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Evaluation of Extracts of *Qualea paraensis* Ducke for their Antimicrobial, Toxic and Anticholinesterase Activities

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ABSTRACT

Popularly known as red mandioqueira, '*mandioqueira vermelha*', *Qualea paraensis* Ducke is a plant species belonging to the family Vochysiaceae, with a natural distribution in the Amazon region. It is used in traditional medicine, by native communities of the Amazon and Bolivia, for the treatment of skin lesions caused by microorganisms. Previous studies of the species have found antimalarial activity in vivo assays. However, studies involving the investigation of numerous biological activities of *Q. paraensis* are incipient. Biological assays already performed with plants of other species of the genus *Qualea* have shown promising biological activities. Therefore, this study describes the evaluation of the biological activities (bactericide, fungicide, toxicity, and anticholinesterase) of an ethanolic extract of the bark of *Q. paraensis* from the state of Roraima, Brazil. For the evaluation of the toxicity of the extract, a system with microcrustacean *Artemia salina* was used. Antimicrobial activity was tested for the pathogenic groups of fungi (*Aspergillus flavus* and *Fusarium proliferatum*), Gram-negative bacteria (*Escherichia coli* and *Salmonella tiphymurium*), and Gram-positive bacteria (*Staphylococcus aureus* and *Streptococcus sanguinis*). The potential of the extract for the inhibition of the enzyme acetylcholinesterase (AChE) was also evaluated. The assays for determining the antimicrobial activity for Gram-positive bacteria revealed satisfactory IC_{50} (29.98µg/mL) inhibition values for *S. sanguinis* strains, showing inhibition of 64.6% of their growth. The assay for *S. aureus*, however, presented low inhibition. For Gram-negative bacteria, there was moderate inhibition of *E. coli* strains. The extract showed low toxicity to *A. salina* and inhibition of 23.66% of the AChE enzyme.

Keywords: Biological activities, Natural products, Amazonia, Roraima.

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INTRODUCTION

Brazil is a country known for its richest biodiversity in the world. Of the 200,000 existing plant species, half of them have therapeutic properties. However, it is assumed that only about 1% of these species have been adequately studied for their medicinal properties. The use of medicinal plants in the treatment of diseases is influenced by diverse cultures such as indigenous, African, and European. Plants are therapeutic sources that contribute to the supply of numerous substances useful in the treatment of various diseases. [1]

The family Vochysiaceae, composed of trees and shrubs, has about eight genera and 200 species, but only six genera and approximately 150 species are found in Brazil. [2] This family is frequently found in the Cerrado biome, which is considered floristic and phyto-physiognomically important in South America. Such heterogeneity is directly associated with the chemical and physical characteristics of the soil. [3-4] The distribution of the family is amphi-Atlantic, where six of the eight genera are predominantly neotropical. [5] The most important genera are *Salverti, Callisthene, Qualea*, and *Vochysia*. [6]

The species of Vochysiaceae can be classified as the most beautiful and most representative plants of the Cerrado. [7] The genus *Qualea* is present throughout tropical America. The species are popularly known as pau-terra, mandioqueira, or quaruba, and used in traditional medicine for a wide variety of purposes. [8]

Biological assays already carried out with plants of the genus *Qualea* have shown good results. A crude extract of *Qualea grandiflora* demonstrated action in the central nervous system, behaving as an anticonvulsive agent and analgesic. [9] A methanolic extract of *Qualea parviflora* demonstrated gastroprotective action. [10]

Among the most common species of this genus is *Qualea paraensis* Ducke, popularly known as 'mandioqueira vermelha'. In Brazil, *Qualea paraensis* has a natural distribution in the states of Amazonas, Pará, Rondônia, Roraima, and Mato Grosso. It can also be found in French Guiana, Peru, and Colombia. [11] The water from the decoction of the stem bark of *Qualea paraensis* is used in baths for the treatment of scabies by the Chacobos, a native community living in the Amazonian part of Bolivia. [12] An in vivo assay by Muniz *et al.* (2000) found that the species *Qualea paraensis* has antimalarial activity. [13]

Considering the limited number of studies of the species, the present work aims to investigate the antimicrobial activity of an extract of *Q. parensis*, as well as its toxicity and its inhibitory effect on the enzyme acetylcholinesterase.

