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ORIGINAL ARTICLE

Inhibitory Effects of *Pterodon emarginatus* Bean Oil and Extract on *Staphylococcus aureus*

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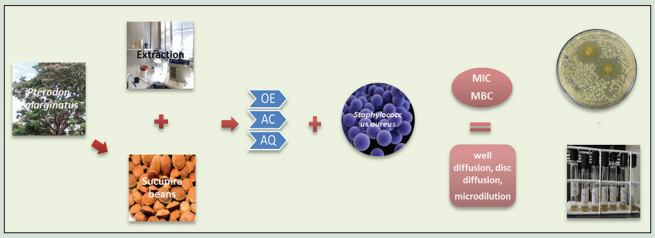
ABSTRACT

Background: *Pterodon emarginatus* is a tree of the Brazilian Savannah. The beans of this tree are used in folk medicine as anti-inflammatory preparations, especially for infections caused by *Staphylococcus aureus*. These bacteria can cause simple infections or serious illnesses such as pneumonia, meningitis, endocarditis, toxic shock syndrome, septicemia, and others. **Objective:** This study had the goal of verifying the effect of the essential oil (OE) from *P. emarginatus* on the inhibition of *S. aureus* in culture medium, i.e., "*in vitro*" tests. **Materials and Methods:** The vegetable material was cut and crushed with a press. The OE was obtained by extraction using hexane, alcohol, and water. The *P. emarginatus* extracts obtained were used to evaluate the antimicrobial effect on *S. aureus* (ATCC 25923) by tests of well diffusion, disc diffusion, and microdilution. The strain used in the assays was maintained in brain heart infusion broth and nutrient agar until testing. Afterward, the bacteria were spread on agar plates with Mueller-Hinton agar medium. In the wells and on the paper discs, the OE suspensions were placed in the following volumes: 10, 15, 20, 25, 30, 40, and 80 µL and subsequently they were incubated at 35°C ± 2°C. After 24 h, the number of colony-forming unit was determined. **Results:** Pure OE and hydroalcoholic extract inhibited the growth of *S. aureus*, while aqueous extract had no effect on bacterial growth in all microbial methods used. **Conclusion:** Thus, the present study showed the potential of sucupira-based extracts against *S. aureus* growth, opening new perspectives for the evaluation of these bioactive compounds as phytopharmaceutical products.

Key words: Antimicrobial test, diterpenes, medical pathogens, phytopharmaceutical extract, *Staphylococcus aureus,* sesquiterpenes, Sucupira-branca

SUMMARY

- Plant extract act as antimicrobials to prevent and reduce bacterial contamination
- Beans of *Pterodon emarginatus* has antibacterial properties
- Extraction with different solvents might implicate on the rate of bacterial death
- The effect of different microbiological methods (well diffusion, disc diffusion and microdilution) was evaluated on reducing CFU
- The results showed by MBC that concentrations superior to 10% (v/v) using AC and 7.5% (v/v) using OE were necessary to eliminate colonies formed
- According to data of MIC, at 2.5% of AC and OE was enough to kill *S. aureus*
- The well diffusion technique demonstrated better performance than disc diffusion test for OE and AC extracts
- Hydroalcoholic and oil extracts of sucupira beans had highest effect against *Staphylococcus aureus*
- Aqueous extract had no effect on bacterial growth in all microbial methods tested
- The sucupira-based extracts is a promising source as herbal drug due to therapeutic value



Abbreviations Used: OE: Essencial oil; AC: Hydroalcoholic oil extract; AQ: Aqueous extracts; MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; CFU: Colony formed unit.

