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Research

The Fixed Oil from the Fruits of Pequi, Popularly Used as An Antiparasitic, has No Activity Against Strains of *Leishmania Infantum*

José Weverton Almeida-Bezerra[1]*, Cláudio Gleidiston Lima da Silva[2], Racquel Oliveira da Silva Souza[2], Maria Elizete Machado Generino[1], Luciano Temoteo dos Santos[2], Viviane Bezerra da Silva[1], Mikael Amaro de Souza[1], Nathallia Correia da Silva[1], Ariston Oliveira Magalhães Filho[3], Maria Ivaneide Rocha[1], Valdilia Ribeiro de Alencar Ulisses[1], Mariana Ferreira da Cruz[1], Henrique Douglas Melo Coutinho[1] and Maria Flaviana Bezerra Morais-Braga[1]

[1]*Universidade Regional do Cariri, Crato, CE, Brasil*

[2]*Universidade Federal do Cariri, Barbalha, CE, Brasil*

[3]*Faculdade de Juazeiro de Norte, Juazeiro do Norte, CE, Brasil*

***Corresponding Author:** José Weverton Almeida-Bezerra, Universidade Regional do Cariri, Crato, CE, Brasil.*

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Abstract

The fruit of the "pequi" (*Caryocar coriaceum* Wittm.) is popularly used for the treatment of infectious and parasitic diseases. Chemically, the fruit is rich in oils consisting mainly of saturated and unsaturated fatty acids. In this study, *C. coriaceum* fixed oil was evaluated for its activity against *Leishmania infantum* strains. Ripe fruits were collected in the municipality of Jardim (Ceará, Brazil) and subjected to cooking in a similar way to the production of extractive oil. After obtaining the oil, the product was evaluated at concentrations of 1,024 and 8,192 µg/mL against promastigote forms of *Leishmania infantum* (C8) strains for 24, 48, and 72 h. After growth, viable cell counts were performed in a Neubauer mirror chamber. At the end of the experiments, the results were subjected to a one-way analysis of variance followed by the Tukey test. Median inhibitory concentrations (IC_{50}) by non-linear regression analysis were also calculated. It was demonstrated that *C. coriaceum* oil has no leishmanicidal effect at concentrations of clinical relevance since its IC50 was 7,300 µg/mL in 24 h of exposure. Although the 8,192 µg/mL oil treatment inhibited the growth of *L. infantum* promastigotes within 72 h, the concentration is clinically irrelevant. Despite the ethnomedicinal use of fixed oil from the fruits of *C. coriaceum* being used for the treatment of infectious and parasitic diseases, this study demonstrated that this product is ineffective at concentrations of pharmacological interest against strains of *L. infantum*.

***Keywords:** Leishmaniasis, Chapada do Araripe, in vitro, Caryocaraceae.*

1. Introduction

Among the nosocomial diseases, those caused by protozoa are the most neglected, since the public health sectors focus on bacterial, fungal, and viral infections. However, some studies point out that parasitic protozoa play an important role in nosocomial infections, especially in immunosuppressed and immunosuppressed patients, since they affect 1% of infections acquired in a hospital environment [1,2,3]. Several protozoa are related to nosocomial infections, among them *Leishmania* spp., *Trypanosoma cruzi, Trichomonas vaginalis, Toxoplasma gondii, Plasmodium* sp., *Babesia* sp., *Acanthamoeba* spp., *Naegleria fowleri, Giardia* spp. and *Entamoeba histolytica* [2].

Of these, the first group, *Leishmania* spp., is composed of heteroxenic unicellular protozoan parasites that present morphological variation in their life cycle, and may present a flagellum (promastigote forms), when present in the vector, or not present such structure (amastigote), when present. are parasitizing mammals. The presence of such parasites in accidental hosts, such as humans, causes the set of diseases known as leishmaniasis, which can be cutaneous/mucosal or visceral, depending on the species of *Leishmania* [4, 5, 6].

The first set, also known as American Cutaneous Leishmaniasis (ACL) is a zoonotic disease that affects humans and several domestic animals in the wild. As it is a polymorphic disease, it has several clinical forms, mainly affecting the skin (cutaneous) and mucous membranes [5, 7, 8]. In Brazil, the Leishmania species capable of causing the mucous form are *Leishmania amazonensis, Leishmania braziliensis, Leishmania guyanensis,* and *Leishmania lainsoni*. This clinical form is characterized by the host presenting ulcerative, painless, single, or multiple lesions.

