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ORIGINAL ARTICLE

Trypanocidal Potentialities of Skimmianine an Alkaloid Isolated from *Zanthoxylum pistaciifolium* Griseb Leaves

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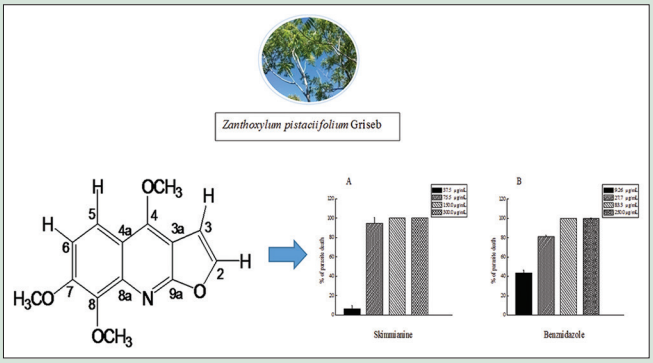
ABSTRACT

Background: *Zanthoxylum pistaciifolium* Griseb (Rutaceae) is an endemic Cuban species traditionally used for respiratory ailment treatments and religious practices. **Objective:** The aim of this study was to evaluate the *in vitro* antimicrobial activity from *Z. pistaciifolium* leaves. **Materials and Methods:** Four phases obtained from the ethanolic extract were tested against bacteria (*Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*), fungi (six *Candida* spp.) and parasites (*Leishmania amazonensis* and *Trypanosoma cruzi*) by microdilution methods. In addition, the cytotoxicity was determined against peritoneal macrophages from BALB/c mice and fibroblast cells L929 using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and resazurin assay. **Results:** The hexane-butanone phase was the most active against *Candida* spp. and exhibited moderate effect on *T. cruzi* trypomastigote (39.3 ± 5.5 μg/mL). The skimmianine isolated from this phase and characterized by nuclear magnetic resonance showed a median inhibitory concentration of 58.0 ± 4.0 μg/mL against *T. cruzi* trypomastigotes and 31.2% ± 6.5% of inhibition on amastigote form at 100 μg/mL. Antiprotozoal activity of this alkaloid was selective with a selectivity index of 9. **Conclusion:** The current study demonstrated for the first time the antimicrobial potential of the leaves of *Z. pistaciifolium*, supporting its ethnobotanical uses. The trypanocidal activity observed for the alkaloid skimmianine could contribute to development future of an herbal medicine for the treatment of Chagas diseases.

Key words: Antifungal activity, hexane-butanone phase, skimmianine, trypanocidal, *Zanthoxylum pistaciifolium*

SUMMARY

- *Zanthoxylum pistaciifolium* Griseb, a Cuban endemic medicinal plant, has a trypanocidal effect. Hexane-butanone phase was the most active by their antifungal activity and moderate effect against *Trypanosoma cruzi*.



Abbreviations Used: NMR: Nuclear magnetic resonance; HBP: Hexane-butanone phase; DP: Dichloromethane phase; BP: Butanol phase; MP: Methanol phase; TLC: Thin-layer chromatography; ATCC: American Type Culture Collection; CFU: Colony-forming units; IC_{50}: Median inhibitory concentration; SI: Selectivity index.

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INTRODUCTION

Natural products have played a significant role in the drug discovery process mainly in the last century. Dissatisfied therapeutic needs in the treatment of infectious diseases have led to the search of new substances with therapeutic applications.[1]

Zanthoxylum pistaciifolium Griseb, endemic of Cuba, known as "palo vencedor," is a tree prevalent in high and dry coasts. The popular literature describes that it is used for aromatic baths,[2] pulmonary infections, and cold.[3] The activity of *Z. pistaciifolium* against different micro-organisms has not been determined; therefore, this study was designed to evaluate the antimicrobial potentialities of its leaves.

