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ANTIMICROBIAL ACTION OF THE LEAF AND FRUIT EXTRACT OF ANDIROBA (CARAPA GUIANENSIS L.)

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ABSTRACT

Andiroba (Carapa guianensis L.) is widely used as an anti-inflammatory, with phytotherapeutic properties coming mainly from its essential oil, extracted from its seed, its extraction and commercialization is common throughout the Amazon region. In addition to being marketed as a herbal medicine, its oil is used in moisturizers and lotions because it has healing characteristics. The main objective of the research was to verify the antimicrobial compounds present in the leaves and fruit of the traditional C. guianensis. For

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this, plant extracts were made to test against strains of pathological bacteria, seeking its antimicrobial potential, the extract was carried out in 3 ways: Cold, staying 5 days on the bench only with the action of solvents and absence of light; Soxhlet, where each extract has undergone 9 cycles of hot extraction in the extractor; Ultrasound, where they spent 20 minutes in an ultrasonic bath. For each method, two organic solvents were used: hexane and ethanol, for each extraction 50g of the leaf and dried fruits were used in the oven at 40°C and crushed. The fruit went through only two extraction methods: Ultrasound and cold. Tests were performed against strains of Escherichia coli (ATCC25922) and Pseudomonas aeruginosa (ATCC27853), which were cultivated in Muller-hinton broth, each test was done in triplicate and gentamicin and dimethyl sulfoxide (DMSO) were used as positive and negative controls, respectively. The halos of bacterial inhibition were observed and measured, those that presented positive results, the extracts that obtained the best results went through the antioxidant tests via the ABTS and DPPH method and chromatographic analysis of the extracts that obtained the best results, which were the leaves static method with ethanol solvent, leaf by the ethanol ultrasound method and leaf by the soxhlet ethanol method.

Keywords: C. guianensis. Amazon. Antagonism. P. aeruginosa and E. coli.

INTRODUCTION

With the adoption of the National Policy of Integrative and Complementary Practices in the Unified Health System (SUS), the demand for knowledge and search for new herbal medicines originated by natural means has been increasing, this need comes from the evolution in research and proof of the effectiveness of herbal medicines as an alternative treatment for various pathologies. Thus, one of the plants best known for its therapeutic properties and widely used by the Amazonian population is *C. guianensis* L., known as andiroba, its pharmacological use has been studied and disseminated by popular knowledge (Aguiar, 2019).

This species belongs to the Meliaceae family, has its origin and strong presence in Latin America and Africa, is popularly known as andiroba, andirova and carapá, its most abundant use is found in the northern region of Brazil, where it has great herbal and commercial importance. The best-known product from *C. guianensis* is its oil, which is rich in fatty acids, with healing, anti-inflammatory, and antiparasitic properties, its main use is for the treatment of tonsillitis (Dias, 2023).

In this sense, andiroba is widely studied and researched by the field of ethnography, however about 90% of this research is focused on the anti-inflammatory action coming from its oil, which is also known to be an excellent accelerator of epithelial healing. However, other parts of andiroba, such as its leaf and fruit, have little research, and its antibacterial potential needs more tests and studies aimed at the alternative search for the treatment of bacterial pathologies, since *C. guianensis* has a strong phytotherapeutic potential (Malheiros, 2023).

Thus, the purpose of the project was to verify the antimicrobial activity of the leaves and fruit of *C. guianensis*, using extracts obtained by soxhlet, ultrasound and static with the use of organic solvents: hexane and ethanol, in addition to making a chemical characterization of the metabolites present in the extracts that showed significant inhibition, in addition to comparing the efficacy of the leaf and the fruit of andiroba.

METHODOLOGY

The leaves and fruits of andiroba (*C. guianensis)* were collected in the forest surroundings of the Federal University of Amazonas (UFAM), and sent to the Microbiology Research Laboratory, at the Institute of Biological Sciences (ICB 1), where they were sanitized, and taken to the closed air circulation greenhouse for the drying of the botanical material for 4 days. The leaves were dried in a closed air circulation oven at 40° C for 4 days, then macerated with the use of a blender so that they remained in smaller fragments.