In the mucocutaneous form, the host may present aggressive and irreversible lesions in the nasopharyngeal regions and is caused by the infection of *L. braziliensis* and *L. guyanensis*. Finally, the diffuse form is recognized for being disseminated throughout the host's body and forming non-ulcerated nodules, with *L. amazonensis* as the etiologic agent [9, 10, 11, 12].

The second set of diseases is known as American Visceral Leishmaniasis (AVL), being more popularized as "kala-azar". Clinically, this disease causes a series of complications for the host, including lymphadenopathy, hepatomegaly, splenomegaly, pallor, anemia, leukopenia, thrombocytopenia, fever, night sweats, weakness, anorexia, asthenia, skin pigmentation and weight loss [13, 14]. Currently, three species of the *Leishmania* complex capable of causing AVL have been described, being *Leishmania donovani, Leishmania tropica*, and *Leishmania infantum*, this being the only one that occurs in Brazilian territory [5, 15].

Such etiological agents are vectorially transmitted by female sandflies of the genera *Phlebotomus* and *Lutzomyia,* known as "birigui", "tatuquira" and "mosquito-palha", during their blood meal [16]. At this moment, the insect transmits the promastigote forms to the host, which will interact with the membrane of the cells of the phagocytic mononuclear system, to be phagocytosed. After phagocytosis, the promastigote strains surrounded by the phagocytic vacuole will undergo biochemical changes until they lose the flagellum and transform into amastigote forms. These will be able to carry out their reproduction by binary division, occupying the entire intracellular space until breaking the cell and infecting new healthy cells, restarting the cycle in the host, or being ingested by the host [17].

This set of diseases has a worldwide distribution, occurring in tropical and subtropical regions, mainly in underdeveloped and developing countries, being classified as a neglected tropical disease [18, 19]. Because it is neglected by public authorities, this disease affects millions of people, since more than 2 million new cases occur annually, and despite having treatment in some cases, more than 70,000 annual deaths still occur [4].

Thus, it is evident that the search for bioactive natural or synthetic products becomes of paramount importance to discover new drugs with action on protozoa. Such products can be fixed oils, essential oils, resins, botanical extracts, waxes, etc., all related to the chemical separation of plants [20]. An oilseed tree species that stands out in the investigation of its antiparasitic potential is *Caryocar coriaceum* Wittm. (Caryocaraceae), which is known in Brazil as "pequi" or "pequizeiro". Its oil is extracted by extractivist from Chapada do Araripe and sold for food and medicinal purposes. Among the medicinal uses of the fixed oil of *C. coriaceum* by the communities that live around Chapada do Araripe, are indigestion, flu, bronchitis, scalp affections, eczema, cough with secretions, wound healing, and sore throat [21, 22, 23, 24].

Some studies have already corroborated the antiparasitic potential of *C. coriaceum*. Alves et al. [25], demonstrated that ethanolic extracts from the bark and pulp of *C. coriaceum* showed significant and promising results against the promastigote forms of *Leishmania amazonensis* (Leishmania), with IC50 of 38 µg/mL and 30 µg/mL respectively, is considered significant since the same effects of the positive controls were observed. Tomiotto-Pellissier et al. [26], evaluated the apolar (ethyl acetate) and polar (methanol) extracts of the leaves of *C. coriaceum* against the promastigote forms of *Leishmania (Leishmania) amazonensis* and showed that the strains are susceptible to the extracts by late apoptosis medium, which showed an IC50 (µg/mL) of 5.25 and 58, respectively.

Based on traditional medicine and the studies mentioned above, the present study hypothesizes that the fixed oil of *C. coriaceum* marketed by extractivists has activity against protozoa that cause parasitosis. Thus, this work aimed to evaluate the effect of fixed oil from *C. coriaceum* fruits against promastigote strains of *Leishmania infantum.*

2. Methodology

2.1 License and Collection of Botanical Material

Ripe and healthy fruits of *C. coriaceum* (Figure 1) were collected in the afternoon (3:00 pm) in February 2021 in an Environmental Protection Area (APA) of Chapada do Araripe, belonging to the municipality of Jardim - CE, Brazil under the coordinates 07° 29' 269'' S and 39° 18' 050'' W at an altitude of 925 m. As it is a conservation area, licenses for the collection and use of genetic heritage were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under registration A4848B1 and the Biodiversity Authorization and Information System (SISBio) under registration 77450-1.

