UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso de Farmácia

SUPERFÍCIES ANTI-INFECTIVAS: USO DE PLANTAS DA CAATINGA COMO FONTE DE COMPOSTOS CONTRA BACTÉRIAS PATOGÊNICAS

JÚLIA CAPP ZILLES

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Resumo: Com o avanço da ciência nas áreas biomédicas, o uso de dispositivos implantáveis vem aumentando. É estimado que 80% das infecções associadas a tais dispositivos são relacionadas a biofilmes. O biofilme é uma estrutura organizada de células microbianas embebidas em matriz exopolimérica, o que dificulta a ação de antibióticos. Portanto, devido às implicações sociais e financeiras de infecções relacionadas a biofilmes e à baixa disponibilidade de fármacos para inibir ou dispersar os mesmos, a busca por novas moléculas capazes de combatê-los é imprescindível. Plantas endêmicas são possíveis fontes de compostos com atividade biológica. A região semiárida do Brasil, conhecida como Caatinga, é um bioma endêmico no qual a flora precisa desenvolver características para sobreviver nesse ambiente rigoroso, com altas temperaturas e baixa pluviosidade. Suas plantas são únicas, sendo uma fonte promissora de metabólitos secundários que podem ter atividade antibiofilme. Após rastreamento da atividade contra duas espécies de bactérias gram-negativas e três gram-positivas com nove plantas endêmicas da Caatinga, a *Schinopsis brasiliensis* (braúna) apresentou pronunciada atividade antibiofilme (80%). Com o intuito de mimetizar dispositivos médicos, superfícies poliméricas foram preparadas com o kit *Sylgard® 184 Silicone Elastomer*, no qual o extrato na concentração de 0,1 mg/mL mostrou uma atividade antibiofilme de 50% contra *Staphylococcus aureus* ATCC 43300, um patógeno de difícil tratamento envolvido em infecções hospitalares. Estudos de caracterização da superfície estão em andamento no intuito de entender as possíveis vias de ação do extrato no processo de adesão bacteriana.

Palavras-chave: biofilme, *Schinopsis brasiliensis*, *Staphylococcus aureus* ATCC 43300, superfície polimérica.

ANTI-INFECTIVE SURFACES: CAATINGA PLANTS AS A SOURCE OF COMPOUNDS AGAINST PATHOGENIC BACTERIA

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Keywords: biofilm; *Schinopsis brasiliensis*; *Staphylococcus aureus* ATCC 43300; polymeric surface.

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Abstract: With scientific advance in biomedical areas, the use of implantable devices has been increasing. It is estimated that 80% of the infections associated to such devices are due to bacterial biofilms. Biofilm is an organized structure of microbial cells soaked in an exopolimeric matrix, which makes it harder for antibiotics to be successful. Therefore, due to social and financial implications of biofilm-related infections and to the low availability of drugs to inhibit adhesion or disperse them, the search for new molecules capable of fighting pathogenic biofilms is essential. Endemic plants are a possible source of compounds with biological activity. The semi-arid region of Brazil, known as Caatinga, is an endemic biome in which the flora has to develop characteristics to survive in this harsh environment, with high temperatures and low rainfalls. Its plants are unique, being a promising source of secondary metabolites that could have antibiofilm activity. After screening the activity against two gram-negative and three gram-positive bacterial strains with nine endemic Caatinga plants, *Schinopsis brasiliensis* (braúna) presented pronounced antibiofilm activity (80%). In order to mimic medical devices, polymeric surfaces were prepared with *Sylgard® 184 Silicone Elastomer kit*, in which the extract of braúna incorporated in 0.1 mg/mL presented antibiofilm activity of 50% against *Staphylococcus aureus* ATCC 43300, a pathogen of hard treatment involved in hospital infections. Surface characterization studies are in progress in order to understand the possible path of action of the extract in the bacterial adhesion process.

Keywords: biofilm; *Schinopsis brasiliensis*; *Staphylococcus aureus* ATCC 43300; polymeric surface.

1. INTRODUCTION

Bacteria can be found in two different forms: planktonic cells and biofilm communities. In planktonic form they are more susceptible to antibiotics and to the host's immune response [1], while in biofilm the inner cells may be less metabolically active, therefore facilitating their resistance [2]. The biofilm is a junction of bacterium cells in an organized structure soaked in exopolimeric matrix [3]. Initially planktonic cells adhere to a surface, which can be biotic (cells or tissues) or abiotic (like plastics or metals), through nonspecific interactions (abiotic surfaces) or specific molecular receptor-binder mechanisms (biotic surfaces). Biofilm then proliferates and maturates, being capable of dispersion, which promotes the infection of other surfaces [3-5].

Infections caused by resistant microorganisms are a challenge for the health system, which can impact in longer hospitalization time and rise in costs. According to the "National Institutes of Health" 80% of infections worldwide are related to micro-organisms that produce biofilm [5]. With the increasing life expectancy, the need for medical devices, as catheters for dialysis, surgeries and biomaterial implants, like joint replacements, also rise. Apart from their composition, they all attract microorganisms that can adhere, proliferate and form biofilm in the abiotic surface, adding risk to human use. Implants and medical devices make it possible to live longer, but infections related to them are a problem to the clinic because they are associated with higher rates of morbidity and mortality, and imply in significant costs, as the most effective response to such infections are the replacement of the implant combined with systemic antibiotic therapy [2, 6].

Antibiotics have five main action mechanisms. They can act in the cell wall, inhibiting its synthesis and disrupting it; in the cell membrane, altering its permeability and causing damage to it; inhibiting the proteic synthesis; inhibiting replication and transcription of DNA and; inhibiting the synthesis of essential metabolites [7].