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INTRODUCTION

Pterodon emarginatus is a Brazilian Savannah ("Cerrado") tree popularly known as "Sucupira-branca" or "Sucupira." It is a medium-sized tree, 8 to 16 m in height and 30–40 cm in diameter [Figure 1]. Fully grown, it can reach 30 m tall with a diameter of 80 cm. This tree is popularly considered to be effective as an antirheumatic, anti-inflammatory, analgesic, anti-infective drug.[1,2] The fruits are used to treat muscle aches, sprains, arthritis, and arthrosis because it is believed to have anti-inflammatory and analgesic action.[3] Due to its pharmacological value, this tree is protected by farmers.[4] It can be found in the Brazilian states of Minas Gerais, São Paulo, Goiás, and Mato Grosso do Sul.[5,6]

Herbal medicine has been used for centuries in human history and has great importance in the popular medicine of many countries, especially in developing countries, where it has been reported that up to 80% people are dependent on traditional medicine as a source of treatment in primary health care.[7] In addition, the Brazilian Cerrado is recognized as

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the richest Savannah in the world, but most of the species of native plants have not been investigated, although more than 220 species have been used for medicinal purposes by native groups. Moreover, because of resistance to commercial antibiotics and the necessity to find new active principles using medicinal plants, there has been an increasing interest in plants with antimicrobial activity as possibilities for the development of a new kind of antibacterial drug.[8]

The essential oil (OE) of *P. emarginatus* has a strong aroma and has been used to fight rheumatism and diabetes. For these reasons, the Brazilian population has been using alcoholic extracts of this plant, made from the beans[6,8,9] for medicinal purposes. The oil has a bitter taste when mixed with water. Even so, it has been used as a mouthwash with positive results against infections and inflammation in human throats.[10]

Bacteria and fungi are the principal threats to the seed pod of the Sucupira.[1] The defense mechanisms of sucupira bean oil eliminated these pathogens in "*in vitro*" tests of effectiveness[6] and also have been effective against Gram-positive bacteria such as *Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus roseus, Micrococcus luteus, Bacillus atrophaeus, Bacillus cereus*, and *Bacillus stearothermophilus.*

The medicinal action of *P. emarginatus* is probably due to the (secondary) metabolites, which are produced by the plant as a defense mechanism against its diseases, caused by microorganisms, insects, and herbivores.[10] Several bioactive compounds metabolized by cell plants have been isolated with medicinal properties from herbal products. These actions are related to antioxidant activities (capacity of scavenging free radicals) and inhibition of enzymes that lead to cause chronic pathologies such as cancer, cardiovascular diseases, diabetes, and neurodegenerative diseases.[11] Moreover, these substances have aided in the control of some infectious agents such as *Escherichia coli*, *Proteus* spp., and *Salmonella* spp.[12] The phytochemical substances of *P. emarginatus* leaves are mainly flavonoids, leucoanthocyanidin, tannins, glycosides, coumarins, saponins, and cardiotonics. In bean oil, there are also diterpenoids, sesquiterpenoids, and isoflavones.[13,14]

S. aureus is a Gram-positive, aerobic, mesophilic bacterium that proliferates at a minimum temperature of 10°C, although higher temperatures are required (>15°C) to produce the toxin.[15] In animals, *S. aureus* can contaminate and produce various abscesses and pyogenic infections such as endocarditis, septic arthritis and osteomyelitis, food poisoning syndrome, desquamation, and toxic shock syndrome.[16,17] Microbial resistance to various drugs is increasing. This generates indecision regarding prospects for the use of antimicrobial drugs in the future and leads to the need to control the use of antibiotics. One alternative to this problem is the use of herbal remedies. Meanwhile, research on the mechanisms of resistance is being carried out to develop new synthetic or natural drugs.[17]

Figure 1: *Pterodon emarginatus*, sucupira tree, native of the Brazilian Savannah ("Cerrado")

Different methods can be used to evaluate the antibacterial, antifungal, and anthelmintic activity of vegetable extracts. The most popular methods are as follows: the well diffusion method, disc diffusion method, and the macrodilution test. To determine the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) of plant extracts, the microdilution broth method is most often used.[18,19] There are few comparative studies reporting the best method to evaluate the antimicrobial activity of plant extracts, even with regard to the methodologies commonly used. Previous studies[14] carried out with "*in vitro*" tests have showed the inhibitory effect of the bean oil *P. emarginatus* on *S. aureus.* It was this finding that has motivated the present research. In this context, the objective of this study was to evaluate the effects of oil and aqueous extracts (AQs) of *P. emarginatus vogel* on the inhibition of *S. aureus* using three different microbiological methods for evaluation of the antimicrobial activity.

