Evaluation of the biological activity of the medicinal plant pata-de-vaca (Bauhinia forficata L.) against the bacterium Escherichia coli, using a flow injection analysis system

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ABSTRACT
Plants for medicinal purposes are widely used worldwide for the treatment, cure and prevention of diseases. Among the numerous plant species of medicinal interest, the plants of the genus Bauhinia are cited a lot, which are normally found in the tropical areas of the planet, and have some important pharmacological activities, for example, hypoglycemic. The objectives of this work were: to evaluate the biological activity of the medicinal plant pata-de-vaca (Bauhinia forficata L.), against the bacterium Escherichia Coli using a flow injection analysis system, coupled with a spectrophotometer, and, in parallel, to carry out the research of secondary metabolites present in the leaves of this plant, through the so-called prospecting tests. Through this system, it was possible to observe its behavior against the bacteria E. coli. The present study demonstrated that there is a range of metabolites present in the leaves of Bauhinia forficata L., which may present significant pharmacological and biological activities. It was also found that there are indications that compounds present in this plant may favor, and others may inhibit, the proliferation of microorganisms, in theory the bacteria Escherichia coli. The ethyl acetate, hexane and aqueous fractions obtained from the crude ethanolic extract of the plant samples were used.

Keywords: Molecular modeling, adsorption, mixture, elastomer.

1 INTRODUCTION

The cow's paw (B*Auhinia forficata* L.) belongs to the family *Fabaceae*, being native to southern Brazil, Paraguay, Argentina and Uruguay, develops mainly around the forests, can be found in the form of shrub or tree that grows from 5 to 9 meters tall. (CARON, et al., 2014). In Brazil it is found mainly in the regions from Rio de Janeiro to Rio Grande do Sul, and has been used by the indigenous and riverine population of these regions for hundreds of years (LUSA, M.G.; BONA, C., 2009).

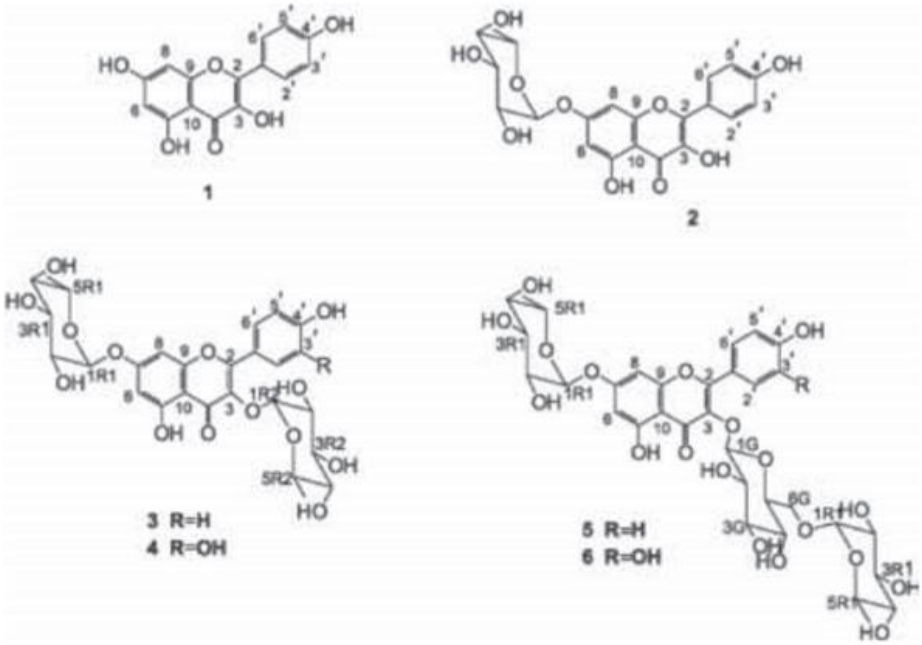
The species is one of the plants selected by the Ministry of Health as an interest to the Unified Health System. It is widely used in folk medicine, as a hypoglycemic, purgative, diuretic among other functions. It has stimulating, expectorant and astringent properties that is often linked with the bark of the stem (LÓPEZ, 2015).

The species *B. forficata* has already been evaluated through the general activity test (TGA), where intense diuresis was observed after ten minutes of administration of the crude extract of the tincture, denoting a great diuretic action (Da SILVA; SON, 2002).

De Souza and other collaborators in 2004 tested the antioxidant potential of a product isolated from a butanolic fraction of *B. forficata*, kaempferol-3,7-O-α-diraminoside, where it was demonstrated that this compound has significant antioxidant activities. In the same year, these same researchers evaluated the antimicrobial activity of extracts and fractions of *B. forficata*, through the method of radial diffusion in agar, and were able to observe that one of these fractions inhibited the growth of *Escherichia coli* and *Staphylococcus aureus* at a concentration of 1000mg mL-1 (NOGUEIRA; ALBINA; SABINO, 2013).

Pizzolatti et al. in 2003, managed to record results of a phytochemical study of the leaves of this species of *B. forficata*, where they identified and described kaempferol and five glycosylated flavonoids containing quercetin as aglycones (Figure 01).

Figure 01 - Canempferol (1) and other glycosylated flavonoids containing kaempferol and quercetin as aglycones.



Source: Adapted from (Nogueira; Albina; Sabino, 2013).

The species *Bauhinia forficata* is the only one in which it seems to present the flavonoid called kaempferitrin in its leaves, and this serves as a great marker for commercial sample analyses, and, in

addition, this flavonoid is related to the hypoglycemic activity of this species. This can be found through Thin Layer Chromatography (MARMITT; REMPEL, 2016).

Secondary metabolites are usually of andcomplex structure, have low molecular weight, and have some remarkable biological activities and different from the primary metabolites, which are present in low concentrations and in certain groups of plants (BERG et al., 2008). This species under study presents as secondary metabolites tannins, flavonoids, glycosides, alkaloids, saponins, among others.

Although by-products have a variety of functions in plants, it is likely that their ecological importance has some relation to potential medicinal effect for humans. For example, by-products involved in plant defense through cytotoxicity to microbial pathogens may be useful as antimicrobial drugs in humans, according to their toxicity (KAUFMAN et al., 1999), and which can be identified using the flow system.

The use of the flow system for the analysis of biological assays presents a huge advantage, because in the case of biological analyses, the determination of the studied sample can be performed using spectrophotometry for turbidimetric detection. And with the system in flux this analysis is done with automation, reducing the amount of waste generated and improving the accuracy of the methods (DE PAULA et al., 2019).