MATERIALS AND METHODS

Plant material

Leaves of *Z. pistaciifolium* were collected from "El Palenque" Siboney, Santiago de Cuba, in October 2016. A specimen of the plant was identified and authenticated by the taxonomist Ing. Felix Acosta Cantillo, and a voucher sample was deposited at the Herbarium of the Eastern Center of Ecosystems and Biodiversity (BIOECO, Spanish acronym), Santiago de

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Cuba, identified by number The taxonomic identification corresponded with the specie reported by Greuter and Rankin to be *Z. pistaciifolium* Griseb.[4]

Phytochemical screening

Phytochemical screening was carried out according to the methodology proposed by Harborne[5] with minor modifications. The air-dried leaves were powdered and successively macerated with *n*-hexane, ethanol 95%, and water. The qualitative determination of secondary metabolites was carried out according to previously proposed methods to identify alkaloids, triterpene and steroids, quinones, coumarins, saponins, reducing sugar, carbohydrates, phenol and tannin, amino acids, amines, and flavonoids.[6]

Extraction, isolation, and spectroscopy

An ethanol extract was prepared by percolation method during 9 days at room temperature, using air-dried and powdered leaves (1000 g). The extract obtained was filtered and evaporated under reduced pressure at 40°C until dryness was achieved. Later, this dry extract was extracted with *n*-hexane, to remove fat and waxes, alkalinized (NaOH 1 mol/L until pH 8.5), and finally partitioned. Partitioning was developed using successive extractions with n-hexane-butanone (50:50 v/v), dichloromethane, butanol, and methanol, obtaining the phases: hexane-butanone (HBP), dichloromethane (DP), butanol (BP) and methanol (MP). The remaining residue was discarded. A yellow solid crystalline precipitated obtained from hexane-butanone phase was purified in methanol, yielding 100 mg.

All phases were analyzed to determine their phytochemical composition by thin-layer chromatography (TLC), using *n*-hexane/chloroform/methanol (15:12:5, v/v/v) as a mobile phase for alkaloids.[7] The plates were observed under ultraviolet (UV) light at 365 nm and under visible light after spraying with Dragendorff reagent. The crystals obtained from liquid-liquid partition were characterized by spectroscopic techniques. ^{1}H-nuclear magnetic resonance (NMR) and ^{13}C-NMR uni- and bidimensional spectra were obtained on a spectrometer (Varian, USA), operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C analysis, respectively. As for the two-dimensional NMR experiments, correlation spectroscopy (COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) NMR experiments were performed, revealing ^{1}H-^{1}H, direct ^{1}H-^{13}C, and indirect ^{1}H-^{13}C connections, respectively. Chloroform-d (99.8% D) was used as a solvent, which was purchased from Sigma-Aldrich (St. Louis, MO, USA). Tetramethylsilane (TMS, Sigma-Aldrich, Germany) was used as an internal standard, while chemical shifts (δ) were expressed in ppm and coupling constants (J) in Hertz. NMR data processing was performed with software MestReNova version 6.1.0-6224 for Windows. UV–visible spectra on spectrophotometer (Shimadzu, model UVmini-1240, Japan) with an emission range from 190 to 1100 nm, infrared (IR) spectra in KBr pellets on spectrometer (WQF-510A, FTIR, Rayleigh, China), and melting points on a Fisatom apparatus (model 431D, Brazil) were also determined.

Micro-organism and reference drug

The *in vitro* antimicrobial activity against three bacteria, six fungi, and two parasites was tested. Bacteria and fungi cultures were obtained from the culture collection of Laboratory of Microbiology, Parasitology and Hygiene of the University of Antwerp, Belgium. The antifungal activity was evaluated against *Candida albicans* ATCC B59630 (azole resistant), *Candida glabrata* ATCC B63155, *Candida kefyr* ATCC B46120, *Candida krusei* ATCC B68404, *Candida parapsilosis* ATCC J941058, and *Candida tropicalis* CDC49. The employed bacteria were *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, and *Pseudomonas aeruginosa* ATCC 9027. All cultures and assays were conducted at 37°C. Miconazole and ampicillin (Sigma-Aldrich, USA) were used as reference drugs for fungi and bacteria, respectively. Parasites were the MHOM/77BR/LTB0016 *Leishmania amazonensis* strain provided by the Institute of Tropical Medicine "Pedro Kourí," Havana, and *Trypanosoma cruzi* (Y and Tulahuen strains) supplied by the Laboratory of Cellular Biology, Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro, Brazil. Pentamidine (Sigma-Aldrich, St. Louis, MO, USA) and benznidazole (LAFEPE, Brazil) were used as a reference drug for *Leishmania* and *Trypanosoma,* respectively.