MATERIALS AND METHODS

Plant material

Bean pods of *P. emarginatus*[20] were collected during the flowering period in April 2014, in Santo Antonio do Descoberto, State of Goiás, Brazil (15° 56' 37" South, 48° 15' 35" West). Samples were stored in a refrigerated chamber in the microbiology laboratory of the Faculty of Anhanguera, in the city of Taguatinga, Federal District. Afterward, samples were sent to the Laboratory of Parasitology and Vector Biology, Faculty of Medicine, University of Brasília, where the microbiological tests were carried out.

Preparation of samples

Beans were removed from the pods of *P. emarginatus* for analysis. The beans were ground (Bosch, Germany) and air-dried prior to every experiment.

Extraction procedures

Conventional oil extraction (essential oil) with the Soxhlet apparatus

OE was extracted from air-dried bean meal (30 g) of *P. emarginatus* by Soxhlet apparatus using hexane, according to the methodology proposed by Silva *et al*.[10] The extraction was performed at normal boiling point for 4 h and afterward the solvent was evaporated until dry. The resulting oil was stored at 4°C until further use.

Hydroalcoholic oil extract

For extraction of the oil from the bean meal with ethanol 70% (v/v), 600 mL of ethanol was added to 100 g of meal in a cylindrical flask with agitation for 1 h. The solution was then stored at 25°C for 4 days. Subsequently, extracts were filtered through gauze and evaporated under reduced pressure by a rotary evaporator. After evaporation of the solvent, the crude extracts obtained were stored at 4°C for further experiment.

Preparation of aqueous extract

Samples of 100 g of bean meal were immersed in a flask of 200 mL of milli Q water and stored at 8°C[13] for 7 days. The samples were then heated in a water bath at 90°C for 4 h, filtered, and stored in a refrigerator until further analyses.[21]

Antibacterial assay

The tests were performed with standard strains of *S. aureus* ATCC 25923. The oil extracts were diluted and evaluated at concentrations varying in volume from 10 to 80 μL. Different methods of antimicrobial evaluation were used

based on the methodology of Ferreira *et al*.[22] and de Bona *et al*.[23] using the disc diffusion method, well diffusion test, and microdilution broth test. Antimicrobial gentamycin was used as a positive control. The determination of antimicrobial activity was evaluated by measuring the diameters of inhibition zone with a millimeter ruler. Each sample was repeated in triplicate, and the mean diameters of inhibition zone were calculated.

Bacterial strains and inoculum

The extracts obtained from *P. emarginatus* were tested against Gram-positive *S. aureus* ATCC 25923 bacteria. Bacterial strains were cultured during 24 h at 35°C ± 2°C on Mueller-Hinton agar (MHA). The concentration of the microorganism was further adjusted to 10^7 and 10^8 colony-forming unit (CFU)/mL and stored at 4°C for later use.

The inoculum of the microorganism was prepared by recovering it from Mueller-Hinton broth and incubating it without stirring for 24 h at 35°C ± 2°C. Subsequently, the inoculum was cultured on MHA plates for 24 h before testing. Suspensions were prepared in culture, diluted in a saline solution (0.85%) using McFarland scale (0.5) to obtain approximately 1.5×10^8 CFU/mL of bacteria.

Disc diffusion method

Prior to the experiments, the bacterial samples were recovered from the broth using brain heart infusion (BHI) at 37°C for 18 h. Antimicrobial susceptibility was evaluated according to the disc diffusion method, following the recommendations of the Clinical and Laboratory Standards Institute,[24] with adaptations. For this evaluation, sterile disc filter paper (6 mm) received an aliquot of 10 µL to 80 µL of each extract to be tested. The discs were then deposited, with sterile forceps, on Petri dishes containing MHA, previously inoculated with the microorganism to be tested. The plates were incubated at 35°C ± 2°C for 48 h. After the incubation period, the diameters of inhibitory zone were measured. Gentamycin was used as a positive control against bacteria. Each experiment was carried out in triplicate.

