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Phytochemical prospection, identification and evaluation of microbiological activity of secondary metabolites of *Annona mucosa* Jacq

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ABSTRACT

The present work describes the results obtained for the phytochemical and microbiological studies of the 70% hydroethanolic extract, its fractions and metabolites isolated from the leaves of the Biribazeiro (*Annona mucosa* Jacq.). Initially, the preparation of the hydroethanolic extract (70%) of the leaves of the plant was carried out and a phytochemical screening was carried out in search of the secondary metabolites of the study. Tests confirmed the presence of tannins, flavonols, flavanones, flavananols, xanthones, leucoanthocyanidins, anthocyanidins, free steroids,
saponins and alkaloids. Then, the organic fractionation of the crude extract was performed, and through thin layer chromatography of the chloroform fraction, the separation of mixtures of alkaloids and flavonoids that were identified by hyphenated techniques of LC-ESI-IT-MS/MS and FIA-ESI-IT-MS^n, such as the alkaloid romucosin and the anthocyanidin delphinidin-3-O-β-glucopyranoside, was performed. In addition to the isolation and identification of secondary metabolites, this work presents results for microbiological evaluation through the technique of diffusion in solid well drilling medium with samples of 70% crude hydroethanolic extract as well as isolated fractions and metabolites. Through these assays, satisfactory results were obtained against the bacterium *Staphylococcus aureaus* ATCC, the fungus *Candida albicans* and the clinical isolate of *Salmonella spp,* attributing these activities to the presence of the identified alkaloids and flavonoids. The phytochemical results obtained corroborate the botanical identification of the studied species Annona mucosa Jacq. as belonging to the Annonaceae family, due to the presence of substances that are commonly found in plants belonging to this family and through microbiological evaluation as an important source of chemical substances in the production of new medicines.

Keywords: Phytochemical prospection, Alkaloids, Flavonoids, Microbiological activity, *Mucosal Annona*.

INTRODUCTION

Annonaceae belong to the order Magnoliales, and this order is subdivided into the families of Annonaceae, Magnoliaceae and Myristicaceae, comprising about 120 species. In Brazil, it is possible to find, of this family, 29 genera and about 392 species, distributed throughout the national territory. The Annonaceae family is distributed in tropical regions, with its highest occurrence in Central and South America, the Asian continent and Africa (BARON, 2010; MAAS et al., 2011) (CHATROU et al., 2012; MAAS et al., 2011) . The vegetables of this family are cultivated because they have good

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commercial potential, in the use of their wood, or in the production of fruits, in addition to obtaining the essential oil from their flowers. (GALASTRI, 2008; JUDD et al., 2002; LARRY; MATOS, 2002)

In folk medicine, several parts of these plants are used in the cure of ailments, and their biological activities are attributed to the presence mainly of alkaloids, acetogenins, terpenes and flavonoids. Studies demonstrate antileishmania, antioxidant and cytoprotective, cytotoxic activity, presented antitumor, anti-inflammatory, antiviral, as well as hypoglycemic potential, attributing these effects to the presence of flavonoids in the extract and fractions evaluated. (SILVA et al., 2009) (RAYNAUD-LE GRANDIC et al., 2004) (BARRECA et al., 2011) (BETANCUR-GALVIS et al., 1999; FORMAGIO et al., 2013a) (FORMAGIO et al., 2013a, 2013b) (BETANCUR-GALVIS et al., 1999) (FORMAGIO et al., 2013b)

The genus Annona is widely studied due to the presence of several constituents synthesized through the secondary metabolism of these plants. The main metabolites studied for this class of vegetables are acetogenins, isoquinoline alkaloids, diterpenes, steroids, monoterpenes, sesquiterpenes, triterpenes, lactones, flavonoids, and ketones. The most common alkaloids are liriodenine, reticulin and anonain, and chemotaxonomic markers of the genus (RODRIGUES, 2016) (Leboeuf et al., 1980) , among acetogenins, exclusive to annonaceae, anossenegaline, xylomaticin, bulatanocin, anomontacin, anonacin, goniolaticimine and isoanacin, isolated from extracts of *Annona muricata*, present toxicity against snails (*Biomphalaria glabrata*) and larvae of the dengue mosquito (*Aedes aegypti*) . Among the flavonoids, flavones and flavonols and their glycosylated derivatives are the most common, being indicated as those responsible for antioxidant, cardioprotective and anti-inflammatory, anticarcinogenic and antiviral activities. (LUNA, 2006) (SAINTS; SALATINO, 2000) (RICE-EVANS et al., 1995; ROY et al., 2022) (ROY et al., 2022) (LIU; JIANG; XIE, 2010) (MIDDLETON; KANDASWAMI; THEOHARIDES, 2000)