In vitro antibacterial and antifungal activity

In vitro antibacterial and antifungal activity was determined by the microdilution method with resazurin (redox indicator, at 50 μg/mL) (Sigma-Aldrich, Germany) in sterile 96-well microplates (Costar, USA).[8] Stock solution of phases was prepared in 100% dimethyl sulfoxide (DMSO, BDH, Inglaterra) at 1 g/mL. In each well, 10 μL of the two-fold water compound dilutions (0.25–4 mg/mL) was added together with 190 μL of bacterial inoculum (5×10^5 CFU/mL) and fungal inoculum (5×10^3 CFU/mL). In the microplates were included untreatedcontrol wells (medium plus cells,100% cell growth) and control wells with medium without cells (0% cell growth). Then, the microplates were incubated at 37°C for 17 h (for bacteria) and 24 h (for fungi). Afterward, 20 μL of resazurin at 50 μg/mL per well (for bacteria) and 10 μL (for fungi) was added. The microplates were incubated under the same temperature conditions (bacteria: 30 min and fungi: 4 h). Microbial growth was determined by fluorimetry method (λ_{ex} = 550 nm, λ_{em} = 590 nm) using a microplate reader (TECAN GENios, Germany). The phases were classified as active, if the bacterial growth inhibition (%) was >50%. The results are expressed as a percentage reduction in bacterial growth/viability compared to control wells. Each experiment was performed by triplicate, and the results were expressed as the mean ± standard deviation.

In vitro antileishmanial activity

Stock solution of phases was diluted in 100% DMSO at 20 mg/mL, and serial dilutions were made to obtain final concentrations between 12.5 and 200 μg/mL. In each well of the 96-well microplate, 2 μL of compound dilutions was added to 98 μL of Schneider's medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) and antibiotics. Afterward, 50 μL of promastigotes was added at 4×10^5 parasite/mL in logarithmic phase. Microplates were sealed with Parafilm and incubated at 26°C for 72 h. Then, 20 μL of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) at 5 mg/mL was added in each well. An additional incubation of 4 h was performed, the supernatant was eliminated, and the formazan crystals were dissolved with 100 μL of DMSO. Finally, the microplate was read in an ELISA microplate reader (Sirio S Reader, 2.4-0, Italy) at 540 nm and 620 nm as reference wavelengths.[9] The median inhibitory concentration (IC_{50}) was determined using SPSS v. 19 software (IBM, Armonk, NY, USA) by lineal dose–response regression analysis. Each experiment was performed by duplicated, and the results were expressed as the mean ± standard deviation.

In vitro trypanocidal activity

Anti-trypomastigote assay

Trypomastigotes forms (Y strain, *T. cruzi*) were incubated in 96-well microtiter plates. One hundred microliter of suspension in RPMI (Gibco, France) and 5% FBS (Invitrogen Corporation, UK) containing 10^7 parasites/mL was added to an equal volume of samples (phases and skimmianine) (diluted into RPMI + 5% FBS) at twice the desired final concentration (from 0 to 300 μg). After 2 and 24 h of treatment at 37°C,

the number of living parasites was determined by microscopy light quantification in a Neubauer chamber. Controls were performed with parasites kept under the same conditions in the absence of treatment. All the experiments were run in triplicate, and the activity was expressed by calculating the IC_{50} value for 2- and 24-h treatment. IC_{50} values were calculated based on the drug concentration that reduced 50% of parasites using SPSS v. 19 software by linear regression.

Anti-amastigote assay

The trypomastigotes of Tulahuen used in this step were obtained from L929 cell lineages (ATCC, CCL-1, originated from a C3H mouse, which were provided by Dr. Maria de Nazaré Correia Soeiro of Laboratory of Cellular Biology, IOC, Fiocruz) with tissue culture-derived trypomastigotes (Tulahuen strain expressing the *E. coli* β-galactosidase gene), following previously established protocols.[10] In a 96-well plate, 4×10^3 cells/well (L929) was seeded in 80 µL RPMI medium (Gibco, BRL) supplemented with 10% FBS, 2% L-glutamine, and phenol red. The plate was incubated "overnight" in an oven at 37°C and 5% CO_2. Cells were infected with 4×10^3 trypomastigotes/well, and 20 µL was diluted in RPMI supplemented with phenol red. The plate was incubated at the same conditions, and after 2 h, the free parasites were removed without phenol-red supplemented RPMI medium. The plate was incubated under the same conditions for 48 h until infection was stablished. Afterward, the culture medium was exchanged for 180 µL medium supplemented RPMI without phenol red plus 20 µL of phase (at 10 µg/mL) or skimmianine (up to 100 µg/mL). The following controls were used per plate: uninfected cells without addition of compounds, infected cells without addition of compounds, cells infected and treated with benznidazole, and infected cells treated with DMSO 1%. The plate was incubated at 37°C and 5% CO_2 for 96 h. Following this period, 50 µL/well of chlorophenol red-*β*-D-galactopyranoside (Sigma-Aldrich, St. Louis, MO, USA) substrate was added, and the plate reading was performed after 16–20 h in ELISA reader (VersaMax tunable microplate reader; Molecular Devices, USA) with 570 nm filter.[10] Benznidazole at concentration of 2.6 µg/mL was employed as a reference drug.

