Human and Animal Health

Vol.61: e18160461, 2018
http://dx.doi.org/10.1590/1678-4324-2018160461
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Phenolic Composition and Leishmanicidal Activity of Red Propolis and *Dalbergia ecastaphyllum* (L.) Taub (Fabaceae) Extracts from Sergipe, Brazil

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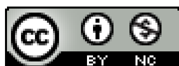
ABSTRACT

Leishmaniasis is a parasitic disease caused by protozoa of the Leishmania *genus. It may manifest in visceral and tegumentary forms, and pentavalent antimonials are the first choice drugs used for the treatment. Frequently these drugs show low efficiency and high toxicity to mammalian host. The present study describes the chemical profile and the* in vitro *leishmanicidal effects of red propolis and* Dalbergia ecastaphyllum *extracts from Sergipe, Brazil, in* Leishmania chagasi *and* Leishmania amazonensis *promastigotes. The phenolic composition of the extracts was evaluated by direct infusion electrospray ionization mass spectrometry (ESI-MS) fingerprinting. The leishmanicidal effect was evaluated by the Resazurin colorimetric method. Similar composition profiles have been found for* D. ecastaphyllum *and propolis samples. The isoflavones formononetin, biochanin A, daidzein and pinocembrin were identified in both extracts. Propolis extract showed leishmanicidal activity in both* L. chagasi *and* L. amazonensis, *with IC_{50} values of 21.54 and 9.73 µg/mL, respectively. The* D. ecastaphyllum *extract presented activity only in L. amazonensis, with IC_{50} of 53.42 µg/mL. These results suggest that red propolis extract from Sergipe has the leguminosae* D. ecastaphyllum *as botanical origin, and that it presents potential leishmanicidal activity, which may be associated with the presence of the phenolic compounds found in its composition.*

Key words: Brazilian red propolis, chemical composition, *Leishmania amazonensis*, *Leishmania chagasi*



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INTRODUCTION

Leishmaniasis is an anthropozoonosis caused by protozoan parasites of the genus *Leishmania*, and remains one of the world's most devastating neglected tropical diseases [1]. Recent data have shown that over 98 countries and territories are endemic to leishmaniasis, and 1.3 million new cases occur each year [2,3]. Clinical manifestations of leishmaniasis are complex, and depend on the parasite species and on the host's immune response [4]. The tegumentary form can be caused by at least fourteen *Leishmania* species, including *Leishmania amazonensis*, which is the etiologic agent of this disease in Brazil [5-7]. Visceral leishmaniasis is caused by species of the *Leishmania donovani* complex, and *Leishmania chagasi* is the main etiologic agent in Brazil [8].

Pentavalent antimonials, patircularly sodium stibogluconate (Pentostan®), and N-methylglucamine (Glucantime®) are the first-choice drugs used in leishmaniasis chemotherapy. Other drugs, such as pentamidine and amphotericin B have been used as second-choice chemotherapeutic drugs. However, both the sodium stibogluconate and the other drugs require long-term parenteral administration, and present serious side effects [9]. In addition, the development of resistance to treatment by the parasites represents an additional and major problem [10]. Thus, there is an increasing demand for new substances that can be used to develop more effective therapies and with fewer side effects.

Propolis is potential source of bioactive compounds. It is a complex mixture of resinous, gummy and balsamic substances collected by honeybees from plant exudates [11,12]. Several biological properties have already been assigned to propolis, including healing, antimicrobial, antifungal, anti-inflammatory, antitumor, antioxidant and hepatoprotective action [13-18]. Furthermore, several studies have demonstrated leishmanicidal action of several types of propolis [19-21].

The biological properties of propolis are directly linked to its chemical composition, which may vary according to the season of collection, but it mainly depends on the climate and on the flora of the region where the apiary is located [22]. In regions of temperate climate, for instance, the propolis derived from the resin of plants of the genus *Populus* is rich in flavonoids, phenolic acids, and esters [23]. On the other hand, in tropical areas, plants of the genus *Baccharis* in Brazil, and of the genus *Clusia* in Cuba and Venezuela are the main sources of propolis. In these cases, the propolis is rich in cinnamic acid derivatives, such as p-coumaric acid and prenylated benzophenones [24,25].