MATERIALS AND METHODS

Plant material

The *Q. paraensis* bark samples were collected in the municipality of Rorainópolis in the state of Roraima, Brazil, in August 2014. The botanical material was identified by Tiago Monteiro Condé, and exsiccates were deposited in the laboratory of Embrapa Roraima and in the Herbarium of the Integrated Museum of Roraima under the code MIRR 6628.

Extraction of the plant materials and sample preparation

The bark samples were dried at room temperature, crushed, and pulverized in a blender, resulting in 1.660 kg of dry powder, which was transferred to a Mariotte bottle. The exhaustive maceration technique was used in the extraction process. The extraction solvents were used in increasing order of polarity; hexane was used first, followed by doubly distilled ethanol. The extracts were filtered and then concentrated under reduced pressure on a rotary evaporator, yielding 26.013 g of ethanolic extract.

Antimicrobial activity

Bacterial and fungal strains

For determination of the antimicrobial activity against fungi and bacteria, the fungi *Aspergillus flavus* and *Fusarium proliferatum*, the Gram-negative bacteria *Escherichia coli* (ATCC 25922) and *Salmonella tiphymurium* (ATCC 14028), and the Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Streptococcus sanguinis* (ATCC 49456) were tested.

Antibacterial bioassay

For the bioassays with Gram-positive and Gram-negative bacteria, the samples were weighed and solubilized in dimethyl sulfoxide (DMSO), resulting in a solution with a concentration of 50 mg/mL for the ethanolic extract. A 40µL portion of this solution was added to a flask containing 960µL of BHI (Brain Heart Infusion) culture medium, generating the working solution. A pre-inoculum was prepared in which the microorganisms stored in test tubes were transferred with a platinum loop and inoculated into test tubes containing 3.0 mL of BHI culture medium. The tubes were incubated in an oven at 37°C for 18 h. A 500µL portion of the pre-inoculum was transferred to the test tubes containing 4.5 mL of sterile distilled water. The solution in the tubes was homogenized and the spectrophotometer adjusted concentration was compared to the standard 0.5 turbidity McFarland scale (10^8 UFC/mL), thus forming the innocuous used in the test. [14-15]

The tests were performed in duplicate on 96 micro well plates. A 100µL portion of the BHI culture medium was added to each well. In the first well, 100µL of working

solution were added. The solution was homogenized and 100µL were transferred to the next well. The process was repeated until eight concentrations of each sample were created. Then, 100µL of the standardized inoculum containing each microorganism to be tested were added to the respective wells. Two controls were performed, one to evaluate the growth of the microorganism, in which there was no addition of the working solution (to verify the cellular viability), and the other one being white, in which the innocuous was not added (to eliminate the effect of colouring the working solution). A control plate containing 100µL of BHI culture medium and 100µL of sterile distilled water was added to the assay as a sterility control of the BHI culture medium. After incubating the microplates at 37°C for 24 h, they were read with an Elisa plate reader (492 nm). For the construction of the graph and the calculation of IC_{50} (mean inhibitory concentration), the program GraphPad Prism 5.0 was used. [14-15]

Antifungal bioassay

In the bioassays for the filamentous fungi *A. flavus* and *F. proliferatum*, the medium used for the growth of the microorganisms was *Sabouraud* broth. The concentration of the inoculated spore suspensions was 5×10^{-5} spores/mL. The samples were weighed and solubilized in DMSO, resulting in a solution with a concentration of 250 mg/mL. The tubes were incubated in an oven at 37°C for 48 h. After incubation, analysis was performed with a microtiter plate reader using a wavelength of 490 nm. [16] Miconazole was used as a positive control. For the statistical treatment of the data, we used the Grubbs test with a significance level of 95%. The percent of inhibition was calculated using the formula

$$\% \text{ inhibition} = 100 - \frac{(EC - CC)\, x\, 100}{CH - CM}$$

EC: test absorbance
CC: sample control absorbance
CH: absorbance of fungus control
CM: absorbance of control of culture medium