The interactions involved in the initial adhesion are reversible and include the Brownian movement or the bacterial flagellum. Physicochemical interactions, like electrostatic, Van der Waals and, hydrophobic interactions, hydrogen bounds, or interactions through adhesins, then take place and can lead to irreversible adhesion [5]. Features of the bacterium, the properties of the materials surface and the micro-environmental interactions are involved in the adhesion process [8]. To prevent such interactions, changes in the materials surface can be done by, for instance, modifying its structure in order to increase electrostatic repulsion between the negatively charged Gram positive bacterial surface and surfaces negatively charged, or by making the surfaces more hydrophilic, reducing the adhesion, are some of the alternatives. [9]

The search of new active molecules from natural products obtained in different environments has been considered, over the last few decades, as a good strategy for the combat of multi-drug resistant bacteria and biofilm formation [10,11]. Some of such molecules have been sought in Brazil. Brazil is the fifth largest country in the world and is known to have vast biodiversity. It has six distinct biomes: Amazônia, Cerrado, Caatinga, Mata Atlântica, Pampa and Pantanal, and each of them have its own fauna and flora. In the northeast of the country, which is in Earth's tropical zone, there is a semi-arid region called Caatinga, which is an exclusive biome of the Brazilian territory. It encompasses ten of the twenty seven Brazilian states and the equivalent of 11% of the national territory. 844,453 km^2 of the country are covered by this unique vegetation

which is widely known and used by local communities for medicinal purposes. In addition to that, being a semi-arid biome, with high temperatures and low rainfalls, vegetation there has to adapt in order to survive, being a source of potential molecules. Furthermore, Caatinga is one of the most neglected biomes of the country, with little more than 1% being under full protection and is also quite unexplored, being a promising source of unknown compounds with secondary metabolites with activity against biofilm [12-14].

The present study aims at the screening of nine endemic plants from the Caatinga, all of them with medicinal usage, to check whether they have antibiofilm activity and then develop an anti-adherent surface for biomedical usage.

2. MATERIALS AND METHODS

2.1 PLANT MATERIAL

Plants (Table 1) were sampled at a national park, Parque Nacional do Catimbau, located in the state of Pernambuco, Brazil, under authorization of the responsible authority Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) using the license SISBIO 16.806. The powdered bark was submitted to extraction with cold water for 24h and filtered with membrane filter of 0.22 µm.

Table 1: Endemic plants used in the experiment.

Popular name	Scientific name	Family
Angico de caroço (ac)	*Anadenanthera colubrina* var. *cebil*	Fabaceae
Aroeira (a)	*Myracrodruon urundeuva*	Anacardiaceae
Quixabeira (q)	*Syderoxylum obtusifolium*	Sapotaceae
Ameixa Recanto (ar)	*Ximenia americana*	Olacaceae
Jatobá (j)	*Hymenaea courbaril*	Fabaceae
Umburana de cheiro (u)	*Amburana cearensis*	Fabaceae
Braúna (b)	*Schinopsis brasiliensis*	Anacardiaceae
Commiphora (c)	*Commiphora leptophloeos*	Burseraceae
Algaroba (al)	*Prosopis juliflora*	Fabaceae

2.2 BACTERIAL STRAINS AND CULTURE CONDITIONS

Staphylococcus epidermidis (American Type Culture Collection (ATCC) 35984), *Staphylococcus aureus* (ATCC 25904), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603) and, *Staphylococcus aureus* (ATCC 43300) were grown overnight in Mueller-Hinton (MH) agar at 37ºC. A bacterial suspension with NaCl 0.9% sterile and optical density (OD) of 0.150 in 600 nm was used in the assays.

2.3 ANTIBIOTIC ACTIVITY ASSAY

The antibiotic activity assay was performed according to [15] with some modifications. A 96-well plate was used, in which 4 control groups were established (sterility, antibiotic, color (white) and growth). 200 µL of tryptone soya broth (TSB) was added to the sterility control group and 40 µL of the same medium was added to the other groups. In the compound and white groups 80 µL of the raw aqueous extract were added in two concentrations: 0.1 and 0.5 mg/mL, while in the antibiotic group 80 µL of antibiotic were added (Gentamicin for gram negative bacteria, Rifampicin for gram positive and Vancomycin for MRSA), and in the growth control group 80 µL of saline solution (0.9%). 80 µL of bacterial suspension were added to all wells but the sterility control and white groups. The white groups were needed in order to discount its reading in the extract groups, as they all have a color that would interfere in the readings. The absorbance was read in time 0h in 600nm. It was then incubated in 37ºC for 24h and read again in time 24h in 600nm. The result was obtained by the difference between readings in time 0h and time 24h. The assay was performed three times, in triplicate.

2.4 ANTIBIOFILM ACTIVITY ASSAY

The antibiofilm activity assay was performed as established by [15] with some modifications. The violet crystal technique was employed. After 24h of incubation (as described before) the content of the wells was removed and the wells were washed three times with sterile saline solution. The plate was incubated at 60ºC for 1h so the remaining attached bacteria could be heat-fixed. After this period a solution of 0.4% violet crystal was added in the wells to stain the biofilm formed for 15 minutes at room temperature. The plate was then washed with running water and ethanol P.A was added to the wells for 30 minutes to solubilize the violet crystal adhered. The plate was then read at 570nm. The growth control group was considered to represent 100% of biofilm formation. The assay was performed three times, in triplicate.