COLD - STEEPING

50g of the dried and crushed leaves and 50g of the dried and macerated fruit were used, placed in a 1000ml Becker and added 500ml of Hexane and Ethyl Alcohol to each one, which was left to rest for a period of 5 days on the bench. After this time, it was taken to the rota-evaporator, for total removal of the organic reagents, the extracts were kept in penicillin bottles and stored in a refrigerator until their use in antimicrobial tests.

HOT - SOXHLET

50g of macerated leaves wrapped in filter paper were used and placed in the two-liter extractor, in a 1000ml flat-bottomed flask 500ml of each solvent, hexane and ethyl alcohol, these flasks are connected to the extractor to start the process. For hexane a temperature of 68°C was used, and for ethanol the temperature was 77°C, in which there were 9 evaporation cycles, the time of each cycle was varied, but the average of hexane was approximately forty minutes and of ethanol one hour, later the extracts went through the separation of the solvent that was also extracted by the route-evaporator.

ULTRASOUND

50g of the leaves and fruits of *C. guianensis* and 500ml of the solvent (hexane and ethyl alcohol P.A) were used in a 20kHz ultrasonic probe, the sample was placed in a water-cooled stainless steel reactor, and then sonicated for a period of 20 minutes each. And later the solvent was evaporated through the rota-evaporator.

EVALUATION OF ANTIBACTERIAL ACTIVITY

For the antimicrobial tests, the bacteria, *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), were inoculated in test tubes containing 5mL of Mueller-Hinton medium (MH) for each of the strains, where they grew in broth for 24h, then were placed in Mueller-Hinton agar culture medium and incubated for 24h. Subsequently, the bacterial suspension by its cell density standardized by turbidity adjusted according to the 0.5 scale was obtained according to the methodology prescribed in the Manual of Clinical Microbiology. American Society for Microbiology (Washington, 1985).

To evaluate the antibacterial activity, bacterial strains were used, namely: *E. coli* and *P. aeruginosa*, the bacteria were reactivated in Muller-Hinton broth (MH) and then spread in Petri dishes containing Mueller-Hinton Agar (MH) culture medium, in three replications, at mg/mL concentrations: C1 (0.010), C2 (0.015), C3 (0.100), C4 (0.150), C5 (0.200), C6 (0.250), C7 (0.300) and C8 (0.350) on the filter paper with 0.5 millimeters each, all were

soaked with ethanolic and hexane extracts of the andiroba leaf from the soxhlet, static and ultrasound processes, with negative control with dimethylsulfoxide (DMSO) and antibiotic (gentamicin) as positive control. The disks were positioned equidistant, maintaining a reasonable distance from each other to avoid interference between the possible inhibition halos. The plates were incubated at 35°C in B.O.D (Biological Oxygen Demand) climatized chambers for 72 hours (three days), during which the development of microorganisms and the appearance of inhibitory halos were evaluated and observed.

THIN LAYER CHROMATOGRAPHIC ANALYSIS

Chromatographic analysis was performed on the extracts that showed better bacterial inhibition. This thin chromatography method was used because it is an example of adsorption chromatography. This technique consists of a stationary phase fixed on a plate (silica gel) and a mobile phase, which is composed of a solvent, which we call eluent. The chemical samples to be analyzed were applied to the stationary phase, which is an adsorbent.

In a chromatographic vat, about 10 ml of hexane was added, and a filter paper cut the size of the beaker, after the addition of this material, the vat was closed with aluminum foil and rested for 10 minutes, so that it was saturated with vapors of the same constitution as the mobile phase.

