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In vitro antileishmanial activity and phytochemical analysis of *Casearia javitensis* Kunth (Salicaceae)

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Abstract

The objective of this study was to carry out phytochemical analysis of the bark extract of *Casearia javitensis* and to evaluate its antipromastigote activity against *Leishmania amazonensis*. The ethanol extract (EE) was fractionated in open-column chromatography to obtain the hexane (FrHex), dichloromethane (FrDcm), ethyl acetate (FrAcOET) and methanol (FrMeOH) fractions. The EE and its fractions were analyzed by thin layer chromatography (TLC). FrDcm was analyzed in high performance liquid chromatography with diode array detection (HPLC-DAD). The antipromastigote assay of *Leishmania amazonensis* and the cytotoxicity test against the acute monocytic leukemia cell line (THP-1) were performed by the MTT colorimetric assay. The phytochemical profile in TLC detected terpenes in all samples. However, in the ultraviolet spectrum (HPLC-DAD) for FrDcm suggested the presence of a phenolic compound. FrHex and FrDcm showed antipromastigote activity (IC_{50} = 116.6 ± 0.9 and 59.4 ± 1.1 µg mL^{-1}, respectively) and low cytotoxicity (CC_{50} = 333.4 ± 3.2 and 241.2 ± 1.9 µg mL^{-1}, respectively). The selectivity index for FrDcm was 4.1. We conclude that the FrDcm of *C. javitensis* has potantial as a leishmanicide, and this activity may be related to the presence of phenolic compound.

Keywords: Leismaniasis. Antipromastigote. Cytotoxicity (THP-1). Phenolic Compound.

Introduction

The leishmaniases are a group of infectious disease, non-contagious, caused by protozoan parasites of the genus *Leishmania*, which affects skin and mucous membranes, transmitted to humans by the bite of infected female phlebotomine sandflies[1]. Endemic in 97 countries, an estimated 1 billion people live in an area at risk for the disease worldwide and there are no optimistic forecasts for its control for the next few years[1,2].

The Amazon was responsible for 57.3% of all cases of leishmaniasis in Brazil, with the states of Pará (30.2%), Mato Grosso (17.3%), Amazonas (15.0%) and Maranhão (13.4%) presented the most cases of Leishmaniasis in this region[3,4].

The treatment of leishmaniasis is mainly carried out with pentavalent antimonial drugs (sodium stibogluconate and meglumine antimoniate), whereas Pentamidine and Amphotericin B are the second choice in therapy[5]. However, the use of these drugs is questionable because of the limitations of toxicity, variable efficacy, requirements for parenteral administration and/or length of treatment regimens, and a high cost[5-7]. In addition, there are reports of parasite resistance, including the global HIV/AIDS epidemic with its accompanying impact on the immune system[8], having high *Leishmania*-HIV coinfection rates reported from Brazil, Ethiopia and the state of Bihar in India[9].

Moreover, in the Amazon, therapeutic failure may be related to the resistance of the parasite to drugs, to the irregular and distant use of individuals who live far enough away from health services[10]. Therefore, it is necessary to search for new therapeutic alternatives, with medicinal plants being an important source of bioactive compounds.

The Amazon region still presents high rates of infectious and parasitic diseases, with an expressiveness of deaths[11] that may be associated with the population's poor access to health services. Given this scenario, traditional knowledge about the use of medicinal plants often symbolizes the only therapeutic resource for these communities[12]. Among the plants, it stands out the genus *Casearia* (Salicaceae), composed of about 180 species throughout the tropical and subtropical regions of the globe, including Africa, Asia, Australia, North America and South America, and the Pacific islands[13], with 70 species belonging to the American continent and 37 present in Brazil[14], they stand out because they have species known for medicinal use in the Amazon, with potential cicatrizant activity[15].

The morphology of this genus is described as shrubs or small trees, found more frequently in forest vegetation[16], and are noted for their medicinal importance, with wide recommendations in traditional medicine for the treatment of wounds, ulcer[17], to cicatrizing[18], in addition to being used frequently for the treatment of different skin diseases[19], which may be related to leishmaniasis.