Well diffusion test

The well diffusion test was performed with adaptations. It differed from the disc test, having three holes of 6 mm in diameter on the agar plates (MHA) in Petri dishes. The plates were inoculated on the surface by the microorganisms with a swab, and then the wells were filled with different volumes of the samples (OE, AQ, and AC) ranging from 10 µL to 80 µL.

Minimum inhibitory concentration and minimum bactericidal concentration assays

The MIC is the lowest concentration of an antimicrobial agent needed to inhibit the growth of microbes and the MBC is the minimal bactericidal concentration. It was determined by a modified microdilution agar method.[25] By this method, the samples of extracts and oil were used in varying volumes from 10 µL to 80 µL serially diluted in broth culture, BHI,[26] with modifications. In eight tubes containing 400 µL of sterilized BHI, the sample extracts were inoculated with *S. aureus*. For each assay (OE, AQ, AC), *Staphylococcus aureus* was used as a positive control plus 10 µL of gentamycin. The negative control used was distilled water.

Cultures were kept at 35°C ± 2°C for 48 h and withdrawn for analysis. After the incubation, the growth of bacteria was determined by serial dilution enumeration (CFU/mL), in which the MBC was interpreted as the lowest concentration that had no bacterial growth. Each value of MIC and MBC was determined by calculating the mean concentration of triplicates.

Statistical analyses

The data obtained in the agar diffusion evaluations were submitted to an analysis of variance and the averages of the halos were compared by Tukey test, both at 5% significance, using the STATISTICA software version 7.0 (Statsoft Inc., Tulsa USA).

RESULTS

The results obtained from the hydroalcoholic extract (AC) and OE had bacteriostatic effects, inhibiting the growth of *S. aureus*. In the present study, independent of extract samples, diameters of the inhibition zone (zi, excluding the diameter of the disc or well) varied significantly from 0 to 6.8 mm. The AQ, however, had no inhibitory effect on bacterial growth by any of the methods used. The results obtained in the different experiments are shown in Table 1. The positive control (gentamycin) had an inhibition zone of 4.2 mm for both the diffusion disc and diffusion wells. As a negative control, saline was used in the same proportions.

The antimicrobial effect of AC was identified by all the three methods tested. In the diffusion well method [Table 1], the inhibition zone ranged from 2 to 6.8 mm, while by the disc diffusion method, the values of diameter zone varied from 1.8 to 3.2 mm [Table 2]. The inhibition suspension method was confirmed by turbidity and CFU counts [Figures 2 and 3c].

Table 1: Antimicrobial activity of the essential oil, hydroalcoholic oil extract, and aqueous extract of the bean oil of *Pterodon emarginatus* on the growth of *Staphylococcus aureus* by well diffusion test

Method	10 µL	15 µL	20 µL	25 µL	30 µL	40 µL	80 µL	CN	CP
AC	2.0±0.01^{a}	2.2±0.01^{a}	4.0±0.01^{b}	4.4±0.01^{b,c}	4.8±0.01^{c}	5.4±0.01^{d}	6.8±0.01^{e}	0±0.01	4.2±0.01
OE	2.0±0.01^{a}	2.4±0.01^{a}	3.0±0.01^{b}	3.4±0.01^{b,c}	3.6±0.01^{c,d}	4.0±0.0^{d,e}	4.6±0.0^{e}	0±0.01	4.2±0.01
AQ	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	4.2±0.01

zi=0: No antimicrobial activity (negative); zi<1 mm: Weakly antimicrobial activity; zi=1 mm: Light antimicrobial activity; zi=2-3 mm: Moderate antimicrobial activity; zi=4-6 mm: High antimicrobial activity; zi=6-9 mm: Strong antimicrobial activity. All the values are expressed as mean ± Standard deviation, and values with different letters are significantly different at $P<0.05$. CP: Gentamycin; CN: Saline 0.85%, diameter of disc used is equal to 6 mm; OE: Essential oil; AC: Hydroalcoholic extract; AQ: Aqueous extract