Thus, in view of the great floristic biodiversity and climatic variations, Brazil has the widest diversity of propolis types [26]. Among the 13 different types of propolis which have been identified and characterized up to now, Brazilian red propolis (BRP) is the most recent. It presents chemical composition distinct from the other 12 types of Brazilian propolis, and constitutes a promising source of new compounds of the classes of the chalcones, pterocarpans, isoflavones and polyphenols [17,27].

Frozza et al.[11] pointed out that red propolis from Sergipe is composed of complex molecules and presents important biological properties related to the antioxidant capacity and inhibition of the proliferation of tumor cells. In addition to the differences in the chemical composition when compared with other types of propolis that have already been identified, Franchi et al.[28] reported that red propolis is more cytotoxic than green propolis in leukemia cell lines. The only report of the leishmanicidal activity of red propolis in the Brazilian Northeast refers to the study of Ayres et al.[20], which shows that red propolis from Alagoas was more effective in reducing macrophage infection by *L. amazonensis* than the three types of green propolis evaluated. Therefore, the present study was carried out to evaluate the chemical

composition of red propolis collected in the Baixo Sao Francisco region, in the State of Sergipe, Brazil, as well as to determine their possible botanical origin. In addition, the leishmanicidal action of the hydroethanolic extract of red propolis was demonstrated in *L. amazonensis* and *L. chagasi* promastigotes.

MATERIAL AND METHODS

Samples

Red propolis and *Dalbergia ecastaphyllum* samples were collected in Brejo Grande (10°28'25"S; 36°26'12" W), located in the Baixo Sao Francisco region, state of Sergipe, Brazil. Red propolis was obtained from the propolis collector installed in the Langstroth beehive. Inner bark and floral branches samples of *D. ecastaphyllum* were collected in the apiary surrounding areas. Voucher specimen was deposited at ASE Herbarium of the Federal University of Sergipe under the registration number 19310.

Extracts Preparation

The hydroethanolic extract of Red Propolis (HERP) was obtained according to Mendonça *et al.* (2015), with modifications. Propolis sample (1 g) was extracted with 12.5 mL of 70% (v/v) ethanol at room temperature for 1 hour in ultrasonic bath. The mixture was subsequently centrifuged at 3,000 rpm for 5 minutes at 24° C. The extract obtained was concentrated by evaporation at room temperature for 48 hours. For the production of the hydroethanolic extract of *D. ecastaphyllum* (HEDe), 1.5 Kg of the inner bark were dried and powdered, and the material obtained was dissolved in 90% ethanol, and kept at room temperature for five days. After this period, the material was filtered and concentrated on a rotary evaporator (LG LOGEN) at 60ºC, with 700 mmHg reduced pressure.

ESI(-)–MS Fingerprints and UHPLC-ESI(-)-MS/MS

The analyses of the ethanolic solutions of the dried extracts (1 mg/mL) were performed on a UHPLC Acquity chromatographer coupled with a TQD Acquity mass spectrometer (Micromass-Waters Manchester, England), with an ESI source according to López et al., (2014). A C18 BEH Waters Acquity column (2.1 mm x 50 mm x 1.7 µm particle size) was used. Solvent A was mili-Q purified water with 0.1% formic acid, and solvent B was methanol. The flow rate was 0.2 mL/min, and 5 µL of samples were injected with a linear gradient starting at 40% methanol, and increasing to up to 100% methanol in 9 min, held until 11 min, and then returning to the initial conditions, followed by column re-equilibration. ESI ionization in the negative ion mode was used under the following conditions: Capillary -3.00 kV, Cone -30 V, Source Temperature 150ºC, Desolvation Temperature 350ºC and Collision Energy 30 V, acquiring data between 100 and 800 m/z. The following authentic standards of phenolic acids and flavonoids were examined: caffeic acid, chlorogenic acid, ferulic acid, p-coumaric acid, protocatechuic acid, trans-cinnamic acid, vanillic acid, apigenin, biochanin A, formononetin, isoliquiritigenin, kaempferol, luteolin, naringenin, quercetin, rutin. All were purchased from Sigma Co. (Sigma, St, Louis, MO, EUA) and had >95% of purity.