2.5 INCORPORATION OF THE RAW EXTRACT IN THE POLYMER

Sylgard 184 Silicone Elastomer Kit was used to create a polymeric surface in which the raw extract was incorporated. The kit consists of two parts, base and curing agent, with a mix ratio of 10 to 1. The assay was performed using a 12 well plate with a final volume of 600 µL. Solutions of the raw aqueous extract were prepared and added to the compound and white wells in 5%, in order to obtain final concentrations in the polymer of 5.0 mg/mL and 0.1 mg/mL. The growth and sterility control groups received water to equal the volume. Both water and extract were dissolved with rapid stirring. The plate was incubated for 1h at 60ºC [16].

The antibiotic and antibiofilm activity assay was adapted to be performed with the polymeric surface. In order to do that, 50 mL falcon tubes were used instead of 96-

well plate and the volumes were also adapted to cover the samples, but the percentage of the items was kept. The assay was carried out one time in quadruplicate.

2.6 COLONY-FORMING UNITS (CFU) COUNTING - ADHESION TEST

At first, the polymeric surfaces (developed as presented in 2.5) were incubated following the antibiotic assay adapted to be performed with the polymeric surface and the usage of falcon tubes. After the incubation period (24 h, 37 ºC), samples were washed three times with sterile saline and transferred to new falcon tubes. 5 mL of sterile saline were added and then vortexed vigorously for 1 minute. Finally 0.1 mL from this bacterial solution was added into a tube with 0.9 mL of sterile saline and serial dilutions were performed. Drops of 10 µL of the bacterial solution and its dilutions were added to Müeller-Hinton agar plates and incubated for 24h, 37ºC. Colony forming units were then counted.

2.7 THIN LAYER CHROMATOGRAPHY (TLC)

The raw aqueous extract was applied in thin layer chromatography plates. The mobile phase used was butanol, acetic acid and water (4:1:5). The plates were revealed with different chemical agents: 1) iodine, 2) natural reagent + polyethylene glycol, 3) ferric chloride, 4) anisaldehyde, 5) ninhydrine, and 6) Dragendorff, to detect, respectively, double bonds, flavonoids, tanins, terpenoids and saponins, amino acids and, alkaloids. 4) and 5) were incubated at 100ºC for 10 minutes. 2) and 4) were visualized under UV light (365 nm). [17]

2.8 EXTRACTIONS

After liofilization, raw aqueous extract (4 g) was extracted with methanol (30 mL) in paper filter. A fraction soluble in methanol (Ox1) and an insoluble one (Ax1) were obtained. The methanolic fraction was evaporated and then water was added to both fractions, which were then frozen and liofilized. Afterwards they were dissolved in water and tested in two concentration, 0.1 mg/mL and 0.5 mg/mL. The same extraction was performed with exhaustion, using methanol three times. A methanol soluble (Ox3) and a methanol insoluble (Ax3) fractions were obtained and the same procedure took place [9].

2.9 STATISTICAL ANALYSIS

Experiments were carried out at least in duplicate and results are shown as percentage mean ± standard deviation. Differences between groups were evaluated by Student's t-test and a $p \leq 0.01$ was considered significant for the screening and a $p \leq 0.05$ was considered significant for the UFC/mL counting.

3. RESULTS

3.1 SCREENING FOR ANTIBIOTIC AND ANTIBIOFILM ACTIVITY

In order to evaluate the activity of the nine selected plants, a screening for antibiotic and antibiofilm activity was performed with the raw aqueous extract. Initially it was tested against *Klebsiella pneumoniae* (ATCC 700603) and *Staphylococcus epidermidis* (ATCC 35984) in the concentration of 0.5 mg/mL. For *Klebsiella pneumoniae* (ATCC 700603) (figure 1), some of the extracts had a discrete antibiotic activity that can be mentioned, but none of them presented a significant antibiofilm activity. For *Staphylococcus epidermidis* (ATCC 35984) (figure 2), the antibiotic activity of four out of the nine extracts tested were relevant, inhibiting more than 94% of bacterial growth, being them from aroeira, ameixa, jatobá and commiphora. As for antibiofilm activity, the braúna is worth mentioning, inhibiting about 40% of its formation. Due to the antibiotic activity of the extracts against the Gram positive bacterial strain, another concentration of the extracts was tested, a lower one, of 0.1 mg/mL. From here on, both concentrations were tested.

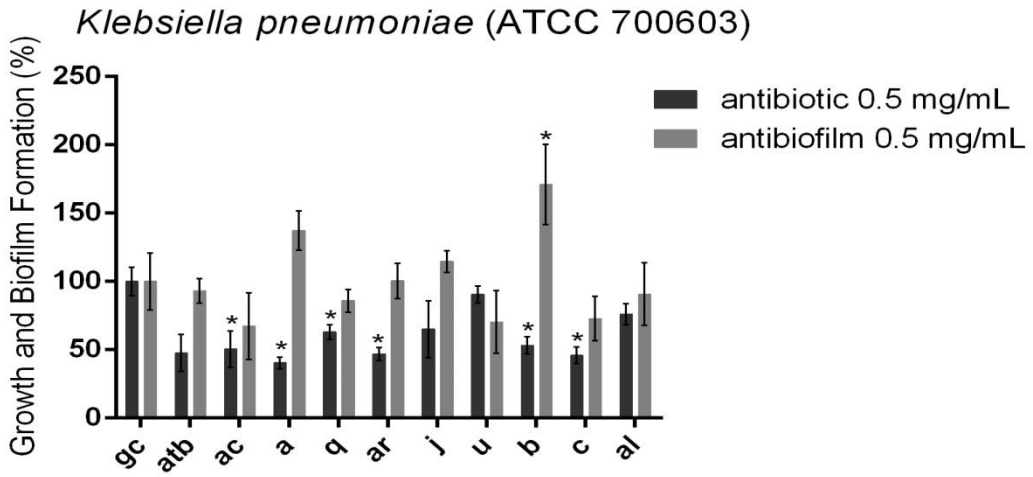

Figure 1: Bacterial growth and biofilm formation (%) of *Klebsiella pneumoniae* (ATCC 700603) in presence of the nine raw aqueous extracts tested. Gentamicin was used as a positive control. The letters in the x-axis represent growth control (gc), antibiotic control (atb), angico de caroço (ac), aroeira (a), quixabeira (q), ameixa recanto (ar), jatobá (j), umburana de cheiro (u), braúna (b), commiphora (c) and algaroba (al).