Cytotoxicity assay

Cytotoxicity assays were performed in parallel to the above antiparasitic assays in order to assess their selectivity. The cytotoxicity effect of phases was assessed on peritoneal macrophages from BALB/c mice (for anti-*Leishmania* assay) and L929 cells from fibroblast mice (for anti-*Trypanosoma* assay) using the tetrazolium dye (MTT, Sigma, St. Louis, MO, USA) colorimetric method and resazurin assay, respectively. Isolated compound was evaluated with resazurin assay on L929 cells.

Peritoneal macrophages: Resident macrophages from the peritoneal cavity of healthy BALB/c mice were collected and washed with RPMI 1640 medium (Sigma, St. Louis, MO, USA), supplemented with antibiotics (penicillin 200 IU and streptomycin 200 µg/mL). Later, $1–3 \times 10^6$ macrophages/mL was seeded in 24-well plates (Costar, USA) and incubated for 2 h at 37°C in 5% CO_2 atmosphere. The nonadherent cells were removed by washing with phosphate buffer solution. Subsequently, RPMI medium and phases at the tested concentrations (12.5–200 µg/mL) were added per well. The plates were incubated for 72 h under the same conditions, and macrophages treated with DMSO were included as a control.[9] The cellular viability was determined by colorimetric assay with MTT (15 µL MTT solution added per well) as described above. Median cytotoxic concentration (CC_{50}) was determined by dose–response linear regression analysis. Each experiment was performed in duplicated, and the results were expressed as the mean ± standard deviation.

L929 cells: Monolayers of mice L929 fibroblasts were cultivated (4×10^3 cells/well in 96-well microplates) at 37°C in RPMI 1 640 medium (pH 7.2–7.4) without phenol red supplemented with 10% FBS and 2 mM glutamine (RPMIS). Uninfected cultures (L929 cells) were exposed to the compounds, and the cytotoxicity level was evaluated using alamarBlue (Thermo fisher, Brazil) reagent. The microplates were incubated during 96 h at 37°C with different concentrations (diluted in RPMI without phenol red) of phases and the isolated compound (up to 1200 µg). Morphology was evaluated by light microscopy. In each well, 10 µL de resazurin was added and the microplates were incubated for 5 h. Finally, the cellular viability was determined by colorimetric bioassay at reference wavelengths of 570 and 600 nm.[10] As negative controls, L929 cells in RPMI medium and RPMI containing each tested compound were included. The results were expressed as the percent differences in the decreases between sample-treated and vehicle-treated cells. The level of CC_{50} was determined by dose–response linear regression analysis.

Selectivity index

A calculated selectivity index (SI) allowed examining the relationship between cytotoxicity and a chosen activity. The SI was then calculated by dividing the CC_{50} (for macrophages or L929 cells) by the IC_{50} (for protozoan).[11] Phases or compounds with a SI ≥10 were considered as selective.[12]

RESULTS

The three extracts generated, i.e., n-hexane, ethanol 95%, and water, demonstrated the common presence of alkaloids, tannins, and flavonoids but with higher abundance in the ethanol extract. Coumarins, quinones, reducing sugars, and triterpenes/steroids were other chemical principles detected. Considering these results, the extract selected to be fractioned was the ethanol extract.

The extract on ethanol 95% yielded 60.1 g of soluble substances after dryness under reduced pressure. Alkaloid fractioning process yields a total of four phases: HBP (18.98 g), DP (7.16 g), MP (2.41 g), and BP (0.65 g). The HBP showed intense positive evidence for alkaloid and phenols qualitatively, followed by DP and BP, whereas MP was negative. Other detected metabolites were flavonoids, coumarins, quinones, triterpenes, and steroids.