In addition, species of the *Casearia* genus present in the literature a study with antiparasitic activity, with activity between trypomastigotes of *Trypanosoma cruzi* (IC_{50} ranging from 0.53 to 2.77 µg mL^{-1}) for clerodane diterpenes (casearins A, B, G, and J) and for *Leishmania infantum* promastigotes were also susceptible to casearins obtained from *Casearia sylvestris*, with IC_{50} values ranging from 4.45 to 9.48 µg mL^{-1}[20], and the

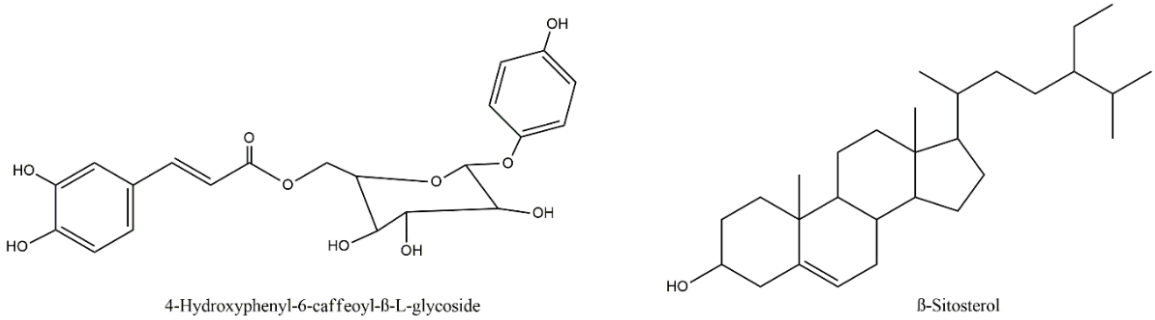
tricine compound, a flavone, obtained from of *C. arborea* demonstrated activity against the intracellular amastigotes of *L. infantum*, with an IC_{50} value of 56 μM[21].

Phytochemical studies on different species of the *Casearia* genus revealed the predominance of terpenoids, emphasizing clerodanic diterpenes[22]. Sesquiterpenes and monoterpenes, phenylpropanoids, steroids, phenolic glycosides, alkaloids and flavonoids have also been isolated[13,23]. Several of these chemical compounds, isolated from plant extracts, have been reported leishmanicidal activity[24], especially the terpene compounds obtained from *Casearia Sylvestris*[20] and flavonic compounds obtained from *C. arborea*[21] present in the genus *Casearia*.

The *Casearia* genus has 180 species, of which 287 compounds have already been isolated, and the terpenoids, in particular the diterpenoids clerodans, are the predominant and representative constituents in the *Casearia* genus[13]. *Casearia javitensis* Kunth is a species found very frequently in the Amazon region[15], Preliminary phytochemical studies detected the presence of terpenes and hexanoic and caproic acids[13,25], besides phenolic glycosides, steroids and flavonoids[23]. In analysis by gas chromatography–mass spectrometry (GC–MS) of the fractions obtained by fractionation of the dichloromethane extract, made it possible to detect triterpene friedelina and β-friedelanol and steroid β-sitosterol[15], but only in one study, 4-hydroxyphenyl-6-caffeoyl-β-L-glucoside and β-sitosterol were isolated from the hexane and methanol extracts of the leaves[26] (**FIGURE 1**).

However, several species of the genus lack validation studies of leishmanicidal activity, especially *Casearia javitensis* Kunth, with limited validation studies of biological activity and cytotoxicity assays.

FIGURE 1: Structures of substances isolated from *Casearia javitensis*.



4-Hydroxyphenyl-6-caffeoyl-ß-L-glycoside

ß-Sitosterol

In summary, despite the existence of phytochemical studies of the species, there are no reports of studies that evaluated the leishmanicidal activity. In view of the above, this work had the objective to evaluate the leishmanicidal potential of *Casearia javitensis*.

Material and methods

Plant material

The trunk bark of a *C. javitensis* individual was collected in an area of the Museu Paraense Emílio Goeldi in January 2016, in the municipality of Belém, state of Pará, Brazil Brazil (01°27'00" S, 48°26'47" W). The exsicata was deposited in the herbarium "João Murça Pires" of the respective museum under registration MG-Etn-00559.