The flow injection analysis (FIA) has as its basic concept the introduction of the sample into a carrier fluid (which can be a reagent solution), whose function is to displace the sample zone introduced throughout the system to the detector (SANTOS and MASINI, 2010). The FIA has some attractive features in which the automation of almost every process stands out, which minimizes both the intervention of the analyst and the possibility that they have contamination. Thus, this automation increases the repeatability of the measurements, as well as making the analysis safer. Among other points that we can mention of the system in flux is the minimization of the consumption of samples and reagents, increased frequency of sampling and versatility (MOREIRA et al., 2014).

Thus, the objectives of this work were: to evaluate the microbiological activity of the medicinal plant pata-de-vaca (*Bauhinia forficata* L.), *analyzing* the leaves and bark, against the bacterium *Escherichia Coli* using a system of analysis by flow injection, coupled with spectrophotometer, and, in parallel, to perform the research of secondary metabolites present in the leaves of this vegetable, through prospecting tests Phytochemical.

2 METHODOLOGIES

2.1 COLLECTION AND IDENTIFICATION OF PLANT MATERIAL

The samples of the leaves of *Bauhinia forficata* were identified and collected in 2019 in the city of Goiânia-GO, with the help of a botanist and the guiding professor Dr. Jonas Alves Vieira. After

this collection, the leaves were subjected to an oven drying at 38ºC with forced ventilation, and later these samples were milled in a conical mill with a determined granulation, this process took approximately one week.

The experiments were carried out with the support of the Chemistry laboratories of the Central Campus – Headquarters: Anápolis – CET and were carried out in 2019 and 2021. The drying of the bark took place in a natural environment under the sun and the grinding was done with the grinding process in a mill with defined granulation.

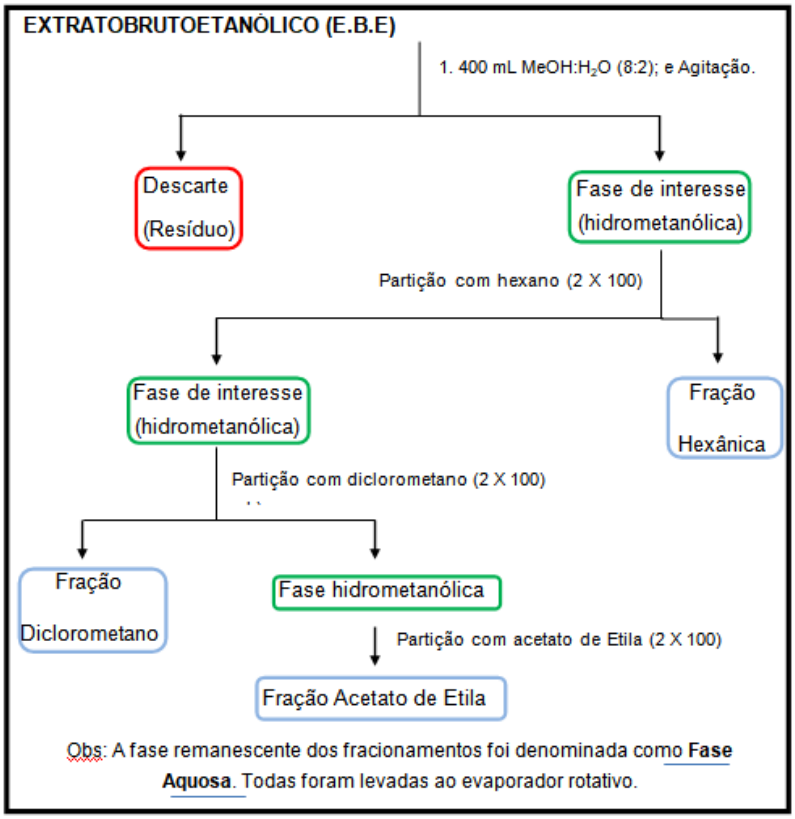
3 OBTAINING THE EXTRACTS

To obtain the Crude Ethanolic Extract (E.B.E) were separated approximately: 300g sample of leaves of *B. forficata* already crushed, which were subjected to cold extraction with 96% Ethanol for seven days. Afterwards, this sample (liquid) was taken to the rotary-evaporator, so that all the Ethanol was evaporated by a distillation process, leaving only one Dry Crude Ethanolic Extract; 300 g sample of the stem bark of *B. forficata* already crushed, and so it was subjected to cold extraction with 96% ethanol for seven days. Then this extract (liquid) was taken to the rotary-evaporator, so that all the ethanol evaporated by a distillation process, leaving only the wet Crude Ethanolic Extract.

4 FRACTIONATION OF CRUDE ETHANOL EXTRACT

To a part of the dry E.B.E was added 400 mL of a MeOH:H solution 2O (8:2 v/v) and stirred. Then, this extractive solution was taken to a separation funnel of 500 mL, and subsequently subjected to liquid-liquid extractions with solvents of increasing polarity: Hexane, Dichloromethane (D.M), and Ethyl Acetate (Act. Ethyl). The organic phases were obtained and taken back to the rotary evaporator (distillation process), leaving only the dry samples (leaves) and without the respective solvents and the wet semi-purified fractions (bark). The compounds produced by the leaves of this plant were adhered by chemical affinity to the phases in question. The fractionation scheme is shown in Figure 02.

Figure 02 – Methodology of the fractionation of the Gross Ethanol Extract.



Source: Adapted (Simões, et al., 1999).

This fractionation step allowed the production of the four phases of interest, three were obtained with the respective solvents (Hexane, Dichloromethane, Ethyl Acetate), and a last one was the remaining phase, which was called the aqueous phase. After this step, it was possible to prepare the samples of interest, in which the bacterium *Escherichia coli was inoculated*.

5 PREPARATION OF TEST SOLUTIONS

After obtaining the Hexane, Dichloromethane, Ethyl Acetate, and Aqueous Phase fractions, roto-evaporated, the preparation of the test solutions began, which were later taken to the flow system, coupled with the spectrophotometer, for a monitoring of the biological activity of these fractions, against the bacterium *Escherichia coli.*

For the evaluation of the biological activity of the compounds of the leaves of *B. forficata* present in these fractions, 103 mg of each extract were weighed separately, and solubilized in 4 mL of DMSO (Dimethylsulfoxide), each one was transferred to its respective volumetric flask of 50 mL, completing the volume with distilled water, thus obtaining four solutions (I) at the concentration 107.98 mg L^{-1} in sample.