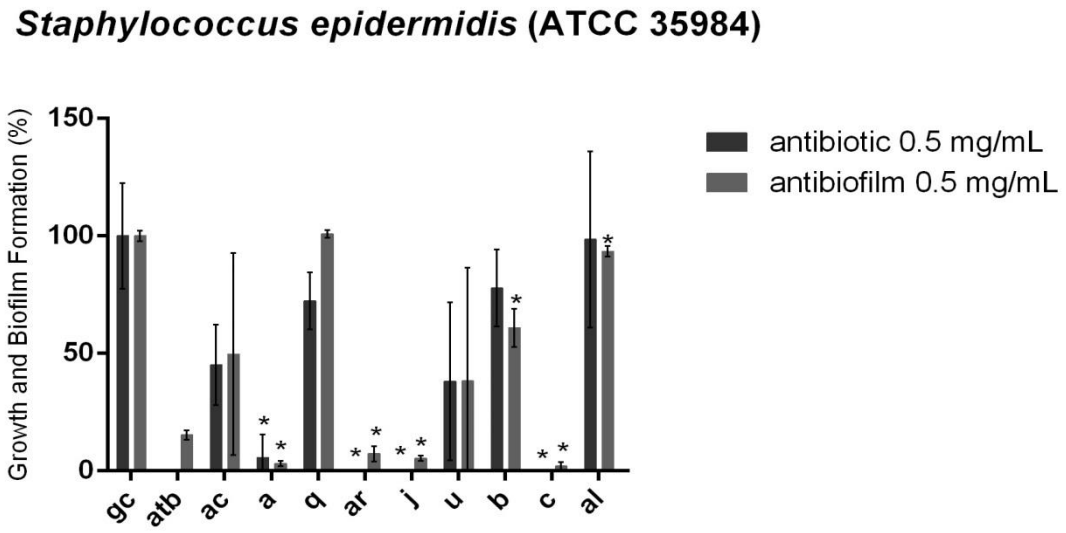

Figure 2: Bacterial growth and biofilm formation (%) of *Staphylococcus epidermidis* (ATCC 35984) in presence of the nine raw aqueous extracts tested. Rifampicin was used as a positive control. The letters in the x-axis represent growth control (gc), antibiotic control (atb), angico de caroço (ac), aroeira (a), quixabeira (q), ameixa recanto (ar), jatobá (j), umburana de cheiro (u), braúna (b), commiphora (c) and algaroba (al).

For *Pseudomonas aeruginosa* (ATCC 27853) (figure 3) both concentrations were tested (0.1 and 0.5 mg/mL) but none of them presented activity against bacterial growth or biofilm formation.

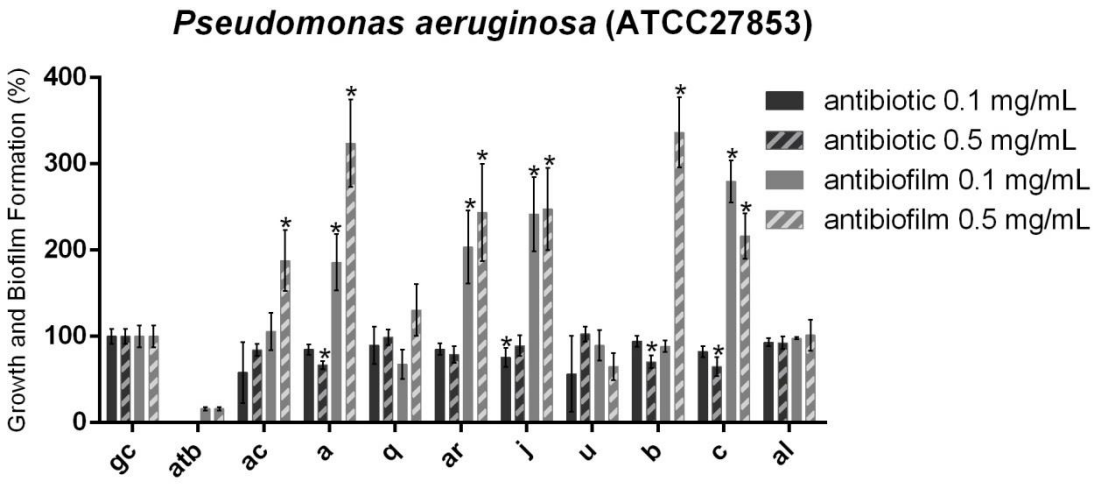

Figure 3: Bacterial growth and biofilm formation (%) of *Pseudomonas aeruginosa* (ATCC 27853) in presence of the nine raw aqueous extracts tested. Gentamicin was used as a positive control. The letters in the x-axis represent growth control (gc), antibiotic control (atb), angico de caroço (ac), aroeira (a), quixabeira (q), ameixa recanto (ar), jatobá (j), umburana de cheiro (u), braúna (b), commiphora (c) and algaroba (al).

