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Phytochemical study and biological activity of the bark of the stem of the plant species araticum bravo (Annona tomentosa R. E. Fr.)

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ABSTRACT

Annona tomentosa R. E. Fr. (Annonaceae), known in Brazil as Araticum-bravo or Araticum de moita, is a small tree found throughout the Brazilian territory, mainly in coastal areas, its distribution is discontinuous, appearing in open fields, in the cerrado of shrubby trees and in the cerradão, where the vegetation is denser. All parts of this vegetable are used in folk medicine, it has been used mainly to combat diarrhea, stomatitis, headache, neuralgia, boils, ulcers and in the eradication of lice in addition to antirheumatics. Based on data obtained by
In a bibliographic survey, this work sought information of chemical natures, through phytochemical and microbiological screening by antimicrobial activity tests. In the phytochemical screening, the presence of substances belonging to the classes of phenols, tannins, anthocyanins, anthocyanidin, flavonoids, xanthones, saponins, steroids, triterpenes and alkaloids were confirmed. Confirmation for alkaloids was performed by chromatoplates. Standard strains (ATCC) of Gram-positive *Staphylococcus aureus* 25923 bacteria and Gram-negative bacteria were used to evaluate the antimicrobial activity *Pseudomonas aeruginosa* 27853, *Escherichia coli* 35218, *Klebsiella pneumoniae* 700603. Clinical isolates of *Acinectobacter baumannii* and the fungus *Candida albicans were used in the microbiological tests*. The antimicrobial potential of the extract was analyzed by the technique of perforation in Müller Hinton medium, the results showed that the peels of *A. tomentosa* have an inhibitory potential of growth on the Gram-positive bacterium, *S. aureus*, and on the clinical microorganisms with emphasis on *A. baumanni* with halo formation of (20mm) respectively.

Keywords: Annonaceae, Phytochemical screening, Antimicrobial activity.

INTRODUCTION

The Annonaceae family stands out for having a large number of species of industrial interest, mainly for food, as they are fruit trees, found mainly in regions of tropical and subtropical climate. This plant family comprises about 2500 species, distributed in 135 genera. The genus (CHATROU et al., 2012) *Annona* L. contains about 162 species of trees and shrubs, 60 of which are found in Brazil, mainly in forests (CHATROU et al., 2012) . This genus is one of the most studied, mainly because of its pharmacological activities, being most of the representatives used in folk medicine, in the cure of various diseases (AMALA DEV; JOSEPH, 2021) .

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Studies with plants of the genus *Annona* demonstrate the pharmacological activities of extracts of leaves, stems, fruits and seeds, being observed anti-inflammatory, antitumor, anti-HIV, anti-protozoal, anti-parasitic, antidiabetic, analgesic, gastroprotective, antihypertensive, and hepatoprotective activities, being attributed these activities mainly to the presence of acetogenins in plant extracts (B; Varadharaj, 2017) .

Several classes of compounds of secondary metabolism are found in plants belonging to the genus, and terpenes such as camphor and borneol isolated from the roots of *A. squamosa*, the acetogenins epomurinin, tucupentol and cornifolina isolated from (Leboeuf et al., 1980) *A. muricata*, *A. montana*, and *A. carnifolia* have been identified, respectively, alkaloids of the benzylisoquinoline classes, such as reticulin, apophynics, such as asimylobin and anonain, and oxoapophynics, such as liriodenine, isolated from (MELOT et al., 2009; SANTOS; BOAVENTURA; PIMENTA, 2006) *A. foetida*, and *A. salzmannii* (COSTA et al., 2013, 2015; DUTRA et al., 2012) . Due to the common occurrence of the alkaloids anonain and liriodenine, they can be considered chemotaxonomic markers of this genus. The presence of flavonoids such as quercetin and kaempferol are also identified. (SAINTS; SALATINO, 2000)

Annona tomentosa, popularly known as araticum bravo or araticum de moita, is a small tree, distributed throughout the Brazilian territory, being found mainly in the biomes of the Amazon, in the Cerrado and in the Pantanal, it has edible fruits, and the tea from its leaves are used in the treatment of inflammations in folk medicine. The study carried out by Santos and Salatino demonstrates the presence of kaempferol-3-O-rhamnosylglycoside, quercetin-3-O-rhamnosylglycoside and luteolin-7-O-glycoside in the extracts of the leaves of this species. (ZAPPI et al., 2015) (CARNEIRO et al., 2017) (2000)

As it is a plant species with few studies, the objective of this work is to identify the main classes of compounds from the secondary metabolism of this plant through a phytochemical screening, and to evaluate the antimicrobial and antifungal activities of the crude extract of the leaves.