Table 2: Antimicrobial activity of the essential oil, hydroalcoholic oil extract, and aqueous extract of the bean oil of *Pterodon emarginatus* on the growth of *Staphylococcus aureus* by disc diffusion test

Method	10 µL	15 µL	20 µL	25 µL	30 µL	40 µL	80 µL	CN	CP
AC	1.8±0.01^{a}	2.0±0.01^{a}	2.2±0.01^{a}	2.8±0.01^{b}	2.8±0.01^{b}	3.0±0.01^{b}	3.2±0.01^{b}	0±0.01	4.2±0.01
OE	0±0.01	2.2±0.01^{a}	2.4±0.01^{a}	2.8±0.01^{a,b}	3.0±0.01^{b}	3.2±0.01^{b}	4.0±0.01^{c}	0±0.01	4.2±0.01
AQ	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	0±0.01	4.2±0.01

zi=0: No antimicrobial activity (negative); zi<1 mm: Weakly antimicrobial activity; zi=1 mm: Light antimicrobial activity; zi=2-3 mm: Moderate antimicrobial activity; zi=4-5 mm: High antimicrobial activity; zi=6–9 mm: Strong antimicrobial activity. All the values are expressed as mean ± Standard deviation, and values with different letters are significantly different at $P<0.05$. CP: Gentamycin; CN: Saline 0.85%, diameter of disc used is equal to 6 mm; OE: Essential oil; AC: Hydroalcoholic extract; AQ: Aqueous extract

The OE of sucupira also presented bacteriostatic effect on the growth of *S. aureus*. The zones of inhibition by well diffusion methodology varied from 2 mm to 4.6 mm. By the disc diffusion method, results from diameter zones were 2–4 mm. Comparisons among the inhibited zones by these two methods are shown in Tables 1 and 2. In the suspension method (microdilution), the colonies were counted and these data are shown in Figure 3a.

The AQ showed no inhibitory effect on *S. aureus* by any of the methods used [Tables 1 and 2]. These results were confirmed using the suspension method and counting of CFUs [Figure 4].

MBC and MIC values of Sucupira extracts tested against *S. aureus* are summarized in Table 3. Regarding MBCs, the data showed antibiotic actions at concentrations superior to 10% (v/v) using AC and 7.5% (v/v) using OE. This difference between those two samples might be due to the polarity of the substances extracted that have some natural antimicrobial activity. However, the values of MICs were equal to 2.5% (v/v) using AC or OE, while samples AQ presented no germicidal activity.

DISCUSSION

The Brazilian ecosystem has an abundant biodiversity with great potential in pharmaceutical industry, but many of these vegetable species have not been properly studied. The use of *Pterodon* (*Fabaceae*), popularly known as "Sucupira," "Sucupira-branca," "Faveira" has been used in folk medicine for their antirheumatic, anti-inflammatory (sore throat) and analgesic activities.[1,13] Usually, the alcoholic extracts from seeds of these plants are prepared and orally ingested by the population, in small quantities, at regular intervals.

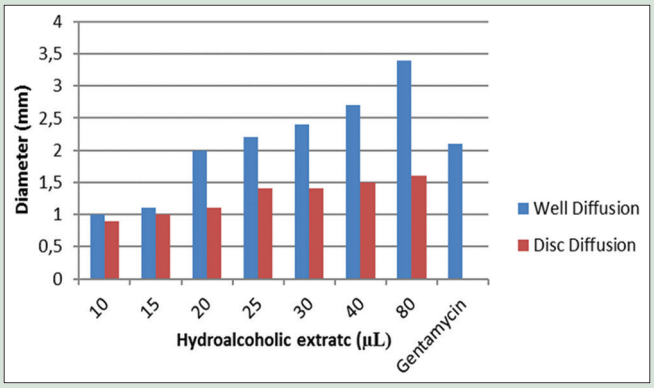

Figure 2: Comparison of the inhibition action of hydroalcoholic extract AC on *Staphylococcus aureus* growth between two different antimicrobial tests