The biribazeiro (*Annona mucosa* sinm. *Rollinia mucosa*) is a medium-sized tree, with yellowish-green flowers, being found in several states of the Brazilian territory. In folk medicine, several parts of this vegetable are used, and its leaves, in the form of teas, are used as a sudorific, carminative, stomachic, antirheumatic and anthelmintic medication, and are also used in the treatment of stomatitis and headaches. For this species, the alkaloids romucosin, anonain, glaucin, purpurein, liriodenine, oxoglaucin, oxopurpurpurein, berberine, and tetrahydroberberine were isolated and identified. Studies have demonstrated the antiplatelet activities of isolated alkaloids, leishmanicidal for the dichloromethane fraction, insecticide, for extracts of leaves, twigs and seeds, and cytotoxic, for extracts and acetogenins isolated from the species. (LORENZI et al., 2006) (CHEN; CHANG; WU, 1996) (KUO et al., 2001) (BARBOZA, 2013; LIMA et al., 2012) (RIBEIRO et al., 2013; TURCHEN et al., 2016) (GU et al., 1997)

The plant species presents documented biological and chemical activities, for some classes of natural compounds present, however, few studies that evaluate the antimicrobial activity of the hydroethanolic extract of the leaves, thus, this work aims to biologically evaluate the crude extract of the vegetable, and identify compounds from secondary metabolism present.

METHODOLOGY

COLLECTION, IDENTIFICATION, OBTAINING OF HYDROETHANOLIC CRUDE EXTRACT AND ORGANIC FRACTIONATION

Leaves of *A. mucosa* were collected in August 2015, in São Luís – MA, Brazil, where the aerial parts were dried under protection from direct sunlight for about one week. After this drying period, the material was separated into leaves and stems, cleaned, crushed, weighed and reserved for a later stage.

The hydroethanolic extract of the leaves (200 g) was obtained by exhaustive cold maceration with an ethanol:water mixture (70:30). The extract obtained was filtered and concentrated under reduced pressure in a rotary evaporator to obtain the concentrated crude 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.

From the concentrated crude extract, a part was separated for organic fractionation with solvents in ascending order of polarity, hexane, followed by chloroform and finally ethyl acetate. The fractions obtained were concentrated in a rotary evaporator and dried completely under a heated air current.

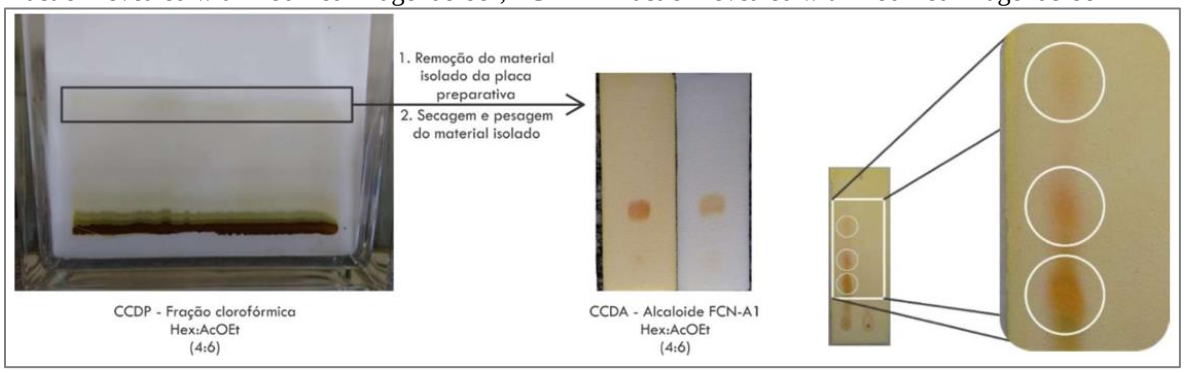
OBTAINING ALKALOID FRACTIONS FOR MICROBIOLOGICAL ANALYSIS

A part of the chloroform fraction (0.44g) was solubilized with 20mL of chloroform, and this fraction was washed in a separation funnel with 25mL of 3% HCl solution, reserving the aqueous fraction. Washing was performed three times consecutively, totaling 100 mL of acid solution.

