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HEALTH SCIENCES

Antimicrobial potential of Copaiba Oil (*Copaifera multijuga* Hayne-Leguminosae) against bubaline mastitis multiresistant isolates

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Abstract: Copaiba oil is a natural product used by Amazonian populations and recognized for its medicinal properties because it has significant antimicrobial activity for several pathogenic microorganisms. The present work aimed to evaluate and characterize the effect of natural oil produced by copaiba – *Copaifera multijuga* against multiresistant isolates of bubaline mastitis. The nitrocefin test was performed with isolates of *Staphylococcus aureus* from bubaline mastitis, which were 100% positive for beta-lactamase enzyme detection. Minimum Bactericidal Concentration (MBC) of 25% to 3.12% was obtained for *Enterococcus faecalis* and Escherichia coli and 50% and 25% for S. aureus, but *Klebsiella pneumoniae* and *Bacillus subtilis* were resistant. MBC with 12.5% and 6.25% oil were obtained for most multiresistant bubaline mastitis isolates from the states of Pernambuco, Ceará, Bahia and Alagoas. The results demonstrated the great potential of using copaiba natural oil in the treatment of buffalo mastitis.

Key words: Amazonian oil, Bacterial resistance, biotechnology, copaiba, *Copaifera multijuga*, mastitis.

INTRODUCTION

Copaiba oil is extracted from the *Copaifera arboreal* vegetable of the Caesalpinaceae family. The oil can be obtained through drilling in the trunk of the Copaibeira being considered a non-aggressive practice, consisting of drilling the trunk with a auger of approximately 2 meters in diameter in two holes. The first should be made 1 meter above the base of the plant and the second from 1 to 1.5 meters above the first (Pieri et al. 2009).

It has been used for medicinal purposes since 1638 when was described the first time by Marcgraf and Piso, this vegetable is popularly known for treating conditions such as cystitis, bronchitis, pneumonia, skin infections such as dermatitis, chronic diarrhea, and other applications (Trindade et al. 2018). Among the properties, the efficacy of copaiba oleoresin is more using in the topical mainly as healing and anti-inflammatory (Ghizoni et al. 2017, Becker et al. 2020). The phytochemical composition of Copaiba oil varies depending on the species from which it is derived. It mainly consists of sesquiterpenes, diterpenes and β-caryophyllene (Veiga & Pinto 2002, Leandro et al. 2012, Becker et al. 2020).

Pharmacological studies of *in vivo* and *in vitro* evaluation have shown that oils from various species of copaifers have anti-inflammatory, healing, anti-dermatological,

antitumor, and bactericidal activity (Ghizoni et al. 2017). The therapeutic properties of herbal principles and medicines are also being used in veterinary treatment, herbalists reveal a high frequency of success in treating parasitic diseases, anti-inflammatory, antitumor, antifungal, antibacterial, infectious diseases including mastitis treatments (Vasconcelos et al. 2008, Huang et al. 2018).

Mastitis can be defined as an acute inflammation of the mammary glands that currently affects a range of mammals (cattle, goats, sheep and buffalo) (Oliveira et al. 2004, Ruegg 2018). This pathology can be fatal when mastitis is from of infectious origin is because, can progress if not eventually treated for sepsis (Gomes & Henriques 2016). In addition, this pathology can cause changes in the milk, compromising the quality of the milk and causing associated damages, such as the reduction of large quantities, economic reduction, increased production cost due to the use of antimicrobial therapy, technical assistance or disposal of contaminated milk or even infected animals (Demeu et al. 2015).

One of the main concerns of the dairy industry is bacterial resistance, as milk produced is one of the main substrates for foods that have potentially increased in recent years, making antibiotics susceptible or ineffective (Heikkilä et al. 2018).

The main microorganism associated with these infections is *Staphylococcus aureus*, however other pathogens may also be involved in the infection, such as *Streptococcus uberis*, *Escherichia coli*, *Klebsiella* spp, *Corynebacterium bovis*, *Mycoplasma* spp., *Streptococcus agalactiae*, *Streptococcus dysgalactiae* (Gomes & Henriques 2016, Marques et al. 2017, Lopes et al. 2020). Furthermore, these animals contaminated are clinic dependent on the capacity and virulence of the strain and the host responsiveness (Acosta 2016). The west region of Brazil, mainly in the Amazon regions, had the largest herd of buffaloes in the country, according to data from the Ministry of Agriculture and Livestock in 2018 (Furlaneto et al. 2020).