For *Staphylococcus aureus* (ATCC 25904) (figure 4) both antibiotic and antibiofilm activity, in the two tested concentrations, must be highlighted. For the concentration of 0.5 mg/mL the aroeira, ameixa, jatobá and commiphora extracts, which had antibiotic activity against *Staphylococcus epidermidis* (ATCC 35984), also had antibiotic activity against *Staphylococcus aureus* (ATCC 25904). Besides those, braúna also presented antibiotic activity. As for the antibiofilm activity, angico de caroço presented significant activity. For the lower concentration, ameixa and quixabeira presented antibiotic activity while braúna presented antibiofilm activity of about 70%.

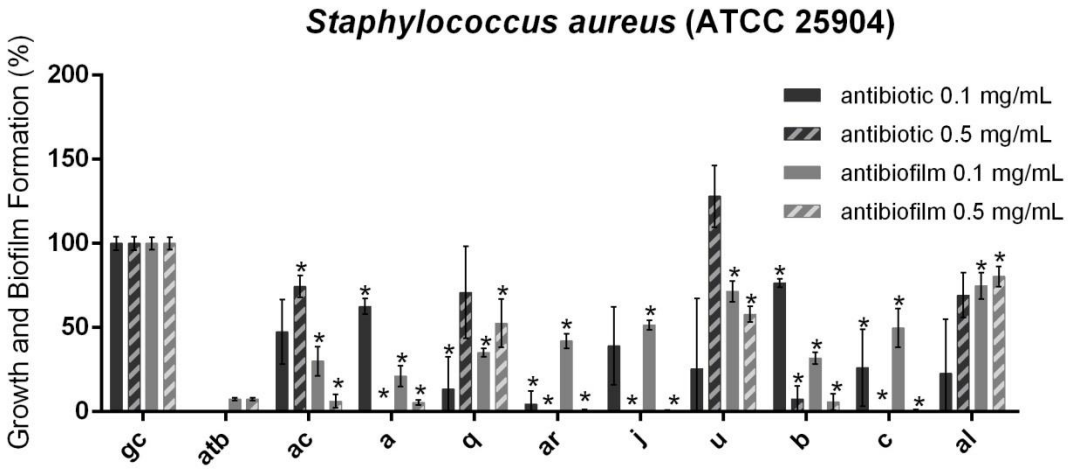

Figure 4: Bacterial growth and biofilm formation (%) of *Staphylococcus aureus* (ATCC 25904) in presence of the nine raw aqueous extracts tested. Rifampicin was used as a positive control. The letters in the x-axis represent growth control (gc), antibiotic control (atb), angico de caroço (ac), aroeira (a), quixabeira (q), ameixa recanto (ar), jatobá (j), umburana de cheiro (u), braúna (b), commiphora (c) and algaroba (al).

For *Staphylococcus aureus* (ATCC 43300) (figure 5), which is a Methicillin-resistant *Staphylococcus aureus* (MRSA), for the higher concentration the same four extracts with antibiotic activity mentioned before also were active for this bacterial strain. Antibiofilm activity also can be mentioned for angico de caroço, quixabeira, umburana de cheiro and algaroba. The lower concentration did not present significant antibiotic activity, but antibiofilm activity of 83% or more was observed in six out of the nine extracts (all of them but ameixa, jatobá and umburana).

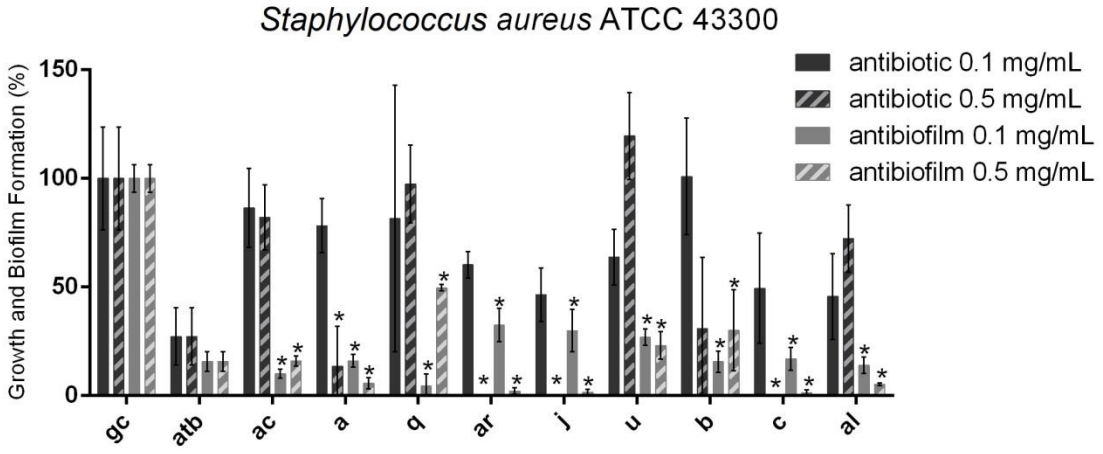

Figure 5: Bacterial growth and biofilm formation (%) of *Staphylococcus aureus* (ATCC 43300) in the presence of the nine raw aqueous extracts tested. Vancomycin was used as a positive control. The letters in the x-axis represent growth control (gc), antibiotic control (atb), angico de caroço (ac), aroeira (a), quixabeira (q), ameixa recanto (ar), jatobá (j), umburana de cheiro (u), braúna (b), commiphora (c) and algaroba (al).

With these results, it is possible to point out five extracts - aroeira, ameixa, jatobá, commiphora and braúna - with important activity against Gram positive bacteria. Among those, the raw aqueous extract of aroeira, commiphora and braúna presented activity against biofilm formation. Because of its potential antibiofilm activity against *Staphylococcus aureus* (ATCC 43300), MRSA, and due to the scarce literature using "*Schinopsis brasiliensis*" and "biofilm", braúna extract was the one used in the subsequent experiments.