2.2 Fixed Oil Obtaining

To obtain *C. coriaceum* oil, the method used by extractivists from Chapada do Araripe was followed, as detailed in the work by Cavalcanti et al. [27]. For that, 1,000 fruits were submitted to manual extraction by a technique called "rolling", which consists of moving a sharp object (knife) against the fruit to remove and discard the peel, consisting of the epicarp and external mesocarp. The remaining parts of the fruits (internal mesocarp, endocarp, and seed) were placed in boilers containing 200 L of drinking water and subjected to constant boiling for 5 h.

After this period, inside the boilers, the fruit parts were rubbed on an artisanal metal grater to separate the inner mesocarp from the other parts of the fruit. At the end of this step, the pits (epicarp + seed) were washed with potable water to remove the impregnated residues, after which the pits were discarded. The boilers remained for another five hours in boiling and constant manual agitation to agglutinate the oil on the surface. The oil was collected, transferred to a container, and boiled for two hours. Subsequently, the oil was filtered with hydrophilic cotton and placed in an amber container at a temperature of 10 °C.

Figure 1: *Ripe fruits of Caryocar coriaceum Wittm. collected in an Environmental Protection Area in the municipality of Jardim – CE (Brazil).*

2.3 Antiparasitic Activity

2.3.1 Parasitic strain

To evaluate the antiparasitic effect of *C. coriaceum* oil, the promastigote strain *Leishmania infantum* (C8) isolated from a dog with visceral leishmaniasis was used. Strain identification was performed by genetic sequencing with the aid of an ABI 3500® Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). The strain was cultured in Schneider medium (Vitrocell-Embriolife, São Paulo, Brazil) supplemented with 10% fetal bovine serum (FBS), 2% sterile human urine, and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) (Vitrocell-Embriolife, São Paulo, Brazil) at 26 °C [28].

2.3.2 Anti-Leishmania Assay

Initially, fixed oil of *C. coriaceum* was diluted in DMSO (Merck, Darmstadt, Germany), obtaining an initial concentration of 15 mg/mL, which was further diluted in phosphate-saline buffer (pH 7.0) to reduce the concentration of DMSO, so that it does not exert any activity on the test cells. The promastigote forms of L. infantum were cultivated in 96-well plates (Kasvi, São José do Pinhais, PR, Brazil) under the same conditions mentioned above and incubated in triplicate in the presence or absence of oil at concentrations ranging from 1,024 to 8,192 µg/ ml. After 24, 48, and 72 h of growth, viable promastigote strains were counted in a Neubauer mirror chamber [28].

2.4 Chemical Composition of *C. coriaceum* fixed oil

Initially, the oil from the fruits of *C. coriaceum* was subjected to transesterification reactions using methanol and KOH as a catalyst. Subsequently, the methyl esters were analyzed using a gas chromatograph coupled to mass spectrometry [29].

The gas chromatography (GC) analysis was performed with Agilent Technologies 6890N GC-FID system, equipped with a DB-5 capillary column (30 m × 0.32 mm; 0.50 µm) and connected to an FID detector. The thermal programmer was 60 °C (1 min) to 180 °C at 3 °C/min; injector temperature 220 °C; detector temperature 220 °C; split ratio 1:10; carrier gas Helium; flow rate: 1.0 mL/min. The injected volume of methyl esters of *C. coriaceum* fixed oil was 1 µL diluted in chloroform (1:10). Two replicates of samples were processed in the same way. Component relative concentrations were calculated based on GC peak areas without using correction factors.

Identification of the constituents was performed based on retention index (RI), determined concerning the homologous series of *n*-alkanes, C_7–C_{30}, under identical experimental conditions, compared with the mass spectra library search (NIST and Wiley), and with the mass spectra literature date Adams [30]. The relative amounts of individual components were calculated based on the CG peak area (FID response).

2.5 Statistical analysis

All tests were performed in triplicate, and their averages and their respective standard errors were calculated. Subsequently, they were submitted to a one-way analysis of variance (One-way ANOVA) using the Tukey test with 95% reliability. Median inhibitory concentrations (IC50) were calculated using non-linear regression analysis. All analyzes were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, United States).

Results & Discussions

After 24 h of observation, the fixed oil from the fruits of *C. coriaceum* showed no anti-*Leishmania* effect at concentrations of clinical relevance (Figure 2a), having an IC50 of 7,300 µg/mL. Total inhibition of the growth of the promastigote forms of *L. infantum* was observed in 72 h of observation at the highest concentration evaluated. However, according to Houghton et al. [31], concentrations greater than 1,000 µg/mL are not considered clinically relevant.