The acidic aqueous fraction was then basified with ammonium hydroxide to pH 11. This fraction was successively extracted 4 times with 30mL of chloroform. After extraction, the alkaloidic fraction was dried, weighed and reserved for further treatments.

The chloroform fraction was separated by preparative thin layer chromatography (DPCC), and separated into two subfractions, FCN-A1 and FCN-A2. The subfractions were revealed with a modified Dragendorff reagent, confirming the presence of alkaloids, as shown in **Figure 01** below.

Figure 01: Separate alkaloid fractions. From left to right: Preparative Thin Layer Chromatography of the chloroform fraction; FCN-A1 fraction revealed with modified Dragenderoof; FCN-A2 fraction revealed with modified Dragenderoof



SAMPLE ANALYSIS BY LC-ESI-IT-MS/MS AND FIA-ESI-IT-MS^N

Preparation of FCN-A1 and FCN-A2 samples

After drying, the samples were solubilized in methanol and filtered through a 0.22 μm PTFE filter to an HPLC vial. The FCN-A2 sample was passed through a C18 cartridge (Chromabond C18ec). The solvent was removed, and after drying, the samples were again plated to confirm the presence of alkaloids in the prepared fractions.

Experimental conditions of LC-ESI-IT-MS/MS and FIA-ESI-IT-MS^n

The analysis of the fractions was performed in a SHIMADZU HPLC system (Shimadzu Corp. Kyoto, Japan), with Luna C18 column, 100 Å, with pores of 5μm, and dimensions 150 x 4.6 mm. The mobile phase consisted of water:formic acid (0.01%) (A) and methanol:formic acid (0.01%) (B). An elution gradient of 10 to 100% B was used in 10 minutes, with a mobile phase flow of 1 mL/min, and an injection volume of 20 μL. For the FIA-ESI-IT-MS n assay, the dry samples were dissolved in 1 mL of methanol, HLPC grade, and the ultrasound bath was used for 5 minutes to solubilize the samples. At the end, the samples were filtered through a 0.22μm PTFE filter and 20μL aliquots were injected directly into the LC-MS for analysis by FIA-ESI-IT-MS^n. The samples were analyzed in a linear *trap ion* analyzer (Thermo Scientific LTQ XL), equipped with an *electronspray* ionization source (ESI), using the positive mode (Thermo Scientific, San Jose, CA, USA).

The full scan analysis was recorded in the m/z range of 100 – 1000. The other fragmentations performed in multiple stages (ESI-MS^n) were performed using the collision dissociation method (CID) against helium for ion activation. The first scanning event was a full mass spectrum to acquire data on ions in this m/z range. The second scanning event was an MS/MS experiment performed with a dependent data scan on the molecules $[M+H]^+$ of the compounds of interest with a collision energy of 30 %, and an activation time of 30 ms. The ions produced were then subjected to new fragmentations under the same conditions as the first fragmentation. until the production of new fragments is not observed. The

identification of the different compounds in the chromatographic profile of the analyzed fractions was done by comparing their retention times with data obtained from the literature, in addition to the data of the fragments obtained through mass spectrometry.

MICROBIOLOGICAL TESTS

For the microbiological assays, the methodology of microbiological diffusion in solid medium was used, the technique used was the drilling of wells. The crude extract concentrated, the fractions hexane, chloroform, acetate, the total alkaloidic fraction and the FCN-A1 fraction were tested.

Microorganisms used

Gram-positive strains of *Staphylococcus aureus* ATCC 25923, and Gram-negative *Klebsiella pneumoniae* ATCC 700603, *Salmonella spp*. (clinical isolate), *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 35218 and the fungus *Candida albicans* ATCC 9002.

Preparation of microbial suspensions

The microorganisms were initially reactivated from their original cultures and maintained in BHI (Brain Heart Infusion) liquid medium at 37 °C for 24 hours. Subsequently, the samples were cultured on Nutrient Agar plates at 37°C for 24 hours. The isolated colonies were then removed from the culture medium, with the aid of a platinum loop, and resuspended in 3mL of sterile 0.89% NaCl saline solution, until reaching an equivalent turbidity on the 0.5 Mc. Farland scale, equivalent to 1.5 x 108 bact/mL.