From these (solutions I) the reading solutions (solutions II) were prepared for analysis in the flow system, at concentrations of 0.191 mg $^{mL-1}$ in the sample, where 2.6 mL of each solution of the fractions (DM, Act. of ethyl, Aqueous, Hexane) were added separately in sterile glass vials, with 24.9 mL of a minimum culture medium, and with 0.5 mL of a suspension containing the bacterium *Escherichia Coli.*

Four samples were then obtained for analysis in the flow system, coupled with a spectrophotometer, where the objective was to compare them with a white sample (containing only culture medium), and with a reference sample (culture medium and bacteria), prepared under the same conditions, to verify if any compound present in the plant extracts (which had affinity to one of the four fractions), it could inhibit bacterial growth or not. In addition, it was also compared with a sample containing the culture medium + bacteria + DMSO, to confirm that this solvent was not inhibiting the growth of *E. coli* in the other samples, since it was used in the solubilization of the fractions.

Before the inoculation of the bacteria under study in these aliquots, a suspension was prepared a day before, with a minimum culture medium (nutritious broth), prepared through a mixture containing sucrose and other chemicals that served as nutrient.

6 MINIMUM CULTURE MEDIUM

The culture medium solution was prepared as follows: 3g of potassium hydrogen phosphate (K2HPO4); 1.5g of potassium dihydrogen phosphate (KH 2PO4), 0.5g of ammonium sulfate ((NH4)2SO4), 0.05g of magnesium sulfate heptahydrate (MgSO4. 7H2O) and 0.25 g of sodium citrate. The mixture was solubilized

with water and transferred quantitatively to a 500 mL volumetric flask, completing the volume with deionized water, and then performed the pH adjustment at 7.22, then the set was heated to boiling and during cooling with monitored temperature, at 70°C 10 g of glucose was added to this solution. This solution was used to promote the population development of *E. coli bacteria (*ATCC 25922), used in the biological assays of the solutions of the samples under analysis (GIMENEZ, 1994).

7 PREPARATION OF SAMPLE AND DMSO SOLUTIONS

- Solution of the Aqueous fraction at a concentration of 2,200 mg L-1. We weighed 110 mg of the aqueous fraction and solubilized in 4mL of DMSO, transferred to a 50 mL volumetric balloon, completing the volume with deionized water.

- Sample solution of the Ethyl Acetate fraction at a concentration of 2,240 mg. L-1· Prepared by solubilizing 112 mg of the fraction in 4 mL of DMSO and transferring to a 50 mL volumetric flask, completing the volume with deionized water. NOTE: In the solutions of the samples of items 01 and 02, the final content in DMSO was 8%.

- 8% DMSO solution, prepared by transferring 4 mL of DMSO to a 50 mL volumetric flask and completing the volume with deionized water. Resulting in a DMSO percentage of 8%.

The DMSO solution was used in order to evaluate whether this solvent in the referred concentrations, could interfere in the development of the *E. coli bacterium* used in the biological evaluation in the sample solutions.

8 READING SOLUTIONS FOR BIOLOGICAL ASSESSMENT

The reading solutions were prepared by removing aliquots from the sample solutions described in item 03. The preparation of these solutions is described in Table 02.

Table 02 - Preparation of the reading solutions, for evaluation of the biological activity in the solutions of the samples of the sample of the bark of the cow's paw plant (2021).

Solutions reading	**Culture Medium (minimum) (mL)**	**Suspension of E. cole (mL)**	**Water (mL)**	**Acete fraction of ethyl (mL)**	**Aqueous fraction (mL)**	**Solution of DMSO 8% (mL)**
Acetate solution 104.5mg L-1	28,1	0,5	-	1,4	-	-
Aqueous solution-102.6mg L - [1]	28,1	0,5	-	-	1,4	-
Acetate Solution 313.6mg L-1	25,3	0,5	-	4,2	-	-
Aqueous Solution	25,3	0,5	-	-	4,2	-

308 mg $^{L-1}$						
White	30	-	-	-	-	-
Reference	28,1	0,5	1,4	-	-	-
DMSO 0,337%	28,1	0,5	-	-	-	1,4
DMSO 1,12%	28,1	0,5	-	-	-	4,2
Volume Final	30 mL					

Descriptions of the solutions in Table 02:

- All solutions presented a final volume of 30 mL.

-Blank solution consisting only of the culture medium used to certify that the culture medium was free from contamination by micro-organisms;

-Reference solution, was used as a means of comparison regarding the development of the bacterium in the solutions of the samples under analysis, to verify the presence of substances harmful or beneficial to the bacterium. Being that, the harmful ones could inhibit the population growth of *E. coli*, while the beneficial ones could favor its proliferation, and may present a similar or even greater development than in the reference solution.

-Two DMSO reading solutions were tested, one at a concentration of 0.373%, and the other at 1.12%, these solutions were prepared from the DMSO solution described in topic 03, in which the objective of its preparation is to verify the possible interference of the solvent in the development of *E. coli* bacteria. The use of this reading solution was necessary, since the samples were solubilized with this solvent.

- Solutions of the aqueous fractions and ethyl acetate in 30 mL for the readings presents concentrations of 102.6 mg L-1 and 104.5 mg $^{L-1}$ respectively.

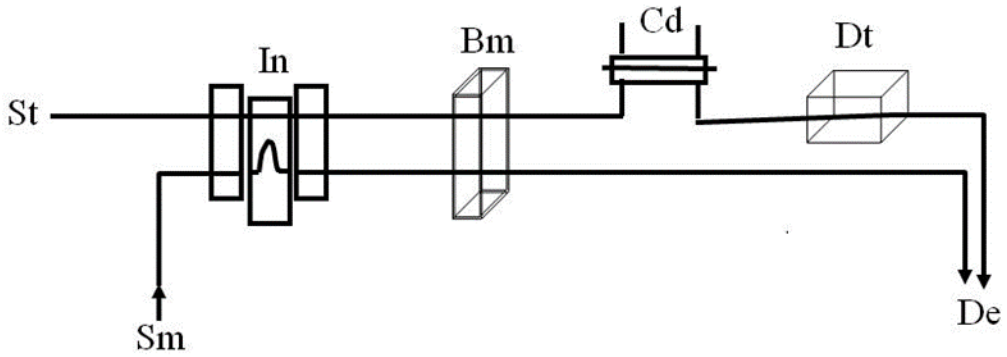
Solutions with three times higher concentration have values for aqueous fraction 308 mg L^{-1} and 313.6 mg L^{-1} for acetate fraction. These solutions were used in order to verify the behavior of the bacterium, whether it would present inhibitory effects or growth of the populations of microorganisms in the specific fractions of the plant.

With the respective solutions, the biological tests were carried out using a continuous flow system using spectrophotometric detection, and the readings were taken at 445 nm. Soon after the first readings, the test solutions were transferred to the water bath, where they were kept between 5 and 6 h at 36.5 °C, to favor the development of *E. coli* bacteria. During this period, readings were performed at 30-min intervals.