Brine Shrimp Lethality Bioassay

The Brine Shrimp lethality bioassay was performed according to Meyer *et al.* [17] with some modifications. The samples were prepared by suspending 100 mg of the extract in saline with the addition of 1% Tween-80 (stock solution). This was further diluted to prepare other solutions of lower concentration (500, 250, 125, 62.5, and 31.25µg/mL). For the control solution, only saline and 1% Tween 80 were used. The pH was adjusted to between 8.0 and 9.0 with 10% Na_2CO_3. Brine shrimp (*Artemia salina*) eggs were placed in a system that was assembled from a glass aquarium with a capacity of 1 L, coupled to an air diffuser pump for 36 hours, left under a luminescent lamp, and kept at a temperature of 25°C. Egg hatching was monitored every 12 hours.

The tests were performed in triplicate. After hatching of the eggs, ten nauplii were transferred to each tube containing the samples and the control. The count of live and dead nauplii was performed after 24 hours. Extracts and fractions are considered active when LC_{50} values are less than 1000µg/mL. [17] The percent mortality was determined according to the formula

$$M\% = \frac{r\, x\, 100}{n}$$

r: number of dead nauplii
n: number of total nauplii in the test tube

Micro-plate assay for the inhibition of Acetylcholinesterase (AChE)

The inhibition of acetylcholinesterase activity was determined using Ellman's colorimetric method. [18] To evaluate the anticholinesterase activity, 25µL of the working solution (10 mg/mL DMSO sample) were added to the wells of the test Elisa plate, including the negative and positive controls. In the first five wells of the positive control column, 25µL of the eserin solution (10 mg/mL in Tris/HCl pH 8.0 buffer) were added. Additionally, 25µL of acetylcholine iodide solution, 125µL of the solution of 5',5-dithiobis-(2-nitrobenzoate) (3 mM) (Sigma), and 50µL of Tris/HCl (50 mM) were added to each well of bovine serum albumin. The absorbance was measured at 405 nm every minute for eight minutes. A 25µL portion of the AChE solution (0.226 U/mL) in Tris/HCl was added to each well. The absorbance at 405 nm was measured 10 times over ten minutes. The percent inhibition was calculated by comparing the sample rates with the eserin control.

RESULTS

Antimicrobial activity

Through the antimicrobial assay, it was possible to verify the activity of the ethanolic extract obtained from the bark of *Q. paraensis* against the strains of the microorganisms evaluated. The ethanolic extract presented inhibitory activity of 64.594% for the Gram-positive *S. sanguinis* bacterium, and was less active against *S. aureus* (33.661%). As for the Gram-negative bacteria, the extract showed activity only against *E. coli* (45.689%). There was no activity against the filamentous fungus *F. proliferatum*, but there was 14.91% inhibition of the *A. flavus* species.

The strains of *S. sanguinis* and *E. coli* were more sensitive to the extracts analysed than *S. aureus* and *S. tiphymurium* when compared with the positive control ampicillin, whose activity reached 100%. Due to the presence of significant inhibition values, the IC_{50} of the ethanolic extract for *S. saguinis* was calculated.