Parasites Cultures

L. chagasi and *L. amazonensis* promastigotes were obtained from the cryobank of Laboratory of Cellular and Molecular Biology of *Leishmania,* Federal University of Sergipe. Cultures were maintained by weekly transfers in a B.O.D. (Biological

Oxygen Demand) chamber at 24°C, in Schneider insect medium (Sigma-Aldrich, St. Louis, MO, EUA), supplemented with 10% fetal bovine serum (Cripion, Brazil), ampicillin (1%), and gentamicin (0.1%) (Sigma-Aldrich, St. Louis, MO, EUA). The parasitic growth curve was obtained from the daily count of parasites in culture for seven days. Parasites harvested during exponential phase of growth (3-day-old culture forms) were employed in the assays with HERP and HEDe.

In vitro Leishmanicidal Activity

Increasing concentrations of HERP (5 to 90 µg/mL) and of HEDe (25 to 1000 µg/mL) were added to microtiter plates (96 wells) containing $1x10^6$ promastigotes/mL of *L. chagasi* or *L. amazonensis* per well. After 24 hours incubation in B.O.D. chamber at 24°C, 50 µL resazurin (3 mM) were added to each well, following 6h incubation. Absorbance was obtained in 570 and 595 nm wavelengths in a Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio Tek) and cell viability was calculated by the following equation:

$$\text{Cell viability} = \frac{\text{Absorbance (test) 570 nm} - \text{Absorbance (test) 595nm x RO}}{\text{Absorbance (control) 570 nm} - \text{Absorbance (control) 595nm x RO}}\ x\ 100$$

Viability values of the treatments were normalized by providing the negative control (cultures underwent all the procedures with no extract addition), and were used to calculate the IC_{50} by regression analyses. Anphotericin B was used as positive control.

Extracts Cytotoxicity Determination in Macrophages

J774 murine macrophages were seeded in 96 wells microtiter plates (2×10^4 cells per well) and maintained for 12 hours in a 5% CO_2 humidified incubator for attachment. After this period, the supernatant was removed, and 200 µl of the HERP was added at concentrations of 40, 60, 80, 100, 120, 140 and 160 µg/mL. After 24 h additional incubation, the cells were washed, and 200 µl of the MTT (3-[4.5-dimethylthiazol l]-2.5- diphenyltetrazolium bromide) solution (0.5 mg/mL) was added to each well, and the plates were again incubated for 3h. Formazan was solubilized by adding 100 µL DMSO to each well, and then quantified by the reading of the plates at 570 nm using in a Synergy H1 Hybrid Multi-Mode Microplate Reader (Bio Tek). The 570-nm absorbance readings were normalized to the control (cultures with no extract addition) to get the percentage of viable cells. Cytotoxic concentration (CC_{50}) was obtained by regression analyses.

Statistical Analysis

Results obtained in this study were analyzed using the Graph Pad Prism 4.0 and Microsoft Excel 2010 software. IC_{50}, and CC_{50} values were obtained by regression analysis. For the plotting, it was used the mean values and the standard deviation obtained from triplicates for each test.

RESULTS AND DISCUSSION

The chemical composition of propolis is related to its botanical origin. Studies based on the behavior of bees and on physicochemical analyses point out trunk exudates of *Dalbergia ecastaphyllum* trees as a potential source of resin for the production of red propolis in the Brazilian Northeast [12,28-31].

In this work, ESI(-)-MS fingerprint results showed similar composition profiles for *D. ecastaphyllum* and propolis samples (Fig. 1). It was observed that propolis has m/z 239, 255, 271 and 283 ions which are also present in *D. ecastaphyllum*, although the ionization intensities vary between samples. However, propolis also has ions that are

not present in *D. ecastaphyllum* (for example, m/z 299 or m/z 301 ions). This was expected since propolis is defined as a complex mixture of compounds [12,30].
Chromatographic analyses by UHPLC-MS along with the analysis of fragmentation profiles allowed the identification of formononetin, biochanin A, daidzein and pinocembrin (Fig. 2). These isoflavones markers have been identified in propolis from several Brazilian states (Alagoas, Sergipe and Paraiba), and in *D. ecastaphyllum* from Paraiba and Alagoas [21,31].