0.1 g of botanical extracts were weighed and diluted in hexane and ethanol and prepared for application, with the aid of a capillary the sample was applied to the chromatographic plate composed of silica, this application was performed in approximately 0.5cm from the lower base. After the application of the samples, the CCD was introduced into a glass vat containing the solvent, the height of the solvent in the vat cannot exceed the line of application of the sample, because the eluent must drag the sample. When the plate was introduced into the vat, the solvent ascended, by capillary phenomenon, to the upper extremity. When ascending, the mobile phase dragged more of the less adsorbed compounds into the stationary phase, separating them from the more adsorbed compounds. The plate was removed from the vat when the front of the solvent reached approximately 0.5 cm from the upper end of the plate. Then, the plate was dried by simple exposure to air. After this procedure, the plate was taken to a UV viewer, because UV light is a non-destructive method of development, where it was possible to observe the separate phases and measure the retention rates, which is the ratio between the distance traveled by the stain of the component and the distance traveled by the eluent, after this visualization the sample was also developed in iodine.

ANTIOXIDANT ASSAY VIA DPPH REAGENT

The DPPH method was used as it is common to measure the antioxidant activity of plant extracts. It involves the capture of the DPPH (2,2-diphenyl-1-picrylhydrazil) radical by antioxidants, producing a decrease in absorbance at 515 nm. To prepare DPPH, 2.8 mg of DPPH were dissolved in 50 ml of methanol, sonicated for 25 minutes and stored in the refrigerator for 1 week. The extracts were diluted in DMSO 10% (Concentration: 1 mg/mL). For the standard curve, a stock solution for Trolox was made in DMSO 10% (Concentration: 1 mg/mL).

For the negative control, 20 µl of DMSO 10% + 280 µl of DPPH were transferred to the plate. In the wells with positive control, 20 µl of Trolox + 290 µl of DPPH were added. For the white, 300 µl of DMSO 10% was added and for the standard 300 µl of DPPH. 20 µl of each extract was added in triplicate to the plate along with 280 µl of DPPH. After this procedure, the plate was incubated for 20 min and then placed in the spectrophotometer for reading at 515nm.

The ABTS method was used to measure total antioxidant activity involving the capture of ABTS 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical in lipophilic and hydrophilic substances such as flavonoids, carotenoids, and plasma antioxidants. This radical is formed by the oxidation of potassium persulfate, which is reduced by hydrogen-donating antioxidants. Trolox was used as the standard antioxidant.

For the preparation of the ABTS solution, 3.84 mg of ABTS were dissolved in 1 mL of distilled water, this solution was stored under refrigeration and protected from light. The potassium persulfate solution was prepared by dissolving 37.84 mg of potassium persulfate in mL of distilled water and stored at room temperature. In the preparation of the ABTS radical, 1 mL of the ABTS solution was mixed with 17.6 µl of the potassium persulfate solution, the mixture was kept in the dark for 16 hours at room temperature. After 16 hours, the mixture was diluted in methanol (ratio of 1:30) until absorbance was obtained around 0.7 (length of 734 nm). The extracts were diluted in DMSO 10% (Concentration: 1 mg/mL). For the standard curve a stock solution for Trolox in DMSO 10% (Concentration: 1 mg/mL) was made.

For the negative control, 20 µl pure water + 280 µl of ABTS were transferred to the plate. In the wells with positive control, 20 µl of Trolox + 280 µl of ABTS were added. For the white, 300 µl of DMSO 10% was added and for the standard, 300 µl of ABTS. 20 µl of each extract were added in triplicate to the plate along with 280 µl of ABTS. After this procedure, the plate was incubated for 20 min and then placed in the spectrophotometer for

reading at 734 nm.

STATISTICAL ANALYSIS

For the statistical analysis, the ANOVA method was used, which is a method used to determine whether there are significant differences between the means of three or more independent groups. This technique was developed by British statistician and geneticist Ronald Fisher in the early twentieth century.