3.2 INCORPORATION OF THE RAW EXTRACT IN THE POLYMER

Followed by the incorporation of the extract in the polymer, the antibiotic and antibiofillm assay was carried out in order to see if the compound was still active once

incorporated. Due to its clinical importance, only one bacterial strain, *Staphylococcus aureus* (ATCC 43300), and two concentrations of the raw aqueous extract, 0.1 mg/mL and 5.0 mg/mL, were tested; the higher one to see if the extract, once incorporated, was still capable of having the activity, and the lower one because it had activity in the antibiofilm assay. Figure 6 shows the braúna raw extract incorporated in 0.1 mg/mL and 5.0 mg/mL. An antibacterial activity of 16.6% was obtained in the braúna 5.0 mg/mL group in comparison to the growth control group. As for antibiofilm activity, the treated sample inhibited 69,7% the biofilm formation. In the lower concentration the extract did not have antibiotic activity but had 55,3% of biofilm inhibition. Such results highlight the antibiofilm activity of braúna against a difficult treatment pathogen often involved in hospital infections.

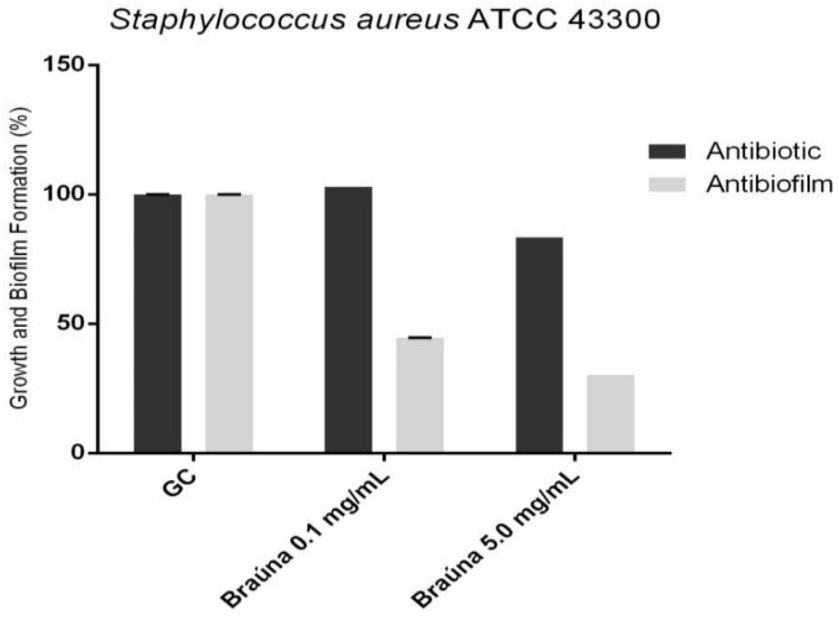

Figure 6: Bacterial growth and biofilm formation (%) of the *Staphylococcus aureus* (ATCC 43300, MRSA) upon braúna raw extract incorporated in the polymer tested in the concentrations of 0.1 mg/mL and 5.0 mg/mL.

3.3 COLONY-FORMING UNITS (CFU) COUNTING - ADHESION TEST

The adhesion test with CFU counting was performed to confirm the results of the antibiotic and antibiofilm assay with the raw extract incorporated. This test was performed two times in sextuplicate. The results represent the colony-forming units per milliliter. It was possible to observe that once incorporated, the bacterial adhesion was lower than the untreated control but it was only significant in the higher concentration ($p \leq 0,05$) (figure 7).

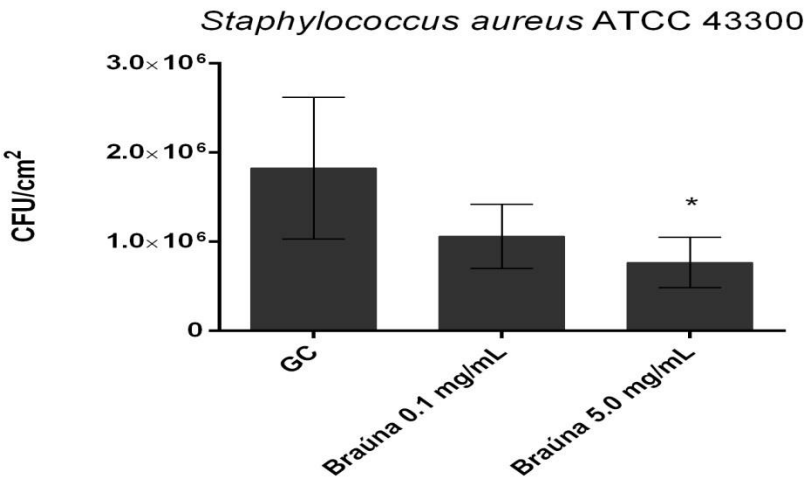

Figure 7: Effect of braúna extract incorporated in the polymer in concentrations 0.1 mg/mL and 5.0 mg/mL against *Staphylococcus aureus* (ATCC 43300). Asterisk represents $p \leq 0.05$. (CFU/cm^2)

3.4 THIN LAYER CHROMATOGRAPHY

The thin layer chromatography was performed in order to elucidate what compounds could be present in the braúna raw aqueous extract. After being revealed

with different chemical agents, it was possible to detect the presence of flavonoids in the extract (figure 8).

