbV: pentavalent antimonial meglumine antimoniate.

et al., 2015; Dos santos et al., 2009). The activity of D2 extract is probably due to the presence of steroids such as diosgenone (Table 2). This situation could also explain the antiplasmodial activity shown by H2 extract from *S. ovalifolium*.

Althouhgh the action mechanism of steroids is unknown, it is accepted that these compounds may be initiated at the cell membrane (Haines, 2001), but also via intracellular receptor binding (Krauss, 2001). In addition, steroids may participate in growth regulation, proliferation and cell death (Devkota et al., 2007; Freilich et al., 2000; Pabón et al., 2002) and redox mechanisms (Pabón et al., 2009). These compounds could participate in a conjugated addition of nucleophilic amino acid residues present in target enzymes such as cysteine proteases on leishmania (Mottram et al., 2004) in a Michael type mechanism. This mechanism has been reported for other α,β-unsaturated compounds such as lactones and chromones (Cardona et al., 2014; Otero et al., 2014).

Another study of *S. arboreum* showed that ethanolic extracts of roots and fruits were active against promastigotes of *Leishmania* sp. (OCR with known characteristics) with IC_{50} of 25.8 and 72.5 µg/ml, respectively. Ethanolic extracts of leaves of this plant were not active (Chinchilla et al., 2014). This result is opposite to what we found in this research, which demonstrates the potential antiprotozoal activity of *S. arboreum*. This result suggests that there is no correlation in activity between promastigotes and amastigotes when it comes to different species of parasites, *L. mexicana vs L. panamensis*, which differ in the clinical forms and treatment response (WHO, 2010). Another explanation could be related to the fact that contents of most of the chemical constituents varied significantly with different seasons (Hussain et al., 2008), collection place (Djouahri et al., 2015), and altitude (Zidorn et al., 2005). A comparative analysis between the ethanolic extracts of *S. arboreum* (Et2) tested here (Table 3) and *S. nudum* (SNMet) evaluated by others (García-Huertas et al., 2013) showed that S. *arboreum* had better antiplasmodial activity than alcoholic extracts of *S. nudum* (IC_{50} 17.3 ± 1.3 vs 54.8 ± 8.1 µg/ml, respectively, data not shown). The poor antiprotozoal activity shown by *S. ovalifolium* could be due to the low concentration of secondary metabolites present in the extracts.

Conclusion

Leishmanicidal, tripanocidal, anti-plasmodial and cytotoxic screening of eight extracts from two *Solanum* species are reported. Based on activity observed on intracellular amastigotes of *L. panamensis* and *T. cruzi* and total forms of *P. falciparum* of dichloromethane D2 and ethanol Et2 extracts of *S. arboreum*, suggests that these extracts may be considered as promising in the search for new antiprotozoal compounds. However, additional studies on toxicity using other cell lines are needed in order to discriminate whether the toxicity shown by these extracts is against tumoral or non-tumoral cells.

Conflict of interest

The authors declare no conflict of interest.

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