Preparation of samples used in microbiological tests

The plant samples tested were the hydroethanolic extract of the leaves, the hexane fractions, chloroform, acetate, the fraction of total alkaloids and the FCN-A1 subfraction. All fractions were dried of their respective organic solvents, weighed, and resuspended with 2mL of 70% ethyl alcohol, in order to keep a control of the concentrations of the samples tested.

Solid Medium Puncture Test

The antimicrobial potential of the samples was evaluated by the technique of drilling wells in Müller Hinton agar medium, for the bacterial assays, and Sabourad 4% dextrose agar, for the fungal sample. Initially, the microorganisms were seeded on the plates with the aid of a sterile *swab*, and then the medium was perforated with 6 mm diameter cylinders. Aliquots of 50 µL of each of the extracts and fractions tested and their respective controls were placed in each of the wells. Chloramphenicol (0.02mg/ml) for bacteria and nystatin suspension was 100,000 IU/mL for fungal samples. The plates were

incubated at 37 ºC for a period of 24 hours. After incubation, the diameter of the growth inhibition halo (mm) was measured, when present (CLEELAND; SQUIRES, 1991) .

RESULTS

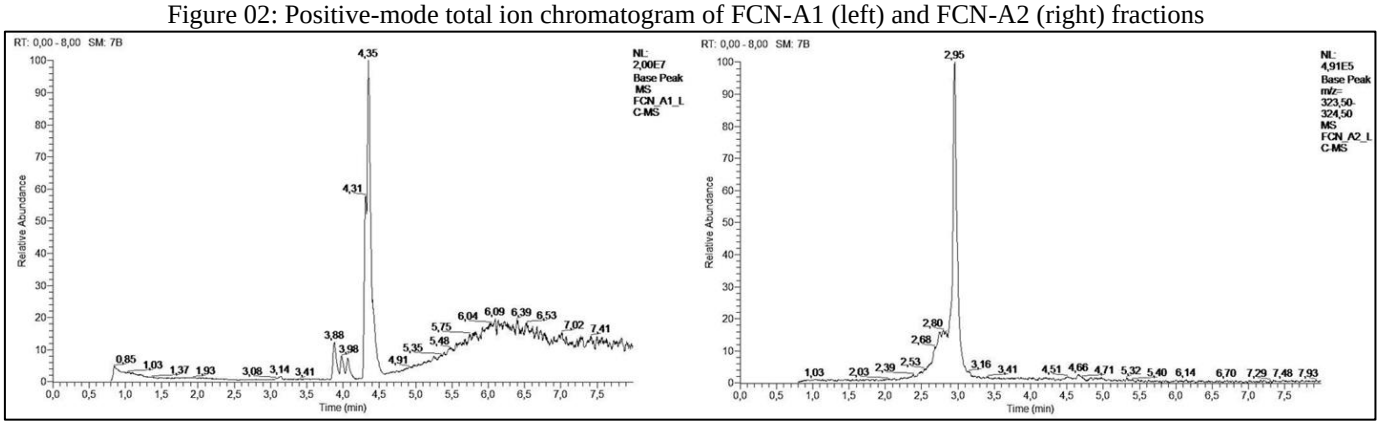
EXTRACTION YIELD AND IDENTIFIED SUBSTANCES

From the means of the masses obtained, it was possible to determine the concentration of the crude extract, which was equal to 0.0670 g/mL. By gravimetric analysis, the extraction yield was calculated to be equal to 16.67 %.

Total ion chromatograms and compounds identified in FCN-A1 and FCN-A2 fractions

In the total ion chromatograms, it is possible to observe the presence of few peaks for the FCN-A1 and FCN-A2 samples.

Figure 02: Positive-mode total ion chromatogram of FCN-A1 (left) and FCN-A2 (right) fractions



The FCN-A1 subfraction was separated by CCDP, weighing 33.1 mg, with an oily appearance and orange color. Despite appearing as a single *spot* when developed on chromatographic plate, the FCN-A1 subfraction presents a total ion chromatogram for a mixture of chemical substances. And because they co-eluted, they did not appear when developed with the modified Dragendorff reagent, or with iodine vapors. From the mass spectra obtained for the signal with retention time (RT) 4.35, 4.31, 3.88 and 3.98, it was not possible to identify the compounds present in this sample.