RESULTS AND DISCUSSION

The antimicrobial activity test was performed, the results were divided into analysis by inhibition of bacteria, the results referring to the inhibition of the ethanolic extracts of the C*. guianensis leaf* against *E. coli* showed positive results, according to the inhibition halo, presenting halos of 18mm, only the ultrasonic extract did not have antimicrobial potential, the extracts obtained by the soxhlet and static methods obtained excellent averages of inhibition halos, especially at higher concentrations.

Table 1: Average of the growth inhibition halos obtained by the ethanolic extracts of the leaf through the ultrasound, soxhlet and static method against the bacterium *Escherichia Coli*

Sample	Concentration (mg/mL)	1.RCE	1.RCS	1.RCU
C1	0,02	13c	14c	-
C2	0,05	14c	14c	-
C3	0,10	16b	15bc	-
C4	0,15	16b	15bc	-
C5	0,20	17b	17b	-
C6	0,25	18ab	18ab	-
C7	0,30	20a	18ab	-
C8	0,35	20a	19ab	-

Legend: 1.RCE: Leaf extract; static method; solvent ethanol. 1.RCS: Leaf extract; Soxhlet method; solvent ethanol. 1.RCU: Leaf extract; ultrasound method; solvent ethanol, equal letters do not differ according to statistics.

The results obtained with the ethanolic extracts performed by the soxhlet and static methods showed significant halos: from 20mm, 18mm and 19mm in the highest concentrations, it was possible to observe that on the first day of the test the halos inhibited the E *. coli bacteria,* as the days went by the bacteria showed resistance and grew superficially through the halos, demonstrating an efficacy for control, but not inhibition.

The ethanolic extract by the ultrasound method did not obtain either an inhibitory or controlling halo against *E. coli*, making a comparison with the article by Silva (2014), in the research an ethanolic extract from the bark of *C. guianensis* tested against the bacterium *Klebsiella pneumoniae* was used,the results at different concentrations were also not positive, demonstrating that Gram-negative bacteria are resistant to these botanical extracts.

The results regarding the inhibition of hexane extracts of the C*. guianensis leaf* against *E. coli* showed positive results, especially at the highest concentrations, as we can observe through the statistics made by Anova through Tukey's test, shown below:

Table 2: Average of the growth inhibition halos obtained by the hexane extracts of the leaf through the ultrasound, soxhlet and static method against the bacterium *Escherichia Coli*

Sample	Concentration (mg/mL)	2.RCE	2.RCS	2.RCU
C1	0,02	14fg	-	-
C2	0,05	18f	-	-
C3	0,10	25d	-	-
C4	0,15	30cd	-	-
C5	0,20	-	17f	32c
C6	0,25	-	17f	33bc
C7	0,30	-	20e	34b
C8	0,35	-	30cd	41a

Caption: 2.RCE: Leaf extract; static method; solvent hexane. 1.RCS: Leaf extract; Soxhlet method; hexane solvent. 1.RCU: Leaf extract; ultrasound method; hexane solvent.

All lower concentrations (0.02, 0.05, 0.1, 0.15) obtained positive results in the extract made with hexane by the static method, the lowest concentrations obtained halos higher than the highest concentrations, a fact explained by chemical interactions of symbiosis. The hexane extraction methods made by ultrasound and soxhlet obtained results at the highest concentrations, surpassing the results found by Monteiro (2017), who used andiroba oil against *E. coli,* the results were similar, there was inhibition at the highest concentrations and at the highest concentrations there was not such a significant result.

The results regarding the inhibition of the ethanolic extracts of the C*. guianensis leaf* against *P. aeruginosa* were not positive, because there were no results in inhibition halos, the extracts obtained small halos on the first day of analysis, however over the days of evaluation the bacterium grew completely in the Petri dishes. In other words, andiroba does

not present any inhibition for the P*. aeruginosa bacterium* at the concentrations tested.