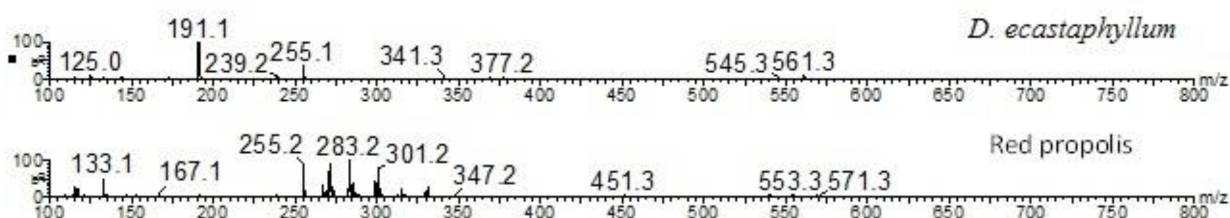

Figure 1 – ESI(-) –MS fingerprint by direct infusion of *D. ecastaphyllum* and red propolis from Sergipe, Brazil. 171x35mm (96 x 96 DPI)

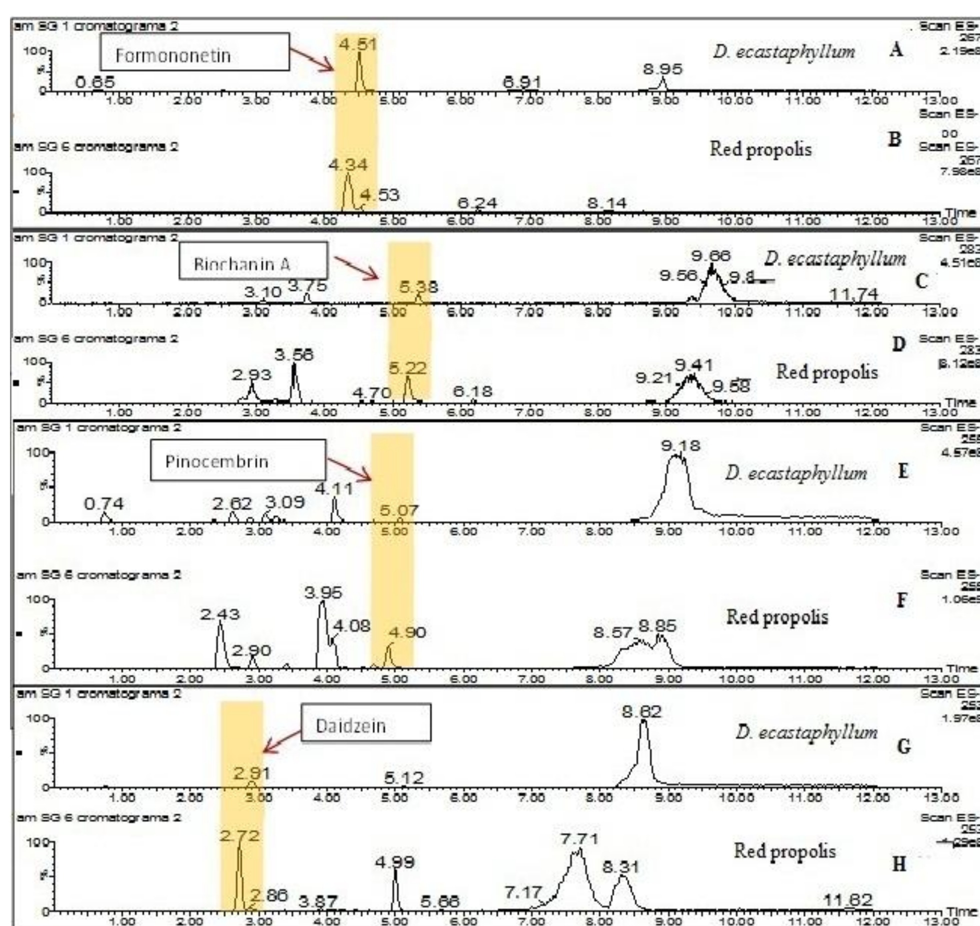

Figure 2 - UHPLC-MS extracted ion chromatograms of the ions m/z 267 –formononetin (A-B), m/z 283 - biochanin A (C-D), m/z 255 – pinocembrin (E-F) and m/z 253 – daidzein (G-H) in *D. ecastaphyllum* and in red propolis from Sergipe. 173x163mm (96 x 96 DPI)