Consequently, HBP and DP were analyzed on TLC plates. Six spots were detected, one of them clearly in majority with an intense fluorescence blue and positive responses to the Dragendorff reagent,

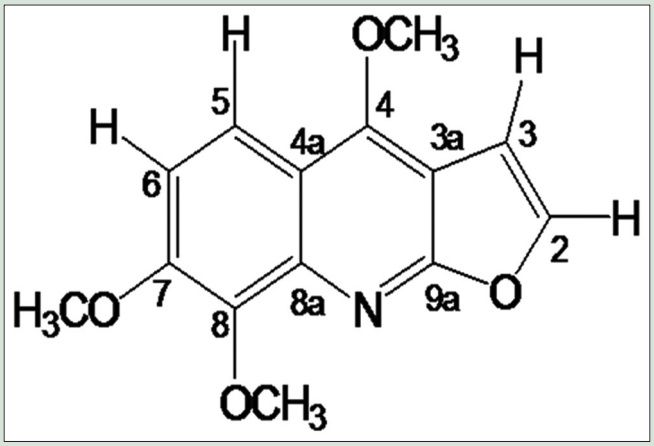

Figure 1: Chemical structure of skimmianine

for the HBP. As a result of the purification, the skimmianine alkaloid was obtained and recrystallized in methanol from HBP (113.2 mg). Its structure was determined by UV, IR, ^{1}H, and ^{13}C NMR spectra and finally compared to data from existing literature[13,14] [Figure 1].

Skimmianine appeared as colorless prism crystals, m.p. 178–179°C.[13] UV λ_{max} nm (log ε), MeOH: 246 (4.9), 320 (4.08) 330 (4.09). IR (KBr): 3147, 3118, 3068, 3006, 2981, 2944, 2835, 1616, 1577, 1490, 1092 cm^{-1}. ^{1}H-NMR (500 MHz, $CDCl_3$) δ_H: 7.89 (1H, d, *J* = 9.4 Hz, H-5), 7.47 (1H, d, *J* = 2.8 Hz, H-2), 7.13 (1H, d, *J* = 9.4 Hz, H-6), 6.92 (1H, d, *J* = 2.8 Hz, H-3), 3.95 (3H, s, 7-OCH_3), 4.04 (3H, s, 8-OCH_3), 4.31 (3H, s, 4-OCH_3); ^{13}C-NMR (125 MHz, $CDCl_3$) δ_C: 164.3 (C/C-9a), 152.1 (C/C-7), 157 (C/C-4), 142.9 (CH/C-2), 141.8 (C/C-8), 141.3 (C/C-8a), 118.2 (CH/C-5), 114.7 (C/C-4a), 111.9 (CH/C-6), 104.6 (CH/C-3), 101.9 (C/C-3a), 56.7 (7-OCH_3), 61.6 (8-OCH_3), 58.9 (4-OCH_3). HSQC ^{1}H-^{13}C ^{1}J (125 MHz, $CDCl_3$) δH/δC: 6.92/104.6, 7.13/111.9, 7.89/118.2, 7.47/142.9, 3.95/56.7, 4.04/61.6, 4.31/58.9. HMBC ^{1}H-^{13}C ^{n}J (*n* = 2,3) (125 MHz, $CDCl_3$) δH/δC: 4.04/141.8 (^{3}J), 3.94/152.1 (^{3}J), 4.31/157 (^{3}J), 7.88–141.23 (^{3}J)/152.07 (^{3}J)/157,10 (^{3}J), 7.47–101.83 (^{3}J)/104.60 (^{2}J)/164.28 (^{3}J), 7.13–114.76 (^{3}J)/141.85 (^{3}J)/152.11 (^{2}J), 6.92–101.88 (^{2}J)/142.84 (^{2}J)/164.29 (^{3}J). COSY ^{1}H-^{1}H ^{1}J (500 MHz, $CDCl_3$) δ_H/δ_H: 7.89/7.13,7.47/6.92.

The inhibitory effect on the micro-organisms (bacteria and fungi) was in the range from 250 μg/mL until 4000 μg/mL. The activity was observed mainly on non-polar phases, while the highest levels of concentration were observed on the polar phases [Table 1].

The phases showed inhibitory effect on six of the evaluated microbial strains, especially against *Candida* spp. HBP and DP demonstrated antifungal activity against *C. albicans*, *C. kefyr*, *C. krusei,* and *C. parapsilosis* at concentration levels below 250 μg/mL, being less active against bacteria. The most susceptible micro-organisms were *C. kefyr*, *C. glabrata, C. krusei,* and *S. aureus*, whereas *C. tropicalis* and *E. coli* were resistant to all phases.