9 EQUIPMENT AND ACCESSORIES

The continuous flow system used was built in the laboratories of the Central Campus – Headquarters: Anápolis - CET, as shown in Figure 03. Consisting of a circular commutator injector constructed of acrylic plates, containing a sampling handle for delimitation of the volume of the sample solution; tygon tubes to promote the pumping of solutions; polyethylene tubes of 0.8 mm internal diameter, for insertion and channeling of sample solutions and reagents in the analytical path of the flow system; a peristaltic pump (Milan, model 204); a UV/VIS BIOSPECTRO ® spectrophotometer model Sp 220 alpha, length 445 -500 nm; a bucket one cm wide (optical path); a gas separation chamber by gaseous diffusion, for the separation and elimination of CO2 produced by the microorganisms (E. coli bacteria) in the medium, aiming to avoid the interference of this gas in the absorbance readings in the *spectrophotometer*. A Maria Solab Bath, Model: SL 155, was used to maintain the temperature of the solutions around 36.5 °C, ideal conditions for the proliferation of the inoculated microorganisms in the reading solutions, an analytical balance of the Gehaka brand and a pH meter with combined glass electrode.

Figure 03 - Flow system analysis module. St = carrier solution, H_{2SO4} 0.2 molL-1; Sm = sample solution; In = circular proportional injector; Bm = peristaltic pump; Cd = separation chamber Dt = detector, spectrophotometer; De = final disposal of the residues from the mixture of the solutions.Source: The authors.



10 DESCRIPTIONS OF THE OPERATION OF THE SYSTEM IN FLOW.

As represented in the analysis module of Figure 03, the carrier solution flows through the gas diffusion chamber, through the detector (spectrophotometer), reaching the disposal. The sample solution (Sm) is injected only at the time of sampling, the solution is inserted until it fills the sampling handle attached to the central part of the rotary injector, where it has a volume of 180 µl. Then the injector is moved by coupling the sampling handle to the path of the carrier solution (H2SO4 0.2 mol $^{L-1}$), where the main objective is to transport the sample solution, but also the acid is used as an aid in cleaning the tubes, further minimizing contamination. Thus, the aliquot of the sample solution travels the path described above, passing through the bucket in the detector, and may generate a change in the reading thus resulting in a variation of the analytical signal, proportional to the turbidity of the solution provided by the proliferation of *E. coli bacteria.*

11 PROSPECTING TESTS

The prospecting tests followed several procedures from the website of the Brazilian Society of Pharmacognosy (http://www.sbfgnosia.org.br/ accessed 2019). Where are all the methods of research of primary and secondary metabolites reported here.

12 RESEARCH OF SIMPLE PHENOLIC COMPOUNDS

Extraction: We weighed on a precision scale, 7.0 grams of the sample of the crushed leaves of *Bauhinia forficata* L., and added 60 mL of ethanol 70% (V/V). It boiled for 5 minutes and filtered on filter paper moistened with 70% ethanol (V/V).

Reaction with Alkaline Hydroxide: Transferred 3.0 mL of the extractive solution to a test tube. Add 1.0 mL of 20% NaOH (w/v) and shake the tube.

Reaction with Aluminum Chloride: Transferred about 5.0 mL of the extractive solution to porcelain capsule. Concentrated halfway and transfer to filter paper spreading all over the surface. Next, he moistened one of the regions of the paper with 5.0% aluminum chloride solution and observed under ultraviolet light.

Reaction with Ferric Chloride: Transferred 3.0 mL of the extractive solution to the test tube. 02 drops of ferric chloride at 4.5% (w/v) were added.

13 COUMARIN RESEARCH

Extract 2.0 g from the crushed leaves of *Bauhinia forficata* L. with 30 mL of (hot) water. Filtered. Added 1.0 mL of HCL 01 N. Transferred to a separation funnel and extract with 10 mL of ether, reduce the volume and drip on the filter paper that will be on a petri dish. Add a drop of NaOH 01 N in one of the regions where the ether extract was applied and observe in UV light.

14 FLAVONOID RESEARCH

Extraction: Boiled for 05 minutes, 7.0 g of powdered drug in 60 mL of 70% ethanol. Filter with filter paper, previously moistened with 70% ethanol.

Cyanidin or Shinoda reaction: There is the reduction of yellow flavonic derivatives, which acquire red coloration and anthocyanic ones, which acquire bluish coloration. This assay produces negative reaction for chalcones and hydroflavones.

Technique: Transferred 3.0 mL of the filtrate to a test tube. Added 01 cm of finely cut magnesium tape. He added 01 mL of concentrated HCL.

Reaction with concentrated H2SO4: There is the formation of oxonium salts, which can be precipitated by the addition of water. The flavonics form solutions from yellow to orange and the chalcones and auronas from red to carmine.

Technique: Added about 3.0 mL of the extractive solution to a porcelain capsule and evaporate to semi-dryness. Add 0.5 mL of H2SO4.

Oxalate-Boric Reactions: In a porcelain capsule, evaporated 5.0 mL of the extractive solution. He added to the semi-dry residue 3.0 mL of the boric acid solution and 01 mL of the oxalic acid solution. It evaporated until dry. He added 7.0 mL of ethyl ether to the dry residue. He watched in ultraviolet light.

Reaction with Alkaline Hydroxides: In alkaline medium, some groups of flavonoids have a specific coloration.

Technique: transferred 3.0 mL of extractive solution to a test tube and added 20% NaOH. He shook the tube.

Reaction with Aluminum Chloride: Flavonoid compounds in the presence of this reactive have variable fluorescence, from yellow to blue green.

Technique: Transferred 5.0 mL of extractive solution to a beaker or porcelain capsule. It concentrated up to about half the volume. He transferred it to a piece of filter paper, spreading it over the surface of it. He moistened one of the regions of the paper with 5% aluminum chloride solution and observed fluorescence over UV light.

14 RESIN RESEARCH

We extracted 2.0 g from the sample of crushed leaves of *Bauhinia forficata* L.com 30 mL of absolute ethanol. Shake and boil for 3 minutes. Filtered and added 10 mL of distilled water.

15 SAPONIN RESEARCH

We weighed 01 g of the sample from the crushed leaves of *Bauhinia forficata* L. and transferred to the erlenmeyer containing 50 mL of boiling water. Kept in moderate boiling for 30 minutes. Cooled and filtered into a 100 mL volumetric flask.

The volume was completed through the filter up to 100 mL. He distributed the decocto obtained in ten test tubes with caps of the same diameter and same height, in successive series from 01 mL to 10 mL and adjusted the volume of the liquid in each tube to 01 mL with distilled water.