METHODOLOGY

COLLECTION, IDENTIFICATION AND PRODUCTION OF CRUDE HYDROALCOHOLIC EXTRACT

The stems of the plant species *Annona tomentosa* were collected at the Bacanga campus of the Federal University of Maranhão in February 2015. The identification of the plant was made at the Rosa Mochel Herbarium, of the State University of Maranhão, with exsicata deposit under number 3773. After collection, the material was cleaned, dried for five days and dehumidified in an oven at 45 °C for 24 hours. Then the material was crushed in an electric mill.

The crude extract of the stem bark (200g) was obtained through exhaustive maceration with an ethanol:water mixture (70:30). The extract obtained was filtered, with 100 mL separated for biological activity and the remainder concentrated under reduced pressure in a rotary evaporator, to obtain the concentrated crude extract.

YIELD DETERMINATION AND PHYTOCHEMICAL PROSPECTION OF THE HYDROETHANOLIC EXTRACT

The extraction yield was determined by transferring aliquots of 1 mL of the extract to previously weighed vials. The material was dried in a heated air circulation and weighed again to obtain the dry mass. The phytochemical screening was performed according to the methodology proposed for the tests indicated below: MATOS, 2009

Test for Phenols and Tannins

5 mL of the hydroalcoholic extract was added to a test tube, where 3 drops of ferric chloride solution ($FeCl_{3)}$ were added. The tube was well agitated and color variation or formation of dark precipitate was observed. Color variation between blue and red indicates the presence of phenols. The formation of a dark precipitate of blue hue indicates the presence of pyrogallic tannins, and if green, the presence of condensed or catechic tannins.

Testing for anthocyanins, anthocyanidins, and flavonoids

5 mL of the hydroalcoholic extract was added to three test tubes. One tube was acidulated at pH 3, and the other two were alkalized at pH 8.5 and 11, respectively. A change in color was observed. A change in color to red in the acid tube suggests the presence of anthocyanins and anthocyanidins or chalcones and aurones. Appearance of lilac coloration in the alkaline tube pH 8.5 suggests the presence of anthocyanins and anthocyanidins. For the tube with pH 11 the appearance of blue-purple color indicates the presence of anthocyanins and anthocyanidins, if yellow, it indicates the presence of flavones, flavonols and xanthones, if red purpura, chalcones and aurones, and if red orange, flavonols.

Test for leukoanthocyanidins, catechins and flavones

5 mL of the hydroalcoholic extract was added to two tubes. One was acidulated at pH 3 and the other alkalized at pH 11. The tubes were heated for 2 minutes, and color changes were observed. In this test, the appearance of a red color in the acid tube indicates the presence of leucoanthocyanidins, and yellowish-brown indicates the presence of catechins. For the alkaline tube, the appearance of a red-orange color indicates the presence of flavones.

Test for flavonols, flavanones, flavanonols and xanthones

To a tube was added 5 mL of the hydroalcoholic extract, a piece of magnesium tape and 0.5 mL of concentrated hydrochloric acid (HCl). At the end of the reaction, color changes were observed. Appearance or intensification of red coloration indicates the presence of free flavonols, flavanones, flavananols, and/or xanthones or their heterosides.

Test for confirmation of catechins

A matchstick was moistened in the hydroalcoholic extract, then the solvent was dried, and again moistened, only on one side, in concentrated HCl. The toothpick was heated for 2 minutes on fire, and the appearance of staining was observed on the acidulated side of the toothpick. The appearance of color indicates the presence of catechins.

Test for steroids and triterpenoids (Lieberman-Burchard)

A beaker was transferred to 10 mL of the hydroalcoholic extract and then evaporated the solvent. Chloroform (CHCl 3) was added three times to the dry residue. The solution was filtered and dried with anhydrous sodium sulfate (Na2SO4), and then transferred to a test tube. 1 mL of anhydrous acetic was added and the tube agitated, then 3 drops of concentrated sulfuric acid ($H2SO4$) were added. The tube was shaken and staining was observed.