Biological material

The parasite used was *Leishmania (L.) amazonensis,* isolated from a human case from Ulianópolis, Pará State (MHOM/BR/2009/M26361). Cell lines from acute monocytic leukemia (THP-1; ATCC Nº TIB 202) were purchased from the cell bank of Rio de Janeiro (BCRJ).

Phytochemical *analysis*

The powder from the barks underwent maceration with ethanol (1:10), and the macerated material was concentrated in a rotary evaporator until residue precipitation. The ethanol extract (EE, 0.125 g) was fractionated in an open chromatographic column, using as a stationary phase silica gel (0.1-0.2 mm/70-130 mesh) and mobile phase gradient solvents: hexane, dichloromethane, ethyl acetate and methanol.

In order to detect the presence of alkaloids and terpenes, EE, hexane fraction (FrHex), dichloromethane fraction (FrDcm), ethyl acetate fraction (FrAcOET) and methanol fraction (FrMeOH) (95:5:0.2) for alkaloids and Hexane, Ethyl Acetate (8:2), for the detection of terpenes the ultraviolet light (365 nm) and reagents from Dragendorff, and Liebermann-Burchard were used as developers[27]. FrDcm was subjected to high-performance liquid chromatography with a diode-array detector (HPLC-DAD).

Biological activity

Promastigotes of *L. amazonensis* were obtained after primary isolation in NNN (Novy–MacNeal–Nicolle) medium. Then the strains were subcultured and adapted to the RPMI (Roswell Park Memorial Institute) medium. The parasite was cultured at 26°C in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (Gibco®, Grand Island, NY, USA), penicillin (100 U mL^{-1}) and streptomycin (100 µg mL)[28].

The culture of log phase promastigotes was adjusted to $5x10^6$ parasites 0.1 mL^{-1}. Susceptibility test determination was performed on 96-well plates. The extract and fractions were analyzed in triplicate in a concentration gradient (200 to 3.125 µg mL^{-1}). The negative control was performed only with parasites and the incubation medium. The positive control was made with amphotericin B (25 to 0.3906 µg mL). After 24 h of incubation at 26°C under 5 %CO_2 atmosphere, 10 µl of tetrazolium salt (5 µg mL) was added to each well, and the parasites were quantified in an enzyme-linked immunosorbent assay reader[28]. The half-maximal inhibitory concentration (IC_{50}) was determined by linear regression (Graph Pad Prism versão 5.04).

To determine cell viability, the colorimetric MTT (tetrazolium dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) metabolic activity assay was used[29]. THP-1 cells ($4x10^5$ cells 0.1 mL^{-1}) were grown in RPMI-1640 medium (Sigma Aldrich®, EUA), supplemented with 5% fetal serum, maintained under 5% CO_2 at 37°C. Cells were treated with extract and fractions at different concentrations (between 25 and 500 µg mL^{-1}). After 24 h additional incubation MTT (5.0 mg mL^{-1}) was added. The plate was incubated at 37°C in a 5% CO_2 atmosphere for 4 h. Dimethylsulfoxide was added to each well to solubilize the formazan crystals. Optical density was determined at 490 nm (Stat Fax 2100 microplate reader, Awareness Technology, Inc., USA). The cytotoxic concentration (CC_{50}) was determined by linear regression[29].

To determine the selectivity index (SI), the ratio of the CC_{50} value of the cytotoxic activity to the IC_{50} value of the antiprotozoal activity was calculated. When the SI value is >1, hat compound presents more active against protozoa and less active against mammalian cells[30].

Results and discussion

The yield was of 3.67% (11.025 g) of EE. Fractionation of referred extract on an open chromatographic column generated fractions of different polarities, such as higher yield for methanol fraction (FrMeOH 91.95% - 7.356 g; **TABLE 1**).

TABLE 1: Yields and thin layer chromatography of *Casearia javitensis*.