16 TANNIN RESEARCH

Procedure: For the extraction of the possible tannins present in the pulverized sample were weighed on a precision balance, 02 g grams of the sample of the crushed leaves of *Bauhinia forficata* L. 50 mL of distilled water was added and boiled for 05 minutes. He then filtered the still-hot mixture using filter paper. Make up to 100 mL (extractive solution) volume. On a shelf, 12 test tubes were

organized. In 06 of the 12 test tubes added 05 mL of the extractive solution. In the remaining 06 tubes, the positive control was added: 05 mL of 0.5% tannic acid (w/v).

Sequence the following reactions:

Reaction with Gelatin: Added to the first tube (control sample), 05 drops of gelatin solution at 2.5% (w / v) in sodium chloride solution at 05% (w / v). Compare both tubes. The presence of tannins in the sample is seen by the appearance of a white precipitate, as these metabolites react with the amino acids of gelatin (proline, for example) by the amine grouping forming a precipitate.

Reaction with Alkaloids: Added to the second turbo (sample and control), 05 drops of 1% quinine sulfate solution (w / v) in sulfuric acid 5% (w / v). To the third tube (sample and control). Added 05 drops of 1% brucin solution in Sulfuric Acid 5% (w / v). He compared the tubes. The presence of precipitate indicates the existence of tannins in the sample. Tannins react with alkaloids because they contain the amine group in the molecule.

Reaction with Metal Salts: In the fourth tube (sample and control), added 05 drops of Copper Acetate at 4% (w / v). To the fifth tube (sample and control), two drops of 2% (w/v) Ferric Chloride were added. Compare the coloration of the tubes and the occurrence of precipitate formation. Tannins have the property of reacting and precipitating with metal salts, such as Lead, Copper Zinc, Chromium and Iron.

Reaction with Alkaline Hydroxides: To the sixth tube (sample and control) added 5 drops of 20% Potassium Sodium Hydroxide solution (W/V). The coloration of both tubes was compared. The presence of tannins in the sample was observed by the darkening of the solution. The tannins do not precipitate by the alkaline hydroxides, because the phenolic groups react to form soluble phenates.

Stiasny reaction: Identification between hydrolyzable tannins and condensed tannins: Submit to reflux for 30 minutes 50 mL of the extractive solution (sample) + 15 mL of Stiasny reactive.

Stiasny reactive: 05 mL of concentrated HCl + 10 mL of formaldehyde, 20 minutes on reflux.

The condensed tannins form red precipitate (flobafens). The hydrolyzable tannins remain in solution and can be detected as follows: 10 mL of the filtrate + 05 g of sodium acetate + (02 – 04) drops of 1% FeCL3 solution in methanol.

17 TERPENE RESEARCH

Extraction: 02 g of sample, 30 mL of a 50% hydroalcoholic solution and 10 mL of 10% lead acetate were added. It boiled for 04 minutes. It was filtered and 10 mL of chloroform was added. 03 mL of the chloroform phase was removed and 01 mL of the Liebermann-Burchard reagent was added.

18 RESULTS AND DISCUSSIONS

In the prospecting tests, several metabolites were found, which are produced by the vegetable (Chart 03), and which may provide some antimicrobial activity.

Table 03 - Metabolites found in the crushed leaves of *Bauhinia forficata* L (2019).

METABOLITES	PRESENCE OR ABSENCE
Simple phenolic compounds	Positive
Coumarins	Positive
Flavonoids	Positive
Tannins	Positive for hydrolysables
Triterpenes and/or Salkowski steroids	Positive
Alkaloids	Positive
Resins	Positive
Saponins	Positive

Among the metabolites researched, we can highlight a large amount of saponins that have emulsifying activity, saponifying, and hemolytic. In addition, it was also observed the presence of simple phenolic compounds, coumarins, flavonoids, hydrolyzable tannins, triterpenes, alkaloids and resins, which may exert various pharmacological activities. These experiments were able to demonstrate the great richness of compounds produced by this vegetable, which can be explored in various ways in the medicinal and scientific branch.

Subsequently, the steps for preparation were initiatedof the E.B.E., where 96º ethanol was used as an extracting solvent, because this is very indicated for this activity, since this substance is one of the most appropriate, being a solvent with polar and nonpolar characteristics, which would increase the chance of removing the highest percentage of metabolites present in the leaves of *Bauhinia forficata* L. (Figure 04). This solvent was then evaporated so that we could obtain this dry E.B.E in a device called a rotary evaporator and subsequently perform its fractionation.

Figure 04 – Cold extraction of crushed leaves of *Bauhinia forficata* with 96% Ethanol.

Source: The Authors (2019).

The equipment used to remove the solvent (rotary evaporator represented in FIgura 05) eliminated it through a simple distillation process, which was very easy to perform, and made it possible to obtain the E.BE. Dry, which was later fractionated.

Figure 05- Rotary evaporator equipment, containing E.B.E.

Source: The authors (2019).

After obtaining the Dry E.B.E, and its dilution with a methanol/water solution, it was possible to perform the fractionation of the organic phase obtained in the phase separation, with the solvents: Hexane, Dichloromethane, and Ethyl Acetate, which are some of the most used in this process, due to their polarity characteristics (the remaining phase was called aqueous because no compound had affinity for the solvents used). Thus, the four fractions shown in Figure 06 were obtained, which were able to extract the compounds produced by the plant, through chemical affinity between its molecules, which would also facilitate a future chemical identification of compounds.

Figure 06 – Solutions obtained in fractionation. From left to right: Hexane, Dichloromethane, Ethyl Acetate, and Aqueous Phase respectively (à).

Source: The Authors (2019).

After obtaining these phases, we moved to the next step, which was to obtain these samples without the liquid solvent (Figure 07) and, therefore, they were packed in round-bottomed balloons, and submitted again to the distillation process, in the rotary evaporator equipment, which took approximately 02 months to be completed due to the difficulty of evaporation of some solvents that still contained water. Evaporation was necessary so that these solvents would not interfere with the analytical results (inhibiting bacterial growth) when manipulated to the samples to be analyzed