Test for saponins

From the previous test, the chloroform-insoluble part was dissolved in 10 mL of distilled water, the solution being filtered and transferred to a test tube. The tube was shaken strongly for 2 minutes and foaming was observed.

Test for confirmation of saponins

To the contents of the tube used in the previous test, 2 mL of concentrated HCl were added and kept in a water bath for 1 hour. After the tube was allowed to cool to room temperature, neutralized, and again stirred for observation of foaming.

Alkaloid test

Ammonium hydroxide (NH4OH) was added to one third of the remaining hydroalcoholic extract up to pH 11, followed by an organic extraction with three successive portions of 30, 20 and 10 mL of an ether:chloroform solution (3:1). The organic fraction was dried with anhydrous sodium sulfate and then washed with an acid solution of hydrochloric acid at 0.1 mol/L. The organic fraction was discarded, and

the acid fraction was divided into three test tubes. In tube one, three drops of Hager's reagent were added, in tube 2, three drops of Mayer's reagent, and in the last tube, three drops of Dragendorff's reagent. The formation of precipitates indicates the presence of alkaloids. By me, a chromatoplate run was performed with the organic fraction (ether:chloroform), and developed with modified Dragendorff reagent.

ASSESSMENT OF MICROBIOLOGICAL ACTIVITY

For the microbiological evaluation, standard ATCC strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), and Gram-negative *bacteria Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), and clinical isolates of *Acinectobacter baumannii* and the fungus *Candida albicans were used*.

To perform the assays, the microorganisms were reactivated from their original cultures and maintained in BHI liquid medium at 37 °C for 24 hours for the bacterial strains and 48 hours for the fungal strains. Subsequently, the strains were cultured on nutrient agar plates at 37 °C for 24 hours for the bacteria and for 48 hours for the fungal strains. The isolated colonies were then resuspended in 3 mL of sterile 0.89% NaCl saline solution until a turbidity of 0.5 on the McFarland scale was reached, equivalent to 1.5 x 10^8 bacteria/mL.

The antimicrobial potential of the hydroalcoholic extract was evaluated using the technique of drilling wells in Müller Hinton medium. Initially, the microorganisms were seeded and then the medium was perforated with 6 mm diameter cylinders. In the wells, 100 μL of the extract was added in addition to the positive controls chloramphenicol (0.4 μg/mL) and ketoconazole (80 mg/mL). The plates were incubated at 37 °C for 24 hours for bacterial strains and for 48 hours for fungal strains. After incubation, the diameter of the growth inhibition halo was measured, when present (CLEELAND; SQUIRES, 1991).

RESULTS

EXTRACTION YIELD AND PHYTOCHEMICAL PROSPECTING

The dry weight of the hydroalcoholic extract was determined by gravimetric analysis, with a value of 0.046 mg/mL, and a yield of 17%.

The phytochemical prospection of the crude extract of the bark of *Annona tomentosa* showed the presence of several classes of compounds from secondary metabolism. Table **01** below shows the identified compounds.

Table 01: Phytochemical prospection of the crude extract of Araticum bravo (*A. tomentosa*)

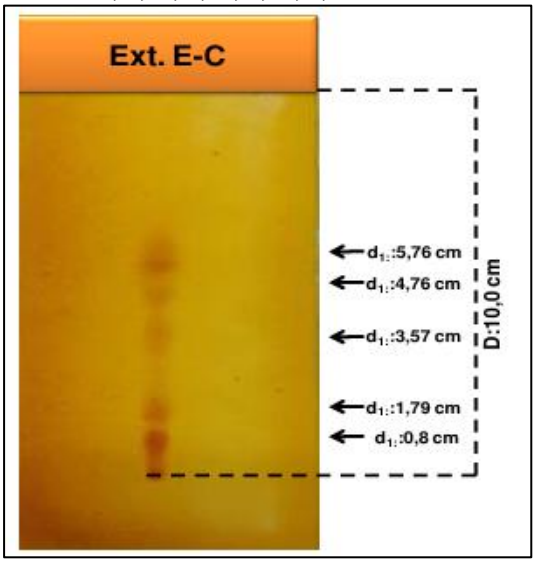
Compound class	**Results**
Condensed or catechic tannins	++