For the FCN-A2 subfraction, separated as a brown amorphous solid, weighing 16.1 mg, it was possible to identify the compounds with signs in retention time 2.95 and 4.21. Figure **03** shows the mass spectrum for a substance with a base peak of m/z: 324 $[M+H]^+$, and other fragments with m/z: 309 $[M+H -CH3]^+$, m/z: 278 $[M+H -CH2O2]^+$ and m/z: 217 $[M+H -C3H7O4]^+$. These data were attributed to the isoquinoline alkaloid romucosin, based on data obtained from the literature. The substance had previously been identified in leaf extracts of *Rollinia mucosa (*sinm. *Annona mucosa*), Annona (CHEN; CHANG;

WU, 1996) *cherimola* (CHEN et al., 2001) and in the extract of the stem of *Annona cassiflora* (ONEDIO; VALVASSOURA; SANTOS, 2013).

In **Figure 04**, below is the spectrogram for a substance with a molecular ion peak m/z: 465 $[M+H]^+$ and peak base m/z: 255. In addition, there are also fragments with m/z: 435 $[M+H -CH2O]^+$; m/z: 347 $[M+H -C4H6O4]^+$; m/z: 301 $[M+H -C6H12O5]^+$. With the fragments obtained, and based on data consulted in the literature, the isolated substance was identified as anthocyanidin, delphinidin-3-O-glucoside (KOH; ONE; KIM, 2014) , which is the first study to indicate the presence of this anthocyanidin in the extracts of the leaves of *Annona mucosa*.

Figure 03: Mass spectrum for the compound identified as romucosin

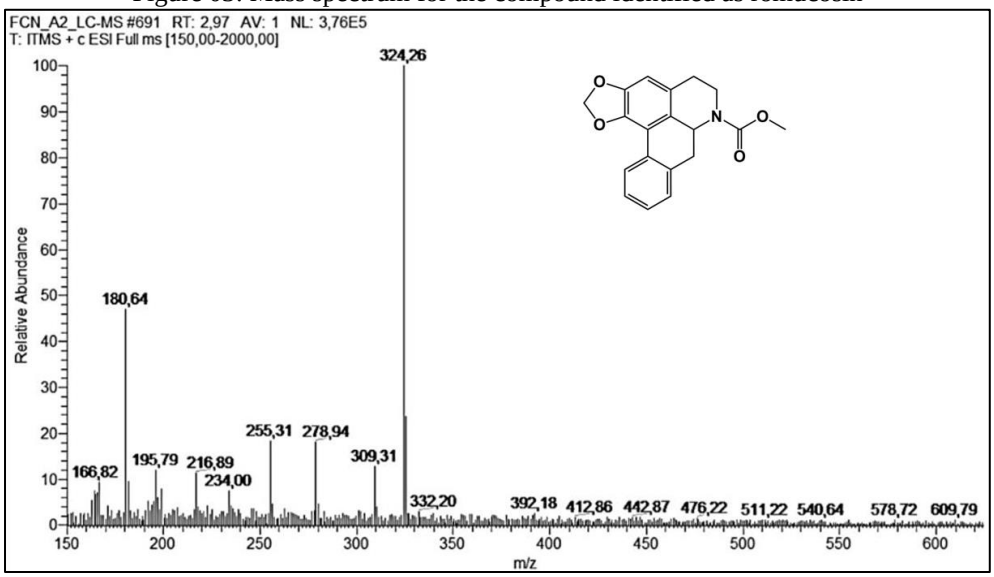
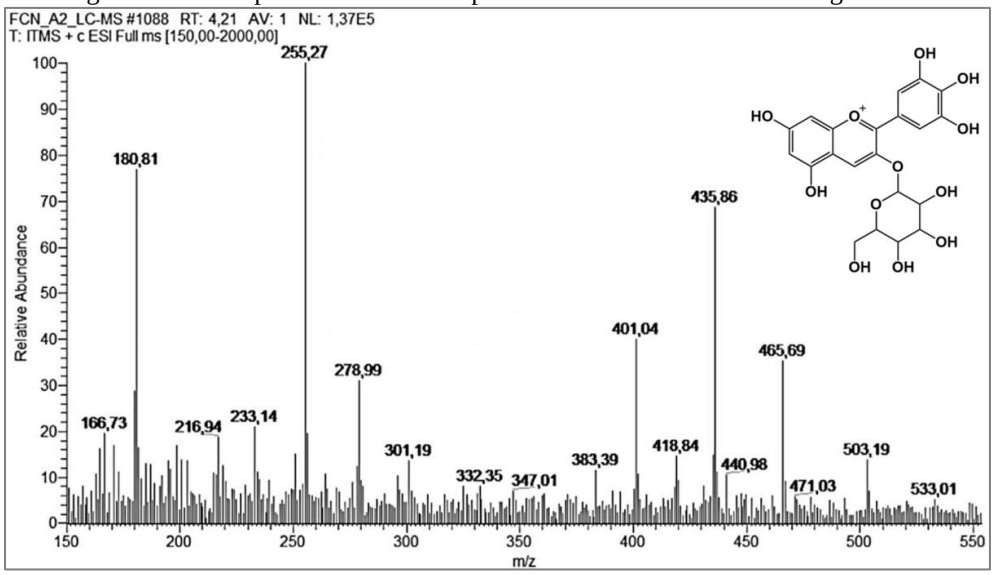