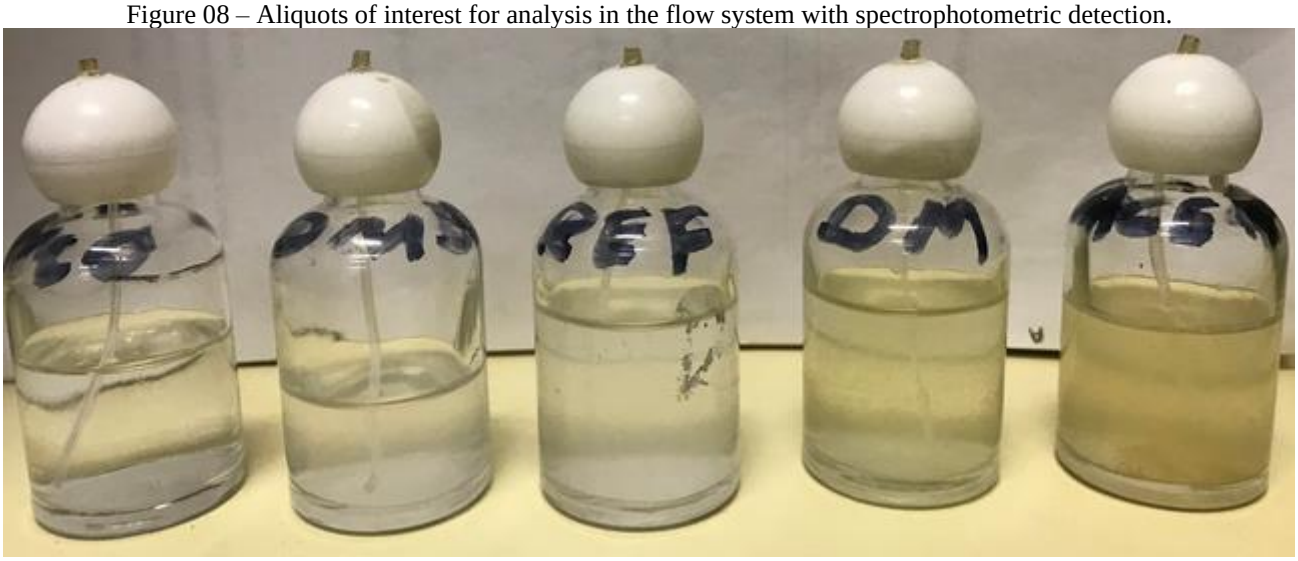
Figure 07 – Solutions obtained in fractionation after evaporation of solvents. Ethyl Acetate, Dichloromethane, Hexane, and Aqueous Phase respectively (à).

Source: The Authors (2019).

The dilution of each fractionation sample (roto-evaporated) was performed with the solvent called Dimethylsulfoxide (DMSO), because it was considered the most appropriate for this stage, making it possible to perform the subsequent preparation of the aliquots of interest, inoculating together the bacteria (*E. coli)* and analyzing them on the spectrum.

The aliquots named in the methodology as: Hexane, Ethyl Acetate, Dichloromethane and Aqueous, could then be analyzed in equipment, to verify the growth of the bacterium over time, comparing them with the aliquots of Reference, Reference of DMSO and White (Figure 08).

Figure 08 – Aliquots of interest for analysis in the flow system with spectrophotometric detection.



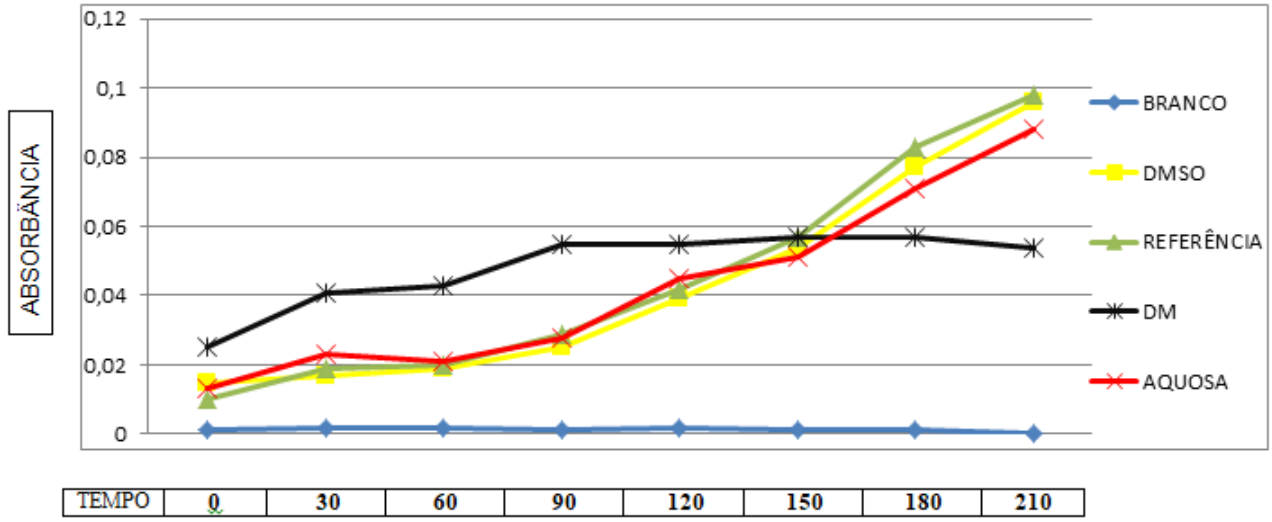
Source: The Authors (2019).

These solutions of the samples represented above were used for the spectrophotometric analysis, where they presented absorbance values according to the growth or not of the bacterium under study (the aliquots of the Aqueous Phase and Hexane Phase are not represented in the image, but were analyzed).

In both graphs of Figures 09 and 10, which demonstrate the results of the tests performed in spectrophotometer, it was possible to observe a curve indicating bacterial growth in the analysis of the reference solution and DMSO, which was already expected, since these were the means of comparison, where the microorganism proliferated without any interference from solvents or external contamination. The white medium in both cases was not contaminated, since asepsis patterns were used in the preparations, and therefore did not present a significant absorbance value. Thus, the analyses can be performed with the solutions of interest, containing the fractions obtained plus the culture medium and the bacterium; comparing them in this way with those mentioned above.

In Figure 09, it can be observed that the Aqueous Phase presented a microbial growth, which could be expected, because this environment (containing a lot of water) sometimes provides a growth of microorganisms such as *E. coli*, therefore, in this case, there was no inhibition of growth, but a favoring of its multiplication. Already with the phase of Dichloromethane happened the opposite, because there was the growth of the microorganism to a certain extent (about 90 minutes), and from this moment its growth remained constant until it began to decay in 210 minutes. Thus, it can be understood that in this fraction, some compound (s) of the leaves of the plant that have action on the microorganism used as a test was adhered to, and that, in higher concentrations, they may have a more immediate antimicrobial action than that presented in these conditions.