Phenols	++
Flavonoides, antocianidinas e antocianinas	++
Leukoantocyanidines, catechins and flavonones	+++
Catechins (confirmation)	+++
Esteroides Livres	++
Saponins (Saponin Heterosideos)	+++
Confirmation of saponins	+++
Alkaloids	+++
Strong: +++, Medium: ++, Weak: +, Suspect: S, Insufficient: -	

The compounds identified in the phytochemical prospection demonstrate the presence of several classes of compounds in the plant extract evaluated, among them, the presence of tannins, flavonoids, saponins and alkaloids in the bark of araticum bravo stand out. Tannins are widely used in leather tanning, in addition to preventing lipid peroxidation and nucleotide degradation and accelerating the healing process. Compounds of the flavonoid class have antioxidant, anti-inflammatory and anticancer activities, saponins, capable of foaming when present in plant extracts, have hemolytic, antiviral and anti-inflammatory activities. Compounds of the alkaloid class have several biological activities, being observed the antitumor, anesthetic, antimalarial, and antidepressant activities, thus being a class of compounds of great pharmacological importance. (ALMEIDA et al., 1998) (LOPES; SCHULMAN; HERMES-LIMA, 1999; PIETTA, 2000) (PANIZZA et al., 1988) (GREENS; BRIGHENTE; PIZZOLATTI, 2005) (SIMÕES et al., 2007) (BRUNETON, 1999)

In **Figure 01** it is possible to observe several *spots* confirming the presence of alkaloids after elution of the ether-chloroform fraction in a chromatographic plate, revealed with the modified Dragendorff reagent.

Figure 01: Plate of the ether-chloroform extract using dichloromethane:methanol (95:5) as a mobile phase, developed with a modified Dragendorff reagent, with its RFs= 0.08; 0,18; 0,36; 0,48; 0,58.