Samples	Yield (%)	TLC			
		Dragendorff	UV	Liebermann-Burchard	UV
EE	3.67	-	-	+	+
FrHex	1.68	-	-	+	+
FrDcm	1.33	-	-	+	+
FrAcOET	4.06	-	-	+	+
FrMeOH	91.95	-	-	+	+

Legend: TLC, thin-layer chromatography; UV, ultraviolet rays; EE, Ethanol Extract; FrHex, Hexane Fraction; FrDcm, Dichloromethane Fraction; FrAcOET, Ethyl Acetate Fraction; FrMeOH, Methanol Fraction.

The solvents influenced the final extraction content, the methanol solvent being the most selective for the extraction of the metabolites present in the casings *Casearia javitensis* and previous studies with *Casearia sylvestris* Sw. observed the presence of flavonic glycosides, with predominance of condensed tannins, metabolism groups[31].

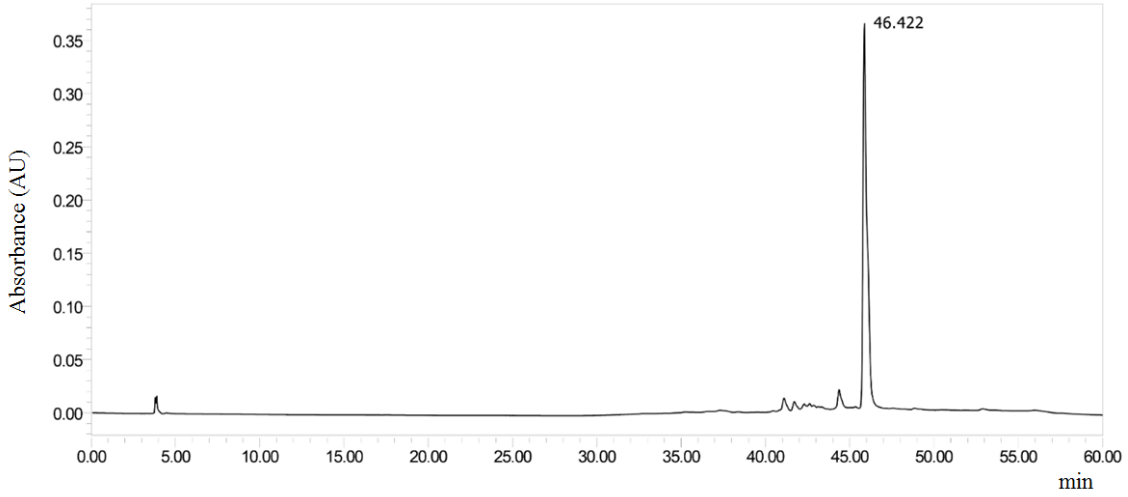
Phytochemical studies on different species of the *Casearia* revealed the predominance of terpenoids, emphasizing clerodan diterpenes[22]. Therefore, this may explain the suggestive results for the presence of terpenes, and absence of alkaloids (**TABLE 1**). In the study of *Casearia sylvestris* Sw. also no alkaloid was observed[31].

Several of these chemical compounds, isolated from plant extracts, have proven leishmanicidal activity, such as terpenoids, aminoglycosteroids, aminosteroids, naphthoquinones, chalcones, iridioid glycosides, flavonoids, lignans and alkaloids[24].

Phytochemical studies of species of the genus, especially *C. corymbosa, C. grewiifolia, C. membranacea* and *C. sylvestris* allowed the isolation and structural characterization of 152 clerodan diterpenes, of which 41 were isolated from *C. sylvestris*[13]. More than 287 compounds have been identified from the *Casearia* genus and it can be said that terpenoids are the predominant class of metabolites, highlighting clerodane diterpenes[13]. For example, two clerodane diterpenes, Casearlucine A and Caseamembrol A also showed activity against *L. amazonensis* amastigote axenic stages (5.98 ± 6.8 and 10.5 ± 0.4 μM, respectively) and promastigote (11.1 ± 0.2 μM, for both)[32].

Phytochemical studies suggest the presence of terpenes in the EE and in all fractions obtained (**TABLE 1**). On the other hand, none of the samples presented a positive result for alkaloids. Following the thin layer chromatography profile analysis, the FrDcm fraction was selected for HPLC-DAD analysis, as it showed a better chromatographic profile when tested with the Liebermann-Burchard reagent and observed in ultraviolet light (UV). The FrDcm submitted to the analysis in HPLC-DAD presented a signal with greater intensity (TR = 46.422 min.) with UV spectrum with λ of 258.3 and 383.4 (**FIGURE 2**).