Figure 09 – Relation of the value of the absorbances of the samples of Dichloromethane and Aqueous Phase after the respective times (min). Absorbance: Intrinsic capacity of materials to absorb radiation at a specific frequency (nanometers); Time: Interval between analyses to ensure microbial growth (minutes); White: Sample containing only culture medium; DMSO: Culture medium plus the Dimethylsulfoxide solution (with a concentration equal to that used in the solubilization of the samples) and the bacterium; Reference: Culture medium plus bacteria; DM: Culture medium + bacteria + dichloromethane fraction; Aqueous: Culture medium + bacteria + aqueous fraction.

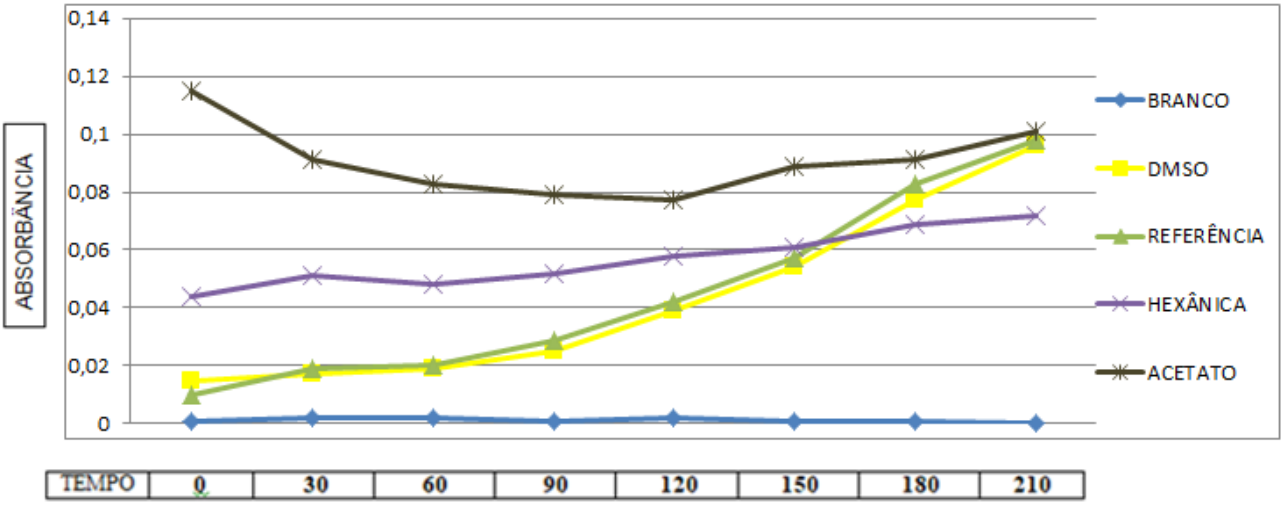


Source: the authors (2019).

In Figure 10, the hexane phase showed a constant microbial growth, but it was lower than the values presented by the reference medium, so this solution should be tested in higher concentrations

to be sure of an inhibition or favoring of *E. coli proliferation.*The phase of Ethyl Acetate, on the other hand, presented high values since the beginning of the analyses, and this is probably due to the amount of chlorophyll present in the sample (more stained), after a certain time, its absorbance value came decreasing up to 120 minutes, and then began to increase again, reaching values close to those of the reference medium; and therefore, A more in-depth investigation is indicated, so that it is possible to know if the microorganism consumed to a certain extent the stained constituents of this medium to use them for its benefit in proliferation (decreasing chlorophyll and consequently the values) or if there really was no microbial growth, and the divergence between the absorbances demonstrated are due to some deviation presented by the spectrophotometer device at the time of analysis.

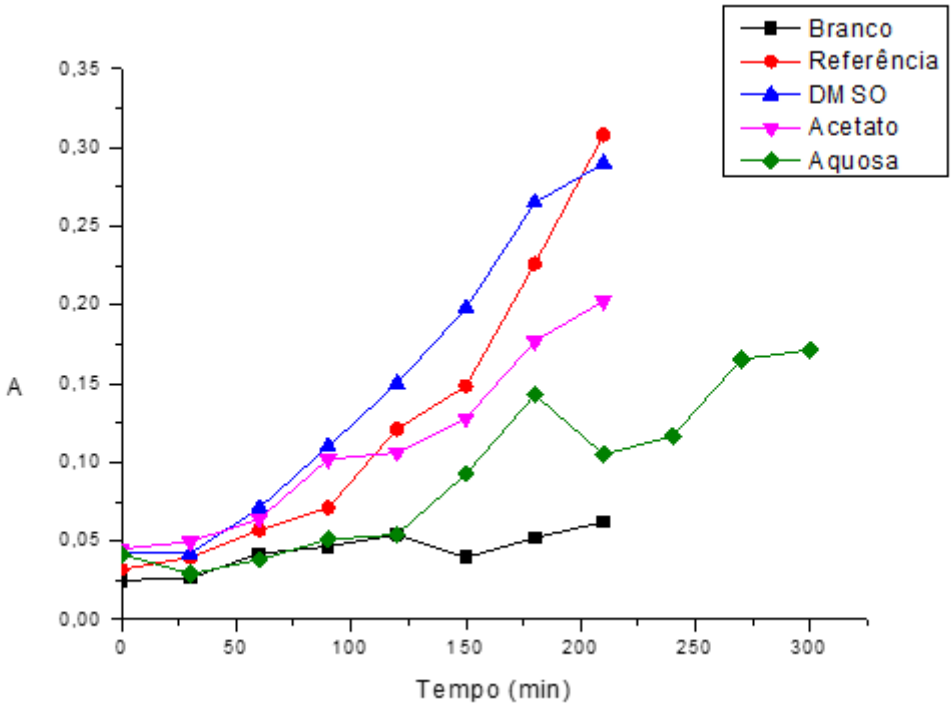
Figure 10 – Relation of the absorbance value of the Hexane phase and Ethyl Acetate samples after the respective times in minutes. Absorbance: Intrinsic capacity of materials to absorb radiation at a specific frequency (nanometers); Time: Interval between analyses to ensure microbial growth (minutes); White: Sample containing only culture medium; DMSO: Culture medium plus the Dimethylsulfoxide solution (with a concentration equal to that used in the solubilization of the samples) and the bacterium; Reference: Culture medium plus bacteria; Hexane: Culture medium + bacteria + hexane fraction; Acetate: Culture medium + bacteria + ethyl acetate fraction.



Source: the authors (2019).

The results regarding the evaluation of the biological activity in the aqueous and ethyl acetate fractions of the sample under analysis are represented in the graphs of Figure 11.

Figure 11 - Results of the evaluation of biological activity in the aqueous and ethyl acetate fractions with concentrations of 102.6 mg L-1 and $^{104.5\ mg}$ L-1 respectively.



Source: the authors (2021).