Brine Shrimp Lethality Bioassay

After exposure for 24 h, live and dead nauplii were counted (Table 2). The degree of lethality was directly proportional to the concentration of the extract. At its highest concentration (1000µg/mL), the mortality was 63%. Based on these results, the ethanolic extract presented low lethality (LC_{50} = 844.35µg/mL) to the microcrustacean, and thus is considered to have low toxicity based on the found LC_{50} value being more than 500µg/mL. [19]

Table 1: Percentage of fungal inhibition

Fungus/control	% inhibition of growth
A. flavus	14.91
F. proliferatum	0
Miconazole	93.72

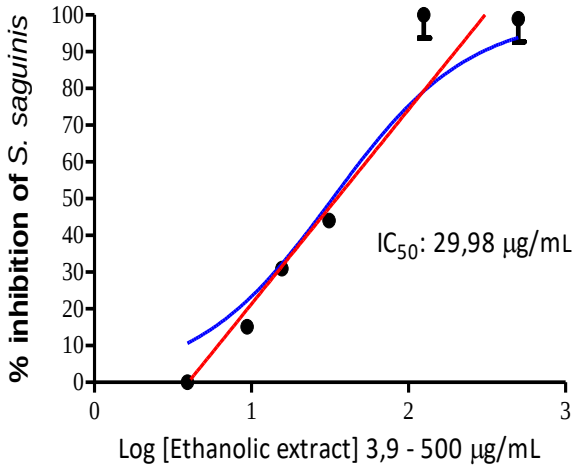

Fig. 1: IC_{50} for ethanolic extract of *Q. paraensis* em *S. saguinis*

Table 2: The number of shrimp nauplii dead after treating with the plant extract and the percentage mortality

Concentration (µg/mL)	Number of dead nauplii after 24 h	% Mortality
1000	6.3 ± 1.52	63
500	2.6 ± 0.57735	26
250	0	0
125	0	0
62.5	0	0
31.25	0	0

Micro-plate assay for inhibition of Acetylcholinesterase (AChE)

The inhibition analysis of the enzyme acetylcholinesterase showed that the ethanolic extract of *Qualea paraensis* has a low degree of inhibition, as shown in Table 3.

Table 3: Percent AChE inhibition for the ethanolic extract of the bark of *Q. Paraensis*

Sample	% inhibition of growth
EEQP	23.66 ± 4.25
Eserin	92.93 ± 0.10

EEQP = ethanolic extract *Qualea Paraensis*

DISCUSSION

The search for new antimicrobial agents is very important, since the high potential for the genetic recombination of bacteria has led to the increase in multi-resistant strains. Pathogens can arise more quickly due to the indiscriminate use of antimicrobial agents and, consequently, making many inefficient antimicrobial drugs available on the market. [20-21]

The assays of the ethanolic extract for the determination of antimicrobial activity of *Q. paraensis* for Gram-positive bacteria revealed satisfactory inhibition values for *S. sanguinis* strains (IC_{50} = 29.98µg/mL), showing inhibition of 64.6%. However, there was low inhibition of *S. aureus*. For Gram-negative bacteria, the extract was able to inhibit only the *E. coli* strains, which showed a moderate inhibition close to 50%. The low activity seen for some bacteria and fungi is interesting, as it presents indications of specificity of the mechanism of action against *S. sanguinis* and *E. coli*.

The Brine Shrimp Lethality assay is considered a preliminary assay because it is a simple, low cost, and efficient method that shows a good correlation with cytotoxicity. [17] The low toxicity presented by the ethanolic extract of *Q. paraensis* demonstrates promising results for its intracellular mechanism of action, and may show good selectivity.

The degree of inhibition of AChE enzyme with plant extracts is classified as potent for values above 50% inhibition, moderate for values of 30-50%, and weak for values below 30%. [22] Therefore, the ethanolic extract of *Q. Paraensis*, which presented a degree of inhibition of the enzyme AChE of 23.66%, is considered a weak inhibitor. However, because it is a mixture of active compounds in low concentration, it is possible that more detailed studies on the chemical composition of the ethanolic extract of *Q. paraensis* can identify, in significant quantities, the active component responsible for the anticholinesterase activity of the extract studied here. Finally, the study contributes to an increase in the knowledge about the Amazonian species studied, bringing unpublished results that will help in the search for new phytotherapeutic drugs and their possible active principles.

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