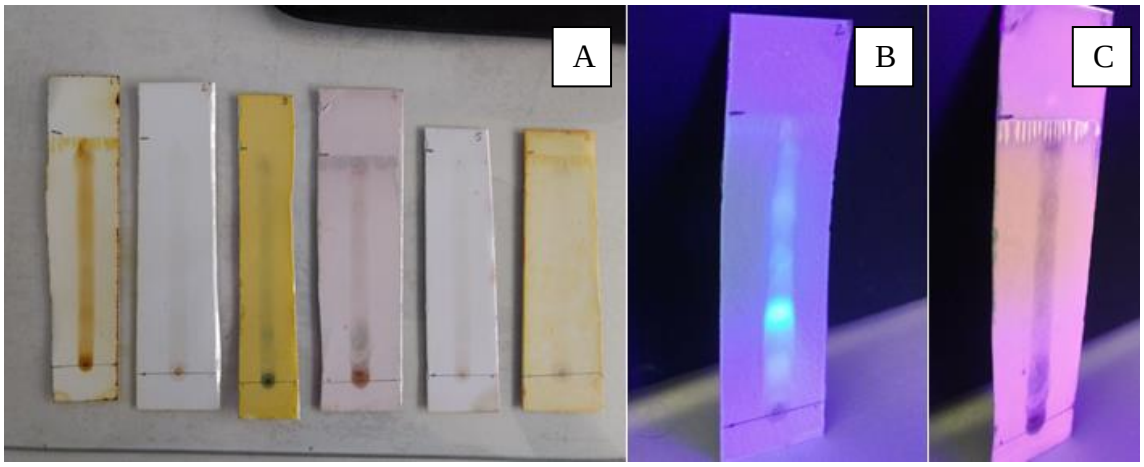

Figure 8: A) TLC plates with the different chemical agents used, from left to right: iodine, natural reagent + polyethylene glycol, ferric chloride, anisaldehyde, ninhydrine, and Dragendorff, reapectively; B) TLC plate revealed with natural reagent + polyethylene glycol under UV light (365 nm); C) TLC plate revealed with anisaldehyde under UV light (365 nm).

3.5 BRAUNA EXTRACTS

A fraction soluble in methanol (Ox1) and an insoluble one (Ax1) were obtained after methanol extraction. The methanol extraction with exhaustion also generated a fraction soluble in methanol (Ox3) and an insoluble one (Ax3). These fractions were, after liofilization, dissolved in water and tested for antibiotic and antibiofilm activity.

In figure 9 results of extraction in which methanol was added just one time (A 0.1x1, A 0.5x1, O 0.1x1 and O 0.5x1) and extraction in which methanol was used with exhaustion (A 0.1x3, A 0.5x3, O 0.1x3 and O 0.5x3) are shown. Results of the raw extract in both concentration (B 0.1 and B 0.5) are also shown to be compared with the

fractions. The fractions were tested against *Staphylococcus aureus* (ATCC 43300). Both the soluble and insoluble fractions were tested in the concentrations of 0.1 and 0.5 mg/mL. As expected, no significant antibiotic activity was obtained in the concentration 0.1 mg/mL, just antibiofilm activity, in both, soluble and insoluble fractions. In the higher concentration, the fraction submitted to extraction with methanol three times showed antibiotic activity, while when it was added just one time such activity did not appear. Such results show that braúna probably has more than one compound responsible for its antibiotic and antibiofim activity.

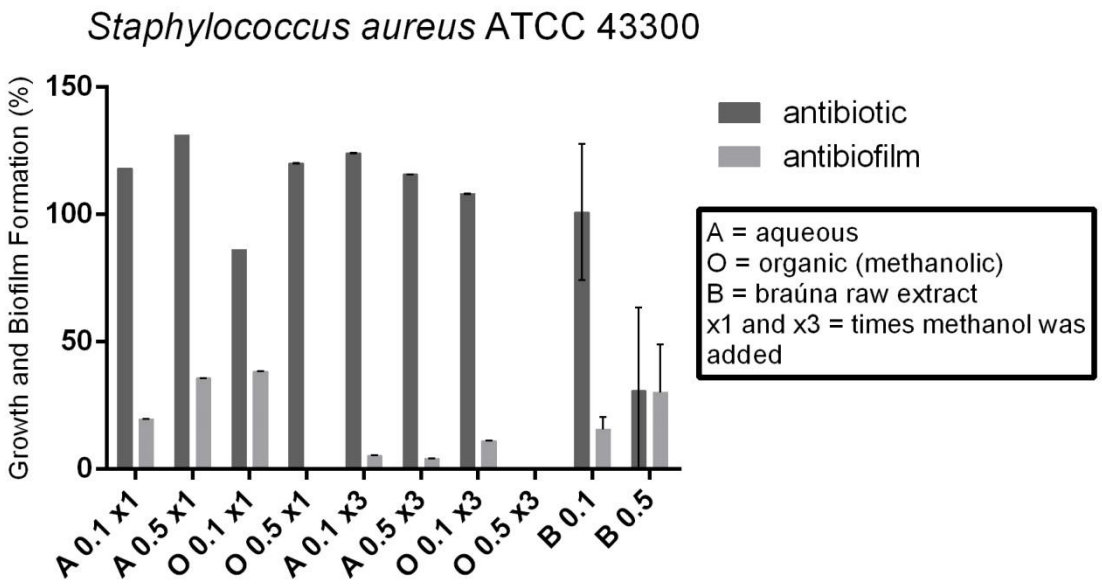

Figure 9: Bacterial growth and biofilm formation (%) of *Staphylococcus aureus* (ATCC 43300) in presence of organic and aqueous fractions, and raw extract.