Figure 11 shows a growing curve of the reference solution, indicating microbial proliferation, where this result was already expected, since this solution aims to compare the analyses of interest. In the blank solution (white medium) there is a constant curve that did not present marked variation in the signals during the analysis, this is due to the asepsis procedures performed so that contamination of the culture medium by microorganisms does not occur. It is also observed a growing curve of the DMSO solution that accompanies the reference solution, presenting a significant result, which demonstrates that the presence of the solvent, in front of the bacterium does not interfere in its proliferation, and thus proves that the DMSO in the experimental conditions does not interfere in the development of the bacterium, since it was used for its solubilization.

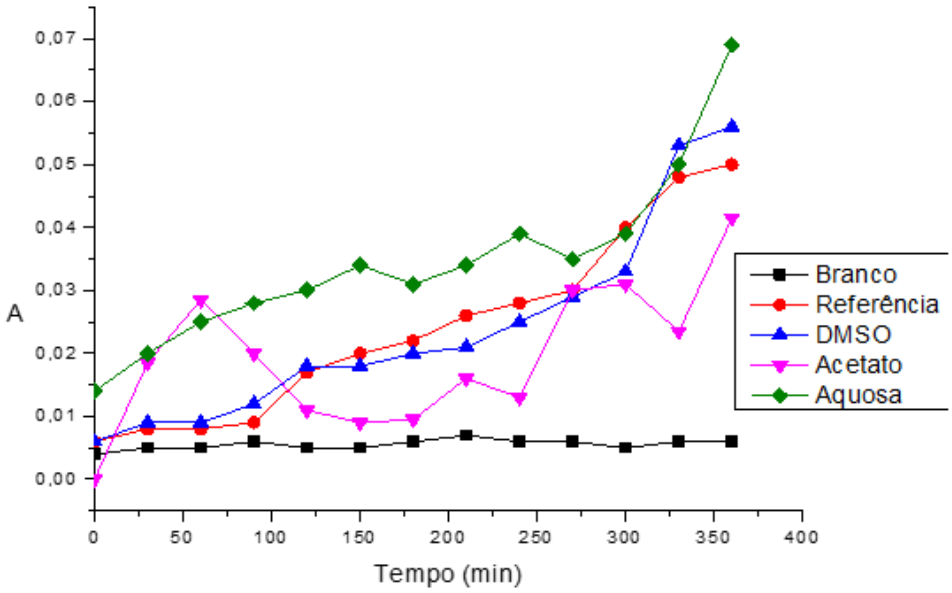
In the solution of the ethyl acetate fraction there is a growing curve, with absorbance signal sensitivity almost in the same order as the reference solution, indicating that possibly the acetate fraction at the concentration of 104.5 mg $^{L-1}$ did not present marked interference in the growth of *E. coli*.

Already in relation to the aqueous fraction in the concentration of 102.6 mg $^{L-1}$ can be observed a marked inhibition in the initial time interval of 0 – 120min in relation to the population development of the bacterium, that is, the sensitivity of the signal in absorbance, accompanied the sensitivity of the blank solution, this inhibition may be an indication of the presence of possible substance with active principle, that can be harmful, that can disable the bacteria or inhibit its population development. These substances can be, for example, vermículo, antibiotic or that contain some toxicity. Subsequently, at the end of the reading period, there was a timid increase in sensitivity, it is believed

that this slight development may have occurred due to low concentrations of substances harmful to the development of the bacterium and possible nutrients present in the environment.

With the intention of achieving better results, from the previous analyses, tests were performed with the reading solutions at the concentration three times higher, Figure 12.

Figure 12 - Results of the evaluation of biological activity in the aqueous and ethyl acetate fractions with concentrations of 308 $^{mg\ L\text{-}1\ and\ 313.6\ mg}$ L^{-1} respectively.



Source: the authors (2021).

It is possible to observe a constancy in relation to the curve of the blank solution demonstrating that there were no contaminations. The reference solutions and DMSO, it is observed that the growth of the bacterium occurred. Already the analysis solutions of the samples occurred incompatibility in relation to the first analysis, it is observed that the fraction of acetate, despite the great variation is noted an increasing in the absorbance signal. The greatest discrepancy is found in the aqueous fraction, where in the first analysis it presented a marked inhibition, but this trend is not observed in the readings made with the higher concentration. These results can be explained due to the delay in carrying out the **analyses, because during the pandemic** it was necessary to paralyze the studies for a long period, which resulted in some losses, both with the bacterium and with the samples in relation to their refrigeration, possibly the substance that had this tendency of inhibition observed in the first analyses, may have suffered degradation thus interfering in the results referring to the latest studies.

19 FINAL CONSIDERATIONS

The results presented in this project indicated that there are indications that compounds present in the plant *Bauhinia forficata L.* may favor and others may inhibit the proliferation of microorganisms, in theory the *Escherichia coli.* Therefore, it is recommended to deepen the studies on these activities presented by this vegetable in this work, performing the tests under the same conditions, but with different concentrations, so that it can evaluate the actual inhibition range of the samples tested. As well as, performing plating on agar plates, containing different dilutions, and testing other microorganisms. It should also be considered the isolation of the compounds present in each of the

fractions of this crude extract, so that they are elucidated in a better way, thus being able to know which probable metabolites are responsible for a given activity, contributing even more to the scientific environment.

In view of the analyses performed, it is possible to observe results that present a favoring and inhibition of the multiplication of microorganisms, in theory E. coli, in the solutions of the samples under analysis. In the solution of the ethyl acetate fraction, it is possible to observe a constant growth of the inoculated bacteria, but there is a smaller signal in relation to the reference, so there is no result that may indicate an interference of the sample in relation to the proliferation or inhibition of the bacteria. The opposite can be observed in the aqueous fraction, which was found a marked inhibition regarding the development of the bacterium, being seen as a possible presence of some active principle in this solution, with some variations at the end of the reading period in relation to time, in which by presenting water in its environment tends to a greater proliferation. By increasing the concentrations, a satisfactory result was expected.

However, due to the timing of the pandemic, the second analysis did not produce a satisfactory result. It is believed that due to the stoppages there were some losses due to the delay in the analyzes as well as in relation to the equipment used to store the samples and this possibly may have caused this discrepancy in the results obtained, especially with the aqueous fraction that there is a tendency of inhibition in the first analysis and did not present the same characteristic in relation to the second, Probably the substance that had this tendency of inhibition observed in the first readings may have suffered degradation and this may have interfered with the results.

Despite the difficulties that were encountered during the accomplishment of this work, it is expected that these studies can be carried out, through the repetition of new biological assays with solutions in higher concentrations of the fractions of the plant or even with the accomplishment of the elucidation of the chemical compounds that confer these characteristics so peculiar.

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