The results of the ethanolic extracts made by the three methods did not show inhibitory halos against the bacterium *P. aeruginosa,* as described by Santos (2015), this bacterium has many biological defense mechanisms, mainly by the production of the enzyme β-lactamases, being very resistant, a fact that explains the results.

The results of the hexane extracts also did not show a halo of inhibition or control, according to Bruice (2006), hexane is a solvent with low polarity, a fact that may be one of the results due to the poor performance of the extract against this pathogen, extracting less polar metabolites that did not produce an effect. Another possible explanation is the high resistance of the P*. aeruginosa bacterium,* which is why it is a pathogen that has been widely studied, as it is the main agent of infections in hospitals.

The results regarding the inhibition of hexane and ethanolic extracts of the fruit of *C. guianensis* against *E. coli* and *P. aeruginosa* showed positive results, a fact that already differentiated the leaf extracts that did not inhibit *P. aeruginosa*, as we can observe through the statistics made by ANOVA through Tukey's test, as demonstrated:

Table 3: Mean growth inhibition halos obtained by the ethanolic extracts of the fruit by the ultrasound method and static against the bacterium *Escherichia coli* and *Pseudomonas aeruginosa*

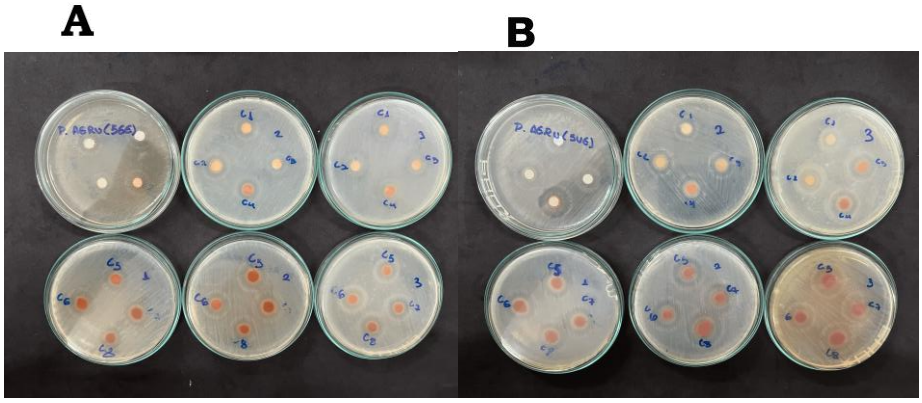
Sample Concentration (mg/mL)	5.EEA	5.EAEU	5.EEP	5.UEP
C1 (0.02)	12e	13th	16cd	12e
C2 (0.05)	16cd	16cd	18c	14d
C3 (0.10)	18c	18c	18c	18c
C4 (0.15)	18c	21b	18c	20bc
C5 (0.20)	19c	22b	20bc	22b
C6 (0.25)	19c	24ab	22b	22b
C7 (0.30)	20bc	25th	23ab	23ab
C8 (0.35)	22b	26th	23ab	25a

Legend: 5.EEE: Ethanolic extract, static method against *E.coli* bacteria; 5.UEE: ethanolic extract, ultrasound method against *E.coli* bacteria; 5. EEP: ethanolic extract, static method against *P. aeruginosa* bacteria; 5. UEP: ethanolic extract, ultrasound method against P*. aeruginosa bacteria.*

As observed through the statistical analysis, all the ethanolic extracts of the andiroba fruit obtained by the two methodologies showed strong inhibitory capacity against the two bacteria: *E. coli and P. aeruginosa,* demonstrating a positive and different result from the leaf extracts that did not obtain satisfactory results against *Pseudomonas aeruginosa.*

As noted, the extracts made with the solvent hexane did not inhibit any of the pathogenic microorganisms demonstrating inhibitory efficacy, these results were similar to those found by Nonato (2018). The fruit of andiroba has a strong antimicrobial potential used by wild animals.