In this context, the present study investigated the antibiotic action of Sucupira' seeds extracts against *S. aureus* by different microbiological tests.[27] *S. aureus* is one of the etiological agents, that cause many human infections and has acquired resistance to many antimicrobial drugs. In this study, gentamycin was used as a positive control. It is an aminoglycoside with a broad bactericidal spectrum of action against *Staphylococcus*.[28] The mechanism of action of amino glycosides on the inhibition of bacteria has not been fully defined. It is believed that ribosome is bound chemically, causing a decrease in protein synthesis and interfering with reading of the genetic code.

The results of this investigation confirmed that the alcoholic extracts (AC) from *P. emarginatus* seeds have antimicrobial effects, comparing to a positive control. Probably, this action is due to the presence of some chemicals compounds such as diterpenes and sesquiterpenes.[3,9,29] It is believed that the mechanism of action of the secondary metabolism product of Sucupira against *S. aureus* is the same as that of gentamycin.

Likewise, the antimicrobial action of AC was greater than OE when determining by well diffusion agar. In general, inhibition extracts promoted by the agar diffusion test by well was higher than the values obtained by disc, regardless of the plant extracts tested. Though, the absence of an inhibition zone does not necessarily mean that the extract is inactive against the tested microorganism, but rather that diffusion has not been completely effective, especially for less polar compounds that diffuse more slowly in the culture.

Comparing the agar diffusion methods tested, the well diffusion technique stood out with the largest inhibition zones. This fact has been confirmed by researchers, who has identified several factors that can influence or alter the effectiveness of disc methodology including culture media, pH, and others.[21,23,27]

The inhibitory zone determined by well diffusion test suggests that this antibacterial activity is due to compounds with intermediate polarity present mainly in alcoholic fractions. These substances are not volatile and easily extracted by nonpolar solvents, producing a variety of biological activities like as phytotoxic, antioxidant, cytotoxic,

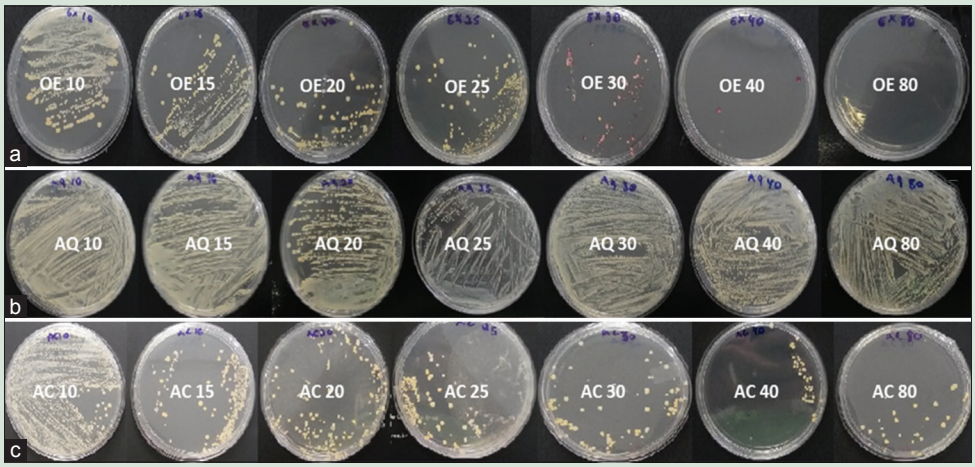

Figure 3: (a) Evaluation of the effect of essential oil, (b) Evaluation of the effect of aqueous extract, and (c) Evaluation of the effect of hydroalcoholic extract

Table 3: Minimal inhibitory concentrations and minimal bactericidal concentrations

Methodology	MIC (%, v/v)	MBC (%, v/v)
AC	2.5	>10.0
OE	2.5	>7.5
AQ	-	-

MIC: Minimal inhibitory concentration; MBC: Minimum bactericidal concentration; OE: Essential oil; AC: Hydroalcoholic extract; AQ: Aqueous extract