Figure 04: Mass spectrum for the compound identified as delfinidine-3-glucoside



IN VITRO *ANTIMICROBIAL ACTIVITY*

Table 01 shows the inhibition halos measured for the hydroalcoholic extract, fractions and the FCN-A1 subfraction. 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 01: Diameter of the bacteria and fungi growth inhibition halos evaluated by the well diffusion test

	Sample/Inhibition Halos Diameter (mm)					
Microorganisms tested	EhF	FH	FC	AGO	FAT	FCN-A1
***Staphylococcus aureus* ATCC 25923**	13	7	10	10	16	16
***Klebsiella pneumoniae* ATCC 700603**	NM	NM	NM	NM	8	NM
***Salmonella spp*. (clinical isolado)**	NM	NM	NM	NM	7	8
***Candida albicans* ATCC 9002**	NM	NM	NM	NM	12	9
EhF – Hydroethanolic extract 70% of the leaves; FH – Hexane fraction; HR – Chloroform fraction; AF – Acetate Fraction; FAT – Total alkaloidic fraction; FCN-A1 – Neutral chloroform fraction, alkaloid 1; NM – Not measurable. Concentrations (in µg/mL): EhF = 67000; FH= 49800; HR= 53500; FA= 56900; FAT= 11000, FCN-A1= 76300.						

From the data obtained, it was observed that the extracts showed microbiological activities for some of the samples tested, especially in the assays carried out against the gram-positive bacterium *Staphylococcus aureus*, where it was observed strong inhibition in growth for the fractions of total alkaloids (FAT) and FCN-A1. No inhibitory activities were observed through the well diffusion methodology in any of the samples tested for the Gram-negative bacteria *P. aeruginosa* and *E, coli*. In addition, it was observed that some of the samples presented inhibition halos of irregular sizes, so that they were not measured, presented as "NM" in **Chart 01**.

The presence of bioactive compounds in the extracts and fractions tested may be responsible for the observed activities, since some types of flavonoids are capable of forming complexes with soluble proteins and the cell wall of bacteria and fungi, thus inhibiting their growth, and that metabolites such as acetogenins, common in nonaceous, and alkaloids, present in the plant extract studied, They also have antimicrobial activities against various types of microorganisms. The microbiological potential of (ARIF et al., 2009; COWAN, 1999) *the species Annona mucosa* against *S. aureaus, P. aeruginosa and E. coli* had already been previously evaluated, demonstrating the activity of the extract against some microorganisms (SIMINSKI et al., 2015) .

The results obtained in this study demonstrate the potential of this species for the study and development of drugs for the control and treatment of *S. aureaus*, since this bacterium is considered an important pathogenic agent due to its resistance to antimicrobials and association with potentially fatal systemic diseases and diseases, and infections of various types (ARDURA, 2009; LOW, 1998) .

FINAL THOUGHTS

Thin layer chromatography techniques allowed the isolation of fractions containing alkaloids as major constituents, and the technique was applied as a separation step of fractions containing such metabolites.

The application of coupled LC-MS techniques and the multiple fragmentations used allowed the structural identification of the alkaloid romucosin, common to the *genus Annona,* and the anthocyanidin delphinidin-3-glucoside, this being the first study in which there was the isolation and identification of this anthocyanidin in the *mucosal species Annona*.

The microbiological assays indicate the presence of active compounds in the total alkaloid fraction, as well as in the other organic fractions, against the clinical isolate of the bacterium *Staphylococcus aureus*, and for the total alkaloidic fractions and FCN-A1 against the fungus *Candida albicans*, allowing the continuation of the study through the characterization and evaluation of the toxicity of these metabolites, in order to evaluate the possibility of drug development.

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