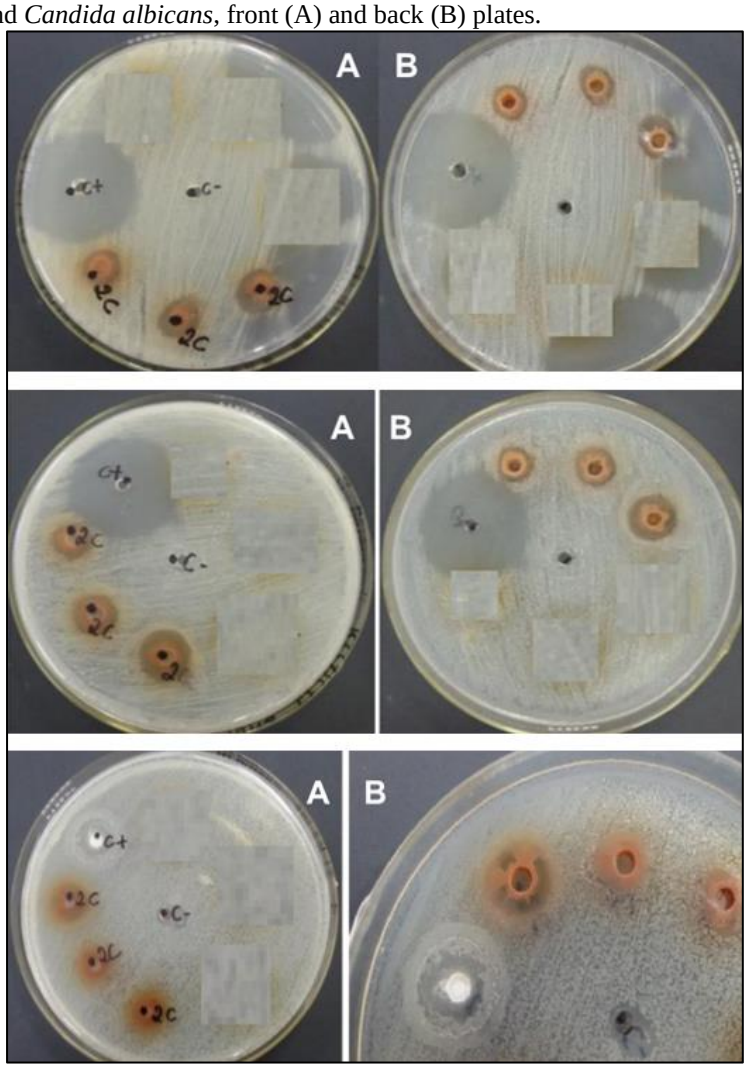
ANTIMICROBIAL ACTIVITY *IN VITRO*

Table **02** shows the inhibition halos measured for the hydroalcoholic extract. The results were evaluated considering as strong inhibition, halos with a diameter greater than or equal to 15 mm, medium inhibition, halos with diameters between 10 and 15 mm, and inactive, when the observed halos are less than 10 mm (CIMANGA et al., 2002) .

Table 02: Results of the perforation test for antimicrobial activity of the hydroalcoholic extract of *Annona tomentosa bark*

Microorganisms	**Inhibition halos(mm)**
	Hydroalcoholic Gross Extract
Escherichia coli ATCC 35218	R
Pseudomonas aeruginosa ATCC 27853	10
Klebsiella pneumoniae ATCC 700603	12
Staphylococcus aureus ATCC 25923	20
Acinectobacter baumannii (clinical isolate)	20
Candida albicans (clinical isolate)	15
A: Durable	

Figure 02: Puncture test plates after 24 (bacteria) and 48 hours (fungus). From top to bottom, *Staphylococcus aureus, Acinectobacter baumannii* and *Candida albicans*, front (A) and back (B) plates.



Compared to the microorganisms evaluated, a better response of the crude extract of the bark of *A. tomentosa* to the Gram-positive bacterium *S. aureus* (20 mm) to the clinical isolates *C. albicans* (15 mm) and *A. baumannii* (20 mm) was observed. Studies have demonstrated the activity of plant extracts from the stems of *Annona crassiflora* against the bacterium *S. aureus*, with MIC of 1.56 mg/mL, and also against (SILVA et al., 2014) *C. albicans* with an inhibition halo of 11 mm (AMARO et al., 2017) . Hydroalcoholic extracts of the species *A. muricata* also showed activity against the bacterium *A. baumannii*, with an inhibition halo of 16 mm. (MEAGHAN BRUSKOSKI et al., 2022)

Studies have shown that the most frequently isolated pathogens in Brazilian hospitals were *S. aureus* (22.8%), *E. coli* (13.8%) and *Pseudomonas aeruginosa* (13.3%), however, fungal infections have also become important causes of nosocomial infections, especially among immunosuppressed patients, or those with other predisposing factors. (SADER et al., 2001) (MIRANDA et al., 2003)

In the experiments, no microbiological activity was observed against *E. coli* bacteria, and studies indicate that the biochemical complexity of Gram-negative bacteria makes them less susceptible to antimicrobial agents. Gram-negative bacteria have an outer membrane that gives this group of microorganisms an effective permeability barrier, restricting the penetration of some compounds. The understanding of this biochemical difference between Gram-positive and Gram-negative bacteria is of the highest relevance for the study of mechanisms of action of chemotherapeutics, pathogenicity and other subjects related to the chemical composition and structure of the bacterial wall. (TADEG et al., 2005) (SCHAECHTER et al., 2002)

With the study carried out, it is not possible to associate the antimicrobial activities observed with any compound present in the extract. Further studies of isolation and identification of the compounds in majoritarians are necessary for the identification of the active ingredients. Given the good antimicrobial activity observed, it is possible to indicate the plant species *A. tomentosa* for more specific chemical and biological studies.

FINAL THOUGHTS

From the experimental data obtained, it is observed the presence of several compounds from secondary metabolism, such as tannins, flavonoids, saponins and alkaloids, identified through the phytochemical prospection of the extract, and the presence of alkaloids is also confirmed through a chromatographic plate developed with modified Dragendorff.

The evaluated extracts showed antimicrobial activity against the ATCC strain of *S. aureus* and the clinical isolate of *A. baumannii* with formation of 20 mm inhibition halos in both assays. Activity was also observed against the clinical isolate of *C. albicans*, with a halo of 15 mm, which demonstrates the pharmacological potential of the hydroethanolic extracts of the bark of the stem of *A. tomentosa*.

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