In a recent study carried out by López et al. [31] 14 red propolis samples from different regions of Brazil and Cuba were characterized by ESI(-)-MS fingerprinting. Such samples were divided into three groups according to the predominance of marker ions, and each group was associated with a different botanical origin. The group with which the red propolis sample collected in the state of Sergipe was associated presented isoflavones biochanin A, formononetin and pinocembrin as marker ions. These markes were also present in the fingerprints of *D. ecastaphyllum*, but they were of low abundance in the spectra of the samples allocated in the other groups.

Jain et al. [32] using a new approach, confirmed through DNA sequencing the presence of components of *D. ecastaphyllum* in red propolis from Sergipe, corroborating with other studies that affirm that this species is the botanical origin of red propolis [12,30,31].

Another evidence which associates the *D. ecastaphyllum* as the botanical origin of the propolis evaluated in this work is the fact that during the samples collections, honey bees were observed collecting red resinous exudates on the surface of the stem of *D. ecastaphyllum* trees present in the apiaries surrounding areas. The fact that the bees from hives located along the northeastern coast of Brazil visited *D. ecastaphyllum* to collect resin on the plant's surface to produce red propolis has been previously reported [12,28].

Although there is a wide range of studies related to the leishmanicidal activity of different types of propolis in different regions of Brazil and the world [20,23,33-40], regarding red propolis, especially the red propolis from Sergipe, there is little information available. In this way, it was evaluated for the first time the effects of different concentrations of hydroethanolic extracts obtained from red propolis from Sergipe and of the inner bark of *D. ecastaphyllum* on the growth of promastigotes of *L. amazonensis* and *L. chagasi*. Figure 3 depicts the dose-dependent effect of HERP and HEDe on *Leishmania* parasites (Fig. 3). In relation to the propolis extract, *L. amazonensis* was about twice more susceptible to HERP than *L. chagasi*, being the IC_{50}/24 h values 9.3 and 21.54 µg/mL, respectively (Table 1). Comparison of the effect of HERP and HEDe on *L. amazonensis* shows that the propolis extract is 5 times more active than the *D. ecastaphyllum* one.

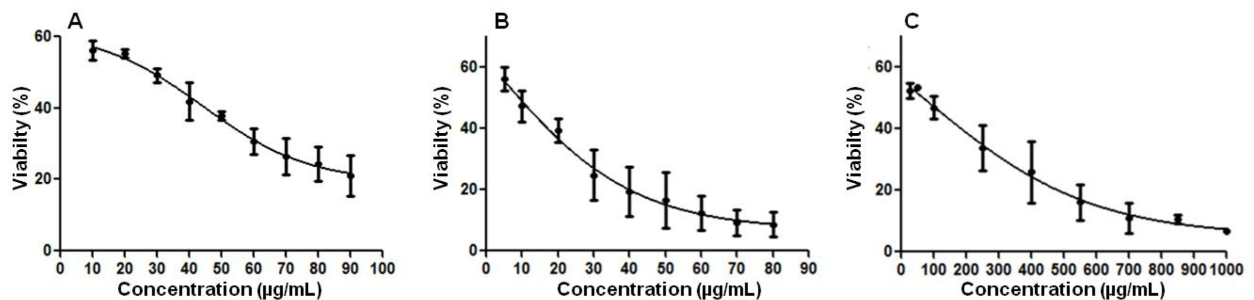

Figure 3 – Effect of the hydroethanolic extract of Red Propolis (HERP) on promastigotes of *L. chagasi* (A) and *L. amazonensis* (B), and of the hydroethanolic extract of *D. ecastaphyllum* (EHDe) on promastigotes of *L. amazonensis* (C). The parasites were cultivated for 24h in the presence of increasing concentrations of extracts, and viability was determined by the Alamar Blue method. The dots represent the mean of three independent experiments, and the bars represent the standard deviation. 350x86mm (96 x 96 DPI)

Table 1 HERP and HEDe IC_{50} values for promastigotes of *L. amazonensis* and *L. chagasi*