The antileishmanial and trypanocidal activity, cytotoxicity, and SI of the phases are shown in Table 2. The DP shows moderate inhibitory activity, with an IC_{50} of 54.4 μg/ml against *L. amazonensis*, whereas HBP, BP, and MP were inactive.

When macrophages were treated with MP and BP, the CC_{50} was higher than 200 μg/mL; therefore, it did not exhibit cytotoxicity. In contrast, for DP and HBP, the CC_{50} was 45.2 and 122.36 μg/mL, respectively [Table 2]. The SI was calculated using the IC_{50} and CC_{50} (for peritoneal macrophages) values obtained from antileishmanial *in vitro* assay. The SI was calculated for the most active phase (DP) and classifies as non-selective due to the small difference between the active and toxic concentrations.

Table 2 shows the activity against trypomastigotes and amastigotes of *T. cruzi*. The highest activity was found in the HBP against trypomastigote form with IC_{50} value of 39.3 μg/mL at 24 h. A SI higher than two was observed after 24 h of treatment but still insufficient regarding the standard protocols. On the other hand, the activity against intracellular form of parasite (Tulahuen strain) was weak for all the phases, since the growth inhibition was <50%. Nevertheless, once again the HBP was the most active.

Considering the isolated compound (skimmianine), an IC_{50} of 58 ± 4 μg/mL for trypomastigote form was noticed after 24 h, when the highest dead parasites percentage at 150 μg/mL was exhibited, while benznidazole did so at 83 μg/mL [Figure 2]. This alkaloid was less active than the reference drug but more active than any other phase. At the same time, it shows less cytotoxicity (CC_{50} = 513 ± 56.7 μg/mL) and therefore a good SI with a value of 8.9. Nevertheless, it was inactive when faced to the intracellular forms of the parasite.

DISCUSSION

Preliminary phytochemical analysis is in agreement with the previous reports of other *Zanthoxylum* species in which alkaloids and phenol compounds are frequently reported.[15]

Table 1: Antibacterial and antifungal activities of *Zanthoxylum pistaciifolium* leaves phases

Micro-organism	Concentration (μg/mL) (percentage inhibition±SD)			
	HBP	DP	BP	MP
Fungi				
Candida albicans (azole resistant)	<250 (96.0±0.5)	<250 (90.5±1.2)	<1000 (61.18±1.36)	NA
Candida glabrata	<250 (87.6±0.9)	<250 (78.5±1.4)	250 (51.19±0.33)	500 (79.2±0.6)
Candida kefyr	<250 (97.1±0.4)	<250 (98.2±0.2)	500 (53.65±1.02)	500 (62.1±1.6)
Candida krusei	<250 (95.7±0.2)	<250 (97.10±1.5)	<250 (59.27±0.44)	1000 (78.4±2.6)
Candida parapsilosis	<250 (96.5±0.3)	<250 (90.2±1.2)	NA	NA
Candida tropicalis	NA	NA	NA	NA
Bacteria				
Escherichia coli	NA	NA	NA	NA
Pseudomonas aeruginosa	NA	NA	4000 (50.18±4.52)	NA
Staphylococcus aureus	250 (50.7±9.0)	<1000 (100.3±0.3)	2000 (53.15±0.99)	4000 (51.3±4.3)

SD: Standard deviation; HBP: Hexane-butanone phase; DP: Dichloromethane phase; BP: Butanol phase; MP: Methanol phase; NA: Nonactive

Table 2: Antiprotozoal activity, cytotoxicity, and selectivity index of *Zanthoxylum pistaciifolium* leaf extracts

Phase	*Leishmania amazonensis*			*Trypanosoma cruzi*				
	Promastigotes IC_{50}±SD (μg/mL)	Cytotoxicity CC_{50}±SD (μg/mL)	SI	Trypomastigote (2h) IC_{50}±SD (μg/mL)	Trypomastigote (24 h) IC_{50}±SD (μg/mL)	Cytotoxicity CC_{50}±SD (μg/mL)	SI	Amastigotes Inhibition (%) (10 μg/mL)
HBP	>200	122.3±3.7	-	112.1±8.5	39.3±5.5	111±62	2.8	21.2±9.8
DP	54.4±0.2	45.2±4.2	1.0	>300	210.0±7.1	376±113	1.8	2.7±1.9
BP	>200	>200	-	>300	>300	727±224	-	14.18±7.28
MP	>200	>200	-	>300	>300	>1200	-	16.9±2.1
Reference druga	0.37±0.01	11.7±1.7	32	>100	13.0±9.0	>4800	-	80.7±8.9 (2.6 μg/mL)