Figure 01: Antimicrobial analysis of the antagonism tests. **A**: Result of the inhibitory halos of the ethanolic extract of the andiroba fruit, obtained by the static method against the bacterium *P. aeruginosa*. **B:** Result of the inhibitory halos of the ethanolic extract of the andiroba fruit, obtained by the ultrasound method against the bacterium *P. aeruginosa*.



The result of the thin layer chromatography had a ratio of 3:7 in hexane, the hexane extracts could not elute in the thin layer demonstrating that another methodology should be used.

Figure 02: A: Samples that were applied to the thin layer chromatography plate **B:** Chromatographic tank prepared and saturated for elution.

Through this technique, it was possible to detect the presence of secondary metabolites of the botanical material produced by *Carapa guianensis,* which could be seen through the development of the botanical material produced by Carapa guianensis.

Figure 03: Samples eluted on the chromatographic plate revealed by iodine with respective rf values of the samples: leaf extracts by the static method with ethanol solvent, leaf by the ethanol ultrasound method and leaf by the ethanol soxhlet method. Rf 1: 0.6 and Rf 2 = 0.72 Rf 3 = 0.74.

According to Bruice (2006), the retention factor (RF) in thin layer chromatography is essential to observe and evaluate the separation of substances, with ideal values between 0.4 and 0.6. The results of the leaf extracts by the static method with ethanol solvent, leaf by the ethanol ultrasound method and leaf by the soxhlet ethanol method, respectively had retention factors equal to: 0.6; 0.72 and 0.74.

Table 4: Result of the antioxidant potential of *C. guianensis leaf extracts* by the ABTS and DPPH assays.

AMOSTRA	ENSAIO ABTS (média % inibição e desvio padrão)	ENSAIO DPPH (média % inibição e desvio padrão)
1HE	96,80 ± 0,92	74,19 ± 1,90
1HS	93,16 ± 3,10	64,39 ± 0,97
1HU	95,34 ± 5,27	87,20 ± 1,00
2EE	98,67 ± 0,10	78,76 ± 0,24
2ES	99,45 ± 0,11	83,99 ± 1,02
2EU	95,13 ± 1,08	71,84 ± 5,05
Trolox (média % inibição)	0,980	1,078

Legend: 1HE = Hexane extract of andiroba leaf by static methodology; 1HS = Hexane extract of andiroba leaf by soxhlet methodology; 1HU = Hexane extract of andiroba leaf by ultrasound methodology; 2EE = Ethanolic extract of andiroba leaf by static methodology; 2ES = Ethanolic extract of andiroba leaf by soxhlet methodology; 2EU = Ethanolic extract of andiroba leaf by ultrasound methodology;

The antioxidant test performed by the ABTS and DPPH assays demonstrated a strong antioxidant potential of the extracts produced through secondary metabolites of *C. guianensis,* the ABTS method was the one that most evidenced the antioxidant potential of the extracts.

Our results were similar to the research of Luz (2024), his ABTS assay made from andiroba demonstrated a strong antioxidant potential that can be justified by the greater presence of phenolic compounds in the extract such as naringenin, one of the most common flavonoids in leaves (Nishimura, 2013).

CONCLUSION

As a result, we had halos of inhibition against the E. *coli* bacterium, that is, all ethanolic and hexane extracts of the leaf and fruit obtained positive results, inhibiting the

growth of the bacteria. When we used the bacterium *P. aeruginosa*, only the extracts obtained from the fruit obtained a positive result, the other extracts were not able to inhibit the growth of this bacterium. It was possible to observe that the fruit extracts were more effective than the leaf extracts, and in the statistical analyses (ANOVA) the ethanolic extracts showed significant differences when compared to the hexanes, demonstrating better performance of polar compounds. After the microbiological tests, the thin layer chromatographic of the extracts that obtained the best results was performed, which proved the extraction of secondary metabolites and the antioxidant assay by DPPH and ABTS was performed, showing strong potential in the extracts.

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