	IC_{50} (µg/mL)		**CC_{50} (µg/mL)**
	L. amazonensis	*L. chagasi*	Macrophages
Red propolis (HERP)	9.73 ± 1.49	21.54 ± 2.58	72.63 ± 5.89
***D. ecastaphyllum* (HEDe)**	53.42 ± 13.38	ND*	ND*
Amphotericin B	0.37 ± 0.18	0.71 ± 0.08	ND*

*ND Not determined. Results expressed in mean ± standard deviation

Although this is the first study on leishmanicidal activity of *D. ecastaphyllum* extract, several studies have been carried out with different propolis extracts, and the results demonstrate that propolis extracts from different part of the world has demonstrated relevant leishmanicidal activity against different species of *Leishmania*. Duran et al. [38] showed antileishmanial activity for Turkish Hatay and Bursa propolis extracts against *L. infantum* and *L. tropica* promastigotes and demonstrated IC_{50} values from 125.0 to 350.0 µg/mL. Another relevant study performed by Santana et al. [40] revealed good results for brown própolis (BP) originating from the semi-arid region of Piauı, Brazil. The BP ethanolic extract and its fractions showed significant inhibition of the *L. amazonensis* promastigotes growth as well as were effective in reducing infection of murine macrophages and the number of internalised amastigotes in these cells. In another recente study Ferreira et al. [35] showed that treatment with water extract of Brazilian green propolis was able to reduce parasite load in the liver of BALB/c mice infected with *L. infantum*. A comparative investigation into whether leishmanicidal activity of Brazilian and Bulgarian green propolis extracts against four different species of *Leishmania* was carried out by Machado et al. [34] It was shown that Brazilian green propolis extract showed leishmanicidal activity for *L. braziliensis*, *L. chagasi* and *L. major* species with IC_{50} close to 49.0 µg/mL, while the Bulgarian propolis extract showed activity against *L. amazonensis*, *L. chagasi* and *L. major* with IC_{50} between 2.8 and 41.3 µg/mL. Monzote et al. [33] evaluated the effect of 20 Cuban propolis chemotypes regarding the viability of amastigotes forms of *L. infantum*. Among these chemotypes, nine were red propolis, whose IC_{50} values ranged between 3.3 and 16.1 µg/mL. In the same study, it was demonstrated the activity of red propolis in amastigote forms of *Trypanosoma cruzi* and trypomastigote forms of *T. brucei*, showing that the red propolis action extends to other trypanosomatids as well as other types of propolis as previously reported [41-44]. Ayres et al. [20] had already described the potential leishmanicidal activity of red propolis from Alagoas. In that study the authors found that at the concentration of 25 µg/mL, the ethanolic extract of red propolis was able to reduce by 60% the parasite load in macrophages infected by *L. amazonensis*. However, it presented no direct effect on promastigotes or extracellular amastigotes. These results led authors to suggest that red propolis from Alagoas may function through macrophage activation and not by a direct action on the parasite viability [20]. On the other hand, our results showed a direct effect of red propolis from Sergipe on the *Leishmania* promastigotes viability. This difference may be due to differences in the chemical composition of propolis collected in the two states. As showed by our results and, as aforementioned [31], the marker ions of red propolis from Sergipe were the isoflavones formononetin, biochanin A, and pinocembrin. On the other hand, in propolis collected in the state of Alagoas, the markers were benzophenones, such as

guttiferone E and xantochymol, probably originated from plants of the family Guttiferae.
These facts demonstrate the existence of at least two types of red propolis in the northeastern region of Brazil, and that differences in their chemical compositions may reflect on differences in their biological properties.

CONCLUSIONS

The results of this study showed that propolis collected in the Baixo São Francisco region of the state of Sergipe has as main marker ions the isoflavones formononetin, biochanin A, and pinocembrin, suggesting *D. ecastaphyllum* as its probable botanical origin. In addition, the activity of the hydroethanolic extract of red propolis from Sergipe on the viability of promastigotes of *L. amazonensis* and *L. chagasi* highlights the plant as potential source for future investigations into new leishmanicidal compounds.

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Received: February 03, 2016;
Accepted: July 14, 2016