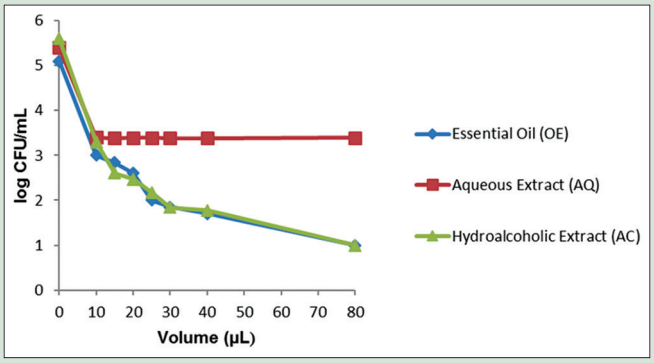

Figure 4: Count of colony-forming units, comparing the amounts of colonies formed, according to the concentrations of the extracts and essential oil obtained from sucupira beans

antiviral and antimicrobial.[9,14] Thus, the AQ had no antimicrobial effect [Tables 1 and 2].

Although this was the best diffusion technique observed in this study, it can be considered that the broth microdilution method was the best option to determine the antibacterial activity, providing quantitative data, besides being more reliable and economical to determine the antimicrobial action of plant extracts in tests *in vitro*.

Similar results were described by Dutra *et al.*,[15] when evaluating the MIC of ethanolic of *P. emarginatus* using broth diffusion methods against *S. aureus*. The authors observed that, in the broth microdilution test, the extract presented antibacterial activity with MIC at 2.5 mg/mL. Differently, Santos *et al.*[1,3] related that ethanolic sample (AC) of *Pterodon* had no antimicrobial effect on the bacteria that act on *S. aureus*.

The oleaginous extract of seeds had also been studied for combating acute and chronic pain in mice caused by plantar incision surgery.[30] Published data have verified that the OE extracted with hexane fraction had steroids, terpenes and acetophenones.[31] However, this mechanism of oil and alcoholic extracts of *Pterodon*' seeds in promoting health benefits are not known, further investigations must be carried out to find them out.[32]

Despite some investigations focusing on antibacterial activities have been reported, there are few works demonstrating the importance on the method adopted for evaluation.[26,27] Our data emphasizes the difference of results obtained by MBC and agar diffusion methods, especially for non-AQs. The effectiveness of oil diffusivity depends on the driving force for diffusion of the solvent and the temperature. Furthermore, the extraction method might implicate in different data for the same sample determination as well the microbial tests. These results showed that nonpolar substances and chemicals with intermediate polarity extracted by *P. emarginatus* were found to be effective against bacteria *S. aureus*, while substances with higher polarity (AQ) failed to show bactericidal activity.

CONCLUSION

The OE and AC of Sucupira bean inhibited *in vitro* the growth of *S. aureus* (ATCC 25923). When comparing the results of the antibacterial activity of AC extracts using different microbiological methodologies analyzed, it was observed that there was significant difference between them. The well diffusion technique demonstrated better performance than disc diffusion test for OE and AC extracts. Possibly, the coefficient of diffusivity of samples on discs was not efficient as expected, due to the extracts' chemical and physical properties.

According to the data presented, the modified microdilution method was the best option for determining antimicrobial activity by enumerating the colonies formed (CFU), providing quantitative data (MIC and MBC). In conclusion, the results obtained in this study showed the need to carry out *in vivo* tests to verify whether these extracts have an effect directly on the reduction of *S. aureus*. Thus, the OE and ACs confirm the popularly believed on antimicrobial action of their healing power used to treat throat infections and other human diseases. Despite the use of Sucupira bean as popular medicine, the herbal market in Brazil is modest, less than 5% of the world phytotherapeutical market. These finds suggest this "Cerrado" plant is a promising source as herbal drug due to therapeutic value and contribute to improve the knowledge of this unexplored area. Furthermore, this study opens new perspectives for the evaluation of these bioactive compounds as phytopharmaceutical products in the future.

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Conflicts of interest

There are no conflicts of interest.

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