Considering the information about the potential of Copaiba natural oil, this study aimed to evaluate and characterize the oil extracted from *Copaifera multijuga* against multiresistant *Staphylococcus aureus* of bubaline mastitis from four dairy farms in the States from Brazil (Pernambuco, Ceará, Bahia and Alagoas).

MATERIALS AND METHODS

Microorganisms

Isolates of Murrah buffalo mastitis previously identified as multiresistant (Medeiros et al. 2009) *Staphylococcus aureus* were isolated from buffaloes from four dairy farms located in the states of Pernambuco (n = 15), Ceará (n = 31), Alagoas (n = 53) and Bahia (n = 118), totaling 217 isolates.

Copaiba Oil

The natural oil of Copaiba (*Copaifera multijuga* Hayne-Leguminosae), from the Green Zone Project of the Rural Producer Fair in Manaus - Amazonas (Brazil), was collected by the natural extraction process. Where a small hole is made in the trunk of the tree, trying to reach the extraction channel where a hose is inserted that leads the oil to a container. After extraction, the hole is sealed with a thread and remains on the trunk to facilitate future extractions, where these containers are sold protected from light, or placed in amber bottles.

Activation of buffalo mastitis isolates

For the microorganism growth (*Staphylococcus aureus*), was used the nutrient broth composed by 0.3% meat extract, 0.3% yeast extract, 1%

peptone and 0.4% glucose. The culture medium was autoclaved at 121ºC/1 atm for 20 minutes. *Staphylococcus aureus* samples were inoculated into test tubes containing the nutrient broth and incubated in a bacteriological greenhouse for 24h at 37° C.

Nitrocefin test for detection of beta-lactamase enzyme produced by bubaline mastitis isolates

For the nitrocefin test, the isolates were seeded on nutrient agar for 24h at 37ºC and the inoculum was standardized to 0.5 on the Mac Farland scale, which corresponded to a concentration of 10^8 CFU/mL. The nitrocefin discs (6 mm diameter) were moistened with a drop of deionized water and placed on the plate containing the microorganisms, one must wait 5 minutes for the reaction to occur. After this time, it was possible to observe the disc color variation from yellow to pink. The pink color indicates the beta-lactamase enzyme production by the microorganism tested.

Disc and Well Diffusion Antimicrobial Activity Test

Antimicrobial activity test was performed by the disk and well diffusion method as described by Ericsson & Sherris (1971). The 6 mm diameter discs received 40 µL of copaiba oil and were tested against microorganisms re4commended by the National Clinical Laboratory Standards Committee - NCCLS (2003). The microorganisms tested with the disc technique were *Enterococcus faecalis* ATCC 6057, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 29665, *Staphylococcus* aureus ATCC 6538, *Bacillus subtillis* ATCC 6633 and *Pseudomonas aeruginosa* ATCC 27853.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

To determine the Minimum Inhibitory Concentration (MIC), the protocol described by the National Clinical Laboratory Standards Committee - NCCLS (2003) was performed. *Staphylococcus aureus* isolates were incubated for 24h at 37ºC in Tryptic Soy Broth (TSB). After growth, the inoculum was standardized (0.5 on the Mac Farland scale) and the tests were performed in 96-well sterile microplates, where the antimicrobial potential of copaiba oil at different concentrations was evaluated (100%, 50%; 25%; 12.5%; 6.25%; 3.12%, 1.56%, 0.78%, 0.39% and 0.19%) diluted in dimethyl sulfoxide (DMSO). Positive control, chlorhexidine (0.12%) and negative control dimethyl sulfoxide were also performed. Subsequently, the plates were incubated in a bacteriological oven at 37° C for 24h.

MIC (bacteriostatic effect) was considered the lowest concentration able to limit bacterial growth, using as reference the control group, but after the removal of the antimicrobial agent, the microorganisms returned to growth. The minimum bactericidal concentration (MBC) is the minimum concentration of an antimicrobial drug that is bactericidal. It is determined by re-culturing (sub culturing) broth dilutions that inhibit growth of a bacterial organism (i.e., those at or above the MIC). The broth dilutions are streaked onto agar and incubated for 24 to 48h. The MBC is the lowest broth dilution of antimicrobial that prevents growth of the organism on the agar plate (Pardon & Deprez 2018). Failure of the organism to grow on the plate implies that only nonviable organisms are present.