FIGURE 2: Chromatographic HPLC profiles of the FrDcm fraction from stem barks of *Casearia javitensis*.



The chromatogram suggests that the major peak of FrDcm is a phenolic compound, since the absorption at 258.3 nm is suggestive of that of the band II (ring A, benzoyl portion). While the peak at 383.4 nm may be related to the Band I (ring B, cinamoyl portion) of the flavonoid[33,34]. From the maximum absorption value of Band I, it is possible to infer about the nature of the flavonoid, especially between flavones (304-350 nm) and flavonols (352-385 nm)[33,35]. The maxima absorption obtained from the FrDcm suggests that this compound is a flavonol.

Casearia javitensis extract was inactive against promastigote forms of *L. amazonensis*, presenting an CI_{50}> 200 µg mL^{-1}. FrHex (CI_{50} 116.6 ± 0.9 µg mL^{-1}) was considered moderately active. FrDcm (IC_{50} 59.38 µg mL^{-1}) obtained the best result among the samples tested, being considered active. FrAcOET (CI_{50}> 200 µg mL^{-1}) and FrMeOH (CI_{50}> 200 µg mL^{-1}) were inactive (**TABLE 2**). Thus, the results of the antipromastigote activity showed that the fractionation process contributed to the improvement of the biological activity, with a medium polarity profile (FrDcm) presenting better biological response.

TABLE 2: The anti-promastigote, activity Cytotoxicity and selective index of *Casearia javitensis*.

Samples	*L. amazonensis* CI_{50} (µg mL^{-1})	THP-1 cell line CC_{50} (µg mL^{-1})	SI
EE	>200	88.77 ± 2.8	Nd
FrHex	116.6 ± 0.9	333.4 ± 3.2	2.85
FrDcm	59.38 ± 1.1	241.2 ± 1.9	4.06
FrAcOET	>200	30.5 ± 5.3	Nd
FrMeOH	>200	101.4 ± 3.1	Nd
Amphotericin B	0.1699 ±0.0	162.8 ± 3.7	958.2

Legend: THP-1, Human *monocytic leukemia cell line*; Nd, Not determined; SI, selective index; EE, Ethanol Extract; FrHex, Hexane Fraction; FrDcm, Dichloromethane Fraction; FrAcOET, Ethyl Acetate Fraction; FrMeOH, Methanol Fraction.

In the cell viability assay, EE (CC_{50} 88.77 µg mL^{-1}), FrAcOET (CC_{50} 30.5 µg mL^{-1}) and FrMeOH (CC_{50} 101.4 µg mL^{-1}) showed toxicity to THP-1 monocytic cells. FrHex and FrDcm showed low cytotoxicity at the concentrations tested (CC_{50}> 200 µg mL^{-1}; **TABLE 2**). Thus, selective index results (**TABLE 3**) show that FrHex and FrDcm are 2.8 and 4.06 fold less toxic to THP-1 cells than to promastigote forms. Thus, it can be observed that fractions of low and medium polarity have secondary metabolites with low capacity to cause damage to the cell.

The activity observed in FrDcm may be related to the presence of flavonoids detected in HPLC-DAD. Two important members of the flavonoid family, namely quercetin and luteolin, have been reported to have marked leishmanicidal activity. Studies have shown that quercetin may induce the death of *L. amazonensis* by increasing the production of reactive oxygen species and by collapsing the mitochondrial potential[36]. Quercetin has also been reported to have multiple targets, including arginase, which is an important enzyme in the polyamine biosynthesis pathway[37], topoisomerase II in kinetoplasts, which induces DNA cleavage leading to apoptosis[38] and iron metal, which is important for parasite growth and replication[39].

It is worth mentioning that the observed values were obtained from fractions, in this way, new studies are required using the bioguided fractionation to aid in the isolation of the substances responsible for leishmanicide activity, which may improve by the inhibitory capacity of the parasite and consequently its selectivity.

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