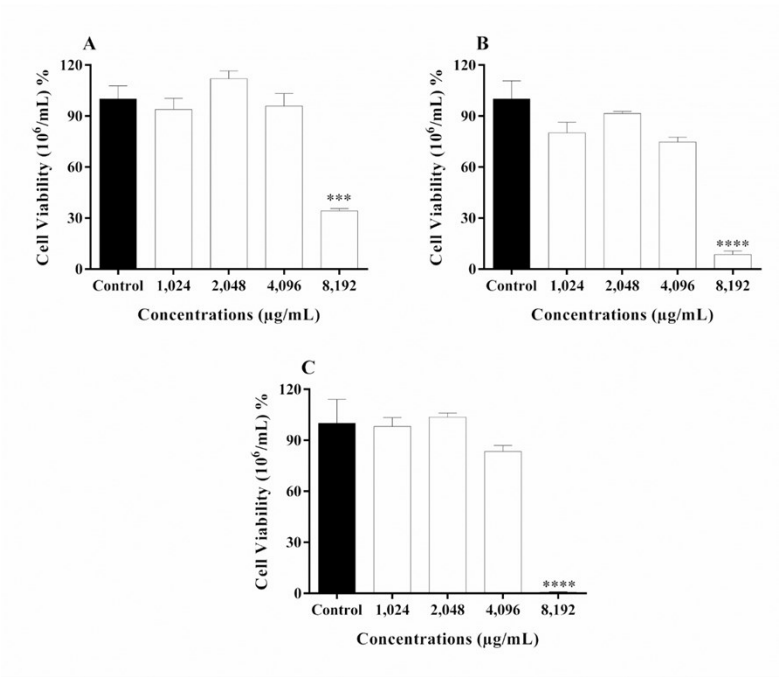

Figure 2: *Percentage of antipromastigote activity of Caryocar coriaceum Wittm fixed oil. against strains of Leishmania infantum (C8). Values are means ± standard error (n = 3). *** = p < 0.001 and **** = p < 0.0001 (Tukey, test). A: 24 h exposure to C. coricaeum oil; B: 48 h exposure to C. coriaceum oil; C: 72 h of exposure to C. coriaceum oil.*

Although the fixed oil of *C. coriaceum* has no leishmanicidal effect, [25] showed that ethanol extracts from the bark and pulp of *C. coriaceum* showed significant and promising results against the promastigote forms of *Leishmania (Leishmania) amazonensis* (MHOM/BR/1989/166MJO). Which had an IC50 of 38 µg/mL and 30 µg/mL respectively. These results were significant, as they had the same effects as the positive controls (pentamidine and meglumine antimoniate).

The gas chromatography revealed that the fixed oil of *C. coriaceum* has unsaturated and saturated fatty acids as the main compounds, oleic acid (59.46%) and palmitic acid (33.58%). Totaling 93.04% of the chemical composition of the fixed oil (Table 1).

Table 1- Chemical composition of the fixed oil of Caryocar coriaceum Wittm.

Fatty acid	**Yield (%)**
Palmitic acid (C16:0)	33.58
Stearic acid (C18:0)	2.96
Oleic acid (C18:1)	59.46
Linoleic acid (C18:2)	2.33

Chemically, the oil from the fruits of *C. coriaceum* has saturated and unsaturated fatty acids, including oleic acid, palmitic acid, stearic acid, linoleic acid, and behenic acid, lignoceric acid, 11-eicosenoic acid and methyl 18-methyl-nonadecanoate [29, 32]. There are no studies evaluating the chemical compounds present in the oil of *C. coriaceum* fruits in isolation against *L. infantum*, however, there are studies evaluating the leishmanicidal effect of these fatty acids against other *Leishmania* species.

As, for example, Saini and Rai [33], demonstrated that linoleic acid alone can protect macrophages by providing an immune response against *Leishmania donovani* infection. Such acid acts by inhibiting the release of microvesicles derived from such protozoa, thus promoting a Th-1-type immune response in macrophages.

Conclusion

Despite the ethnomedicinal use of fixed oil from the fruits of *C. coriaceum* for the treatment of infectious and parasitic diseases, including wound healing, one of the clinical manifestations of leishmaniasis, this study demonstrated that this product is ineffective against the species of *L. infantum*. Such inefficiency can be justified by the fact that this parasite causes the visceral form, and not the tegumentary form, which is caused *by Leishmania braziliensis, Leishmania guyanensis,* and *Leishmania amazonenses*. Therefore, studies evaluating the action against these parasites should be carried out.

Conflict of Interest

The authors declare no conflict of interest.

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