[a]Pentamidine for *Leishmania*, Benznidazole for *Trypanosoma*. HBP: Hexane-butanone phase; DP: Dichloromethane phase; BP: Butanol phase; MP: Methanol phase; SD: Standard deviation; SI: Selectivity index; IC_{50}: Median inhibitory concentration; CC_{50}: Median cytotoxic concentration

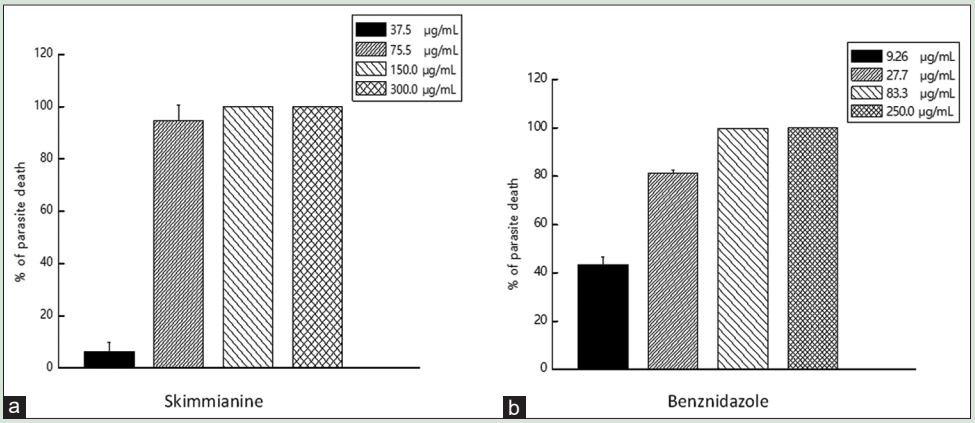

Figure 2: Effect of skimmianine (a) and benznidazole (b) upon bloodstream trypomastigotes (Y strain) *Trypanosoma cruzi* after 24 h of incubation

Phytochemical screening of HBP and DP indicated the presence of alkaloids, phenols, flavonoids, and coumarins. Those metabolites have been associated with antimicrobial activity in other species of the genus. In addition, some isolated alkaloids from species of *Zanthoxylum* genus have been found to possess antifungal activity.[16] Therefore, the antifungal effect observed for HBP and DP could be related to alkaloid presence. Moreover, there is a direct correlation between the antimicrobial effect and the presence of alkaloids because the most active phase (HBP) was the one with higher intensity to alkaloids in the qualitative assays and with the largest amount of spots (reactive with Dragendorff reagent) on the TLC analysis. Three alkaloids isolated of *Zanthoxylum nitidum* had good antifungal activity,[17] while the ones isolated from *Zanthoxylum rhoifolium* (eight) were active against ten micro-organisms.[18] These studies indicated a higher activity for alkaloids of the benzophenanthridine kind over those of the fluoroquinolonic one but confirm that both types are indeed active. Regarding their solubility profile, it looks like they are clearly involved in the antifungal activity.

The results against fungi were also similar to *Zanthoxylum americanum* extracts, with a broad spectrum of antifungal activity associated with the furanocoumarins.[19] Precisely, coumarins are one of the metabolites identified in the HBP and DP and therefore could be also responsible for the antimicrobial activity detected for *Z. pistaciifolium* leaf extracts in a direct or synergistic way.

The essential oils are other of nonpolar metabolites that could be related to the antimicrobial activity in the species, whereas essential oils extracted from the leaves of *Zanthoxylum alatum*[20] and *Zanthoxylum monogynum*[21] show potent antimicrobial activity. At last, phenol compounds had a wide range of biological activities including the antimicrobial, antiviral, antioxidant, anticancer, and other remarkable properties. These pharmacological activities are usually associated to their free radical scavenging efficacies and their ability of binding proteins with a high specificity degree.[22] All these facts suggest us that the antifungal activity observed in HBP and DP could be related to their chemical composition, mainly alkaloids and phenols. Regarding the antibacterial activity, all the studied phases were active only against *S. aureus*. This is a fact already previously reported but in leaf extracts of *Zanthoxylum acanthopodium*.[23] Alkaloids can be also associated to this activity, considering the results reported from isolates of two species of *Zanthoxylum,* being chelerythrine the most active compound.[24]