4. DISCUSSION

Once the data analysis has been made, it is important to point out some of the aspects concerning this study. Firstly, production of medical devices with surfaces less prone to microbial adhesion is an alternative to fight biofilm formation, and that can be achieved by changing their surface physically or chemically. There are four known strategies to fight biofilms: prevention of bacterial adhesion, inhibition of its maturation, disruption of biofilm exopolimeric matrix or killing microorganisms in mature biofilm [10]. In this regard, incorporating substances or compounds with antibiofilm activity to those materials, while keeping them safe and maintaining their activity is a good alternative to fight biofilm. Hospital infections and the ones related to medical devices are frequently associated with two pathogens: *Staphylococcus epidermidis* and *Staphylococcus aureus* [10]. Multi-resistant strains, such as the one tested in this study (*Staphylococcus aureus* ATCC 43300), turn out to be a challenge for the clinic because of their resistance to the majority of antibiotics normally used to treat microbial infections.

Among the raw aqueous extracts tested, some interesting activities were obtained, for Gram negative and, mostly, Gram positive bacteria. Between the two concentrations tested (0.1 and 0.5 mg/mL), more than once the lower concentration had more activity, which can be seen in other studies, such as [18]. This could be explained by the fact that in higher concentrations the bacterial response could be induced, activating defense mechanisms. Another explanation for greater activity in lower concentrations is that the material being tested is the raw aqueous extract, meaning more than one substance can be active and that they can interfere in each other's activity. In

other words, higher concentrations of one molecule can inhibit the molecule responsible for the activity found.

The braúna (*S. brasiliensis*) extract was the chosen one to be carried out in this study. It was incorporated to the polymeric surface, at first, in the concentration of 5.0 mg/mL, a concentration 50 times higher than the active one, to check whether once mixed with the polymer it would still have activity or not. The results obtained showed a discrete antibiotic activity but an interesting antibiofilm formation rate. To confirm such results, an adhesion test was made and the cell counting confirmed that the biofilm formation was indeed lower. Afterwards, the concentration of 0.1 mg/mL, which was the one with antibiofilm activity in the initial screening in the 96-well plate, was tested. It did not have antibiotic activity, but inhibited the biofilm formation in 55.3%, which is a high rate of activity for such a low concentration. The adhesion test with cell counting was also performed and biofilm formation was lower than the untreated control (growth control), but higher than when the extract was incorporated in the final concentration of 5.0 mg/mL. The adhesion test showed significant results only in the higher concentration ($p \leq 0.05$).

Considering that antibiotics have to interact with bacteria to perform its activities, it can be inferred that the braúna raw aqueous extract, once incorporated in the polymer, probably loosens from it and therefore has antibiotic activity, even if discrete, and only in the higher concentration. To confirm such hypothesis, HPLC could be used, incubating the samples and reading aliquots in time 0h and time 24h and comparing them.

Gram-positve bacteria have a thick peptidoglycan cell wall, with teichoic acid. Due to the presence of phosphate groups in teichoic acid, gram-positive bacteria are negatively charged [7]. It is known that *S. brasiliensis* has tannins and flavonoids among its molecules [19], and both of them are rich in hydroxyl groups, what guarantee their negative charge. Once a negatively charged compound is incorporated to a surface, it can change its characteristics and the surface can become negatively charged as well. If a surface is negatively charged and anionic bacteria try to adhere to it a repulsion of the charges will occur inhibiting the bacterial adhesion to the surface [9, 20]. Other studies of anti-infective materials [21] show that cationic surfaces can interact with bacteria and depolarize their membranes, leading to leakage of intracellular constituents. Therefore, the surfaces charge and electrostatic interactions are of great importance to the development of anti-infective surfaces.

In order to isolate the active molecules, extractions with methanol were performed, and insoluble and soluble (methanolic) fractions were obtained. Antibiotic and antibiofilm activity assay were performed in the four fraction samples (insoluble and soluble of the extractions with and without exhaustion). It was possible to see that the in the lower concentration there is an antibiofilm activity in all extracts (figure 9), with the best activity in the higher concentration of the aqueous fraction submitted to extraction with methanol three times, and in organic (methanolic) fraction submitted to methanol once. Also, the methanolic fraction extracted three times presented, in the higher concentration, antibiotic activity, as it did in the raw extract. This is probably due to the fact that in order to achieve antibiotic activity, the compound needs to reach a minimum concentration, otherwise it will only act inhibiting the biofilm formation, either by loosening it or inhibiting its adherence. Moreover, both soluble and insoluble

fractions had activity, meaning that the probability of the raw extract having more than one compound related to the antibiotic and antibiofilm activity is high.

Other studies, have already isolated compounds of *Schinopsis brasiliensis*, such as biflavonoids, tannins and polyphenols [19, 22], but it is still not known which of the plant metabolites is, or are, the one(s) responsible for the antibiofilm activity. The TLC performed in this study with the raw aqueous extract confirms the presence of flavonoids. [23] correlates the presence of tannins and flavonoids with antimicrobial activity, reassuring the potential of braúna's activity. [24] has also demonstrated the antibiofilm activity of braúna against oral microorganisms.

5. CONCLUSION

Infections related to microorganisms that produce biofilm are a problem to the clinic, especially the ones involving multi-resistant bacteria. The results obtained in this study with braúna extracts suggest that it could be a possible candidate to the development of new products that inhibit biofilm formation and when incorporated to polymer generates an anti-infective surface. Studies to isolate and elucidate the antibiofilm compounds of *S. brasiliensis* are needed, as well as toxicity tests *in vitro* and *in vivo*. Additional assays to isolate and purify such compounds and evaluation of the surfaces characteristics are under evaluation.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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