The activity of *Zanthoxylum* spp. extracts against *L. amazonensis* has been less explored. In Costa Rica, extracts from 67 plant species were evaluated and *Zanthoxylum juniperinum* was one of the 16 that showed activity.[25] In Brazil, extracts of *Z. rhoifolium* were also identified as active against promastigote forms.[26] Recently, extracts from *Zanthoxylum armatum* exhibited good antileishmanial activity.[27] The results obtained in this work showed that DP of *Z. pistaciifolium* is the only active and attending their alkaloid composition, and they emerged as possible responsible. Benzophenanthridine alkaloids isolated from *Z. rhoifolium* root bark were active against both parasite forms: promastigotes and amastigotes as well as in *in vivo* studies of cutaneous leishmaniasis in mice.[28] The same occurs to the alkaloids isolated from *Z. chiloperone* against *L. amazonensis*.[29] On the other hand, *in vitro* experiments show that skimmianine alkaloid isolated from *Spiranthera odoratíssima* (Rutaceae) had a significant effect against *Leishmania braziliensis*.[30] Moreover, this alkaloid extracted from *Adiscanthus fusciflorus* showed inhibitory activity against the enzyme adenine phosphoribosyltransferase (APRT) from *Leishmania* of 68%. The authors showed that its alkaloid crystallizes in the centrosymmetric space group P21/c, with one molecule in the asymmetric unit, and has at least two C-H…O intermolecular interactions, leading to the formation of centrosymmetric dimers. The structural characterization of skimmianine provided important information regarding the interaction ways of this compound with APRT. Further investigations will allow to study the interactions between this compound and the APRT active site to explain its action mechanism against *Leishmania* spp. as well as the inhibition capacity of other PRTases.[31] Furanoquinolines alkaloids (common in species from Rutaceae) have been reported as typical DNA alkylation compounds excerpting cytotoxic and antiparasitic properties. Typical DNA damage occurs when DNA-alkylating compounds form covalent bonds with DNA bases. If the DNA enzymes do not repair these alkylations, errors will occur after the next round of replication. Consequences are point mutations and sometimes deletions and frameshift mutations. If such mutations occur in important protein-coding genes, they can lead to the death of a parasite.[32] Considering the presence of alkaloids in the most active phase (DP), it is likely that there is an association between antileishmanial activity and some of the present alkaloids. Nevertheless, skimmianine, the furanoquinoline alkaloid isolated for the first time in this work for *Z. pistaciifolium*, was not active at the doses tested for the HBP where it was isolated. This discrepancy between its inactivity and the previous reports for the skimmianine[30,31] opens a gap for new structural activity relationship studies for the furanoquinoline alkaloids against *Leishmania* spp.

For *T. cruzi*, not too many reports for activity of *Zanthoxylum* spp. extracts exist. Nevertheless, extracts of *Zanthoxylum chalybeum* stem bark exhibited antitrypanosomal activity at very low doses.[33] The canthin-6-one alkaloid isolated from the ethanol extract of *Zanthoxylum chiloperone* reached the same level of *in vitro* activity against trypomastigote and amastigote forms of *T. cruzi* than the reference drug benznidazole.[34] In this study, HBP and

the skimmianine alkaloid both exhibit good levels of activity against *T. cruzi* parasite. Moreover, the SI of skimmianine (close to ten) is considered acceptable, being close to tenfold less toxic than active and opening opportunities for future *in vivo* studies. Michael previously suggested the activity of skimmianine in his review paper about quinoline, quinazoline, and acridone alkaloids.[35] This activity makes the skimmianine producing plants a valuable source of ethnomedicinal formulations.

CONCLUSION

The phytochemical screening revealed the presence of alkaloids, triterpenes, and steroids, reducing sugars, flavonoids, coumarins, phenols, and tannins in *Z. pistaciifolium* leaves. The HBP did not prove to be active against promastigotes of *L. amazonensis* but shows an antifungal potential and moderate effect on *T. cruzi*. On the other hand, DP shows promising activities against fungi and *L. amazonensis*. *In vitro* trypanocidal activity is attributable to skimmianine, a furoquinoline alkaloid isolated in this specie. These findings suggest for the first time that *Z. pistaciifolium* specie could be a potential candidate for the treatment of several infectious diseases in ethnobotanical practices.

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Conflicts of interest

There are no conflicts of interest.

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