Profile of copaiba oil fractions by Thin Layer Chromatography

Thin layer chromatography was performed on 10cm x 5cm silica gel plates (Whatman UV254) to which copaiba oil was applied. The mobile phase used was hexane: ethyl acetate solution (8: 2 v/v). After the run, the plates were observed in UV light at 254 and 327nm. Specific reagents were then tested to detect the classes of compounds present in the oil: NP for flavonoid detection, Liebermann for terpenes and Dragendorff for alkaloids. Retention factor (Rf) was determined for each compound observed in the chromatoplate.

Bioautography

The microorganism *S. aureus* isolated from bubaline mastitis was used to verify the antimicrobial activity of the fractions observed in thin layer chromatography. The concentration of the microorganism was standardized on the Mac Farland 0.5 scale and incorporated into the Müeller Hinton Agar and the medium was poured into a Petri dish over the eluted chromatograms. The plates were incubated for 24h at 37°C, after which time it was developed with 2,3-triphenyl tetrazolium chloride (20 mg/mL). The formation of colorless halos on substance stains indicates inhibition of bacterial growth and thus antimicrobial activity of the substance (s) present on the chromatogram.

Statistical analysis

The collected data was transported to Microsoft Office Excel, where the average and standard deviation were calculated in the program.

RESULTS

All the multidrug resistant bubaline mastitis isolates analyzed were positive for the detection of beta-lactamase enzyme by nitrocefin test. The results obtained to evaluate the antimicrobial activity of copaiba oil using the disc diffusion methodology are presented in Table I. Copaiba oil presented a potential inhibition of bacterial

Table I. Inhibition halos of copaiba (*Copaifera multijuga*) natural oil against different bacteria - disc diffusion method.

Oil concentration (%)	*E. faecalis* ATCC 6057	*E. coli* ATCC 25922	*K. pneumoniae* ATCC 29665	*S. aureus* ATCC 6538	*B. subtilis* ATCC 6633	*P. aeruginosa* ATCC 27853
100.00	NI	NI	NI	NI	NI	NI
50.00	NI	NI	NI	10	NI	NI
25.00	12	10	NI	NI	NI	12
12.50	19	14	NI	NI	NI	12
6.25	19	12	NI	NI	NI	12
3.12	14	10	NI	NI	NI	12
1.56	NI	NI	NI	NI	NI	NI
Chlorhexidine	18	19	15	19	18	14

*** Halos determined in mm. NI = No inhibition halo formation.**

growth on the pathogenic isolates evaluated. Its antimicrobial activity varied according to oil dilution, and at concentrations of 25% to 3.12% (v/v) growth inhibition halos were observed for *E. faecalis*, *E. coli* and *P. aeruginosa* (Figure 1). For *S. aureus* was observed inhibition halos using 50 and 25% copaiba oil concentrations. It is noteworthy that the microorganisms *Klebsiella pneumoniae* and *Bacillus subtilis* were resistant to all concentrations of copaiba oil.

No inhibition halos were observed for all microorganism tested in concentrations of 1.56% copaiba oil. However, for the concentration of 3.12%, only 3 microorganisms (*K. pneumoniae*, *S. aureus* and *B. subtilis*) were not inhibited. The largest inhibition halos were obtained when used 12.50% of the copaiba oil: *E. faecalis* (19 mm), *E. coli* (14 mm) and *P. aeruginosa* (12 mm).

The results obtained from the antimicrobial activity, using the well diffusion technique, with the pure copaiba natural oil and the other DMSO concentrations presented inhibition halos only at the 12.5% and 6.25% concentrations for the majority. Inhibition of multiresistant isolates of bubaline mastitis, from the Brazilian states of Pernambuco, Ceará, Bahia and Alagoas, were evaluated against different concentrations of copaiba oil. It is important to highlight that the size of the inhibition halos formed by the multidrug resistant bubaline mastitis isolates for the states of Pernambuco, Ceará and Bahia were similar, differing only from the isolates of Alagoas State, which presented smaller halo size (Table II). Dimethyl sulfoxide (DMSO) was used as both diluent and negative control due to the favoring of the antimicrobial activity of copaiba oil by this compound. It is noteworthy that neither pure oil nor DMSO alone showed antimicrobial activity (Figure 2).

The turbidity of the culture medium was not possible to correlate with the bacterial growth, due to the copaiba oil in aqueous solution (Müeller Hinton broth) promoting turbidity, therefore it is not possible to differentiate growth inhibition either visually or per microplate reader. Minimum inhibitory concentrations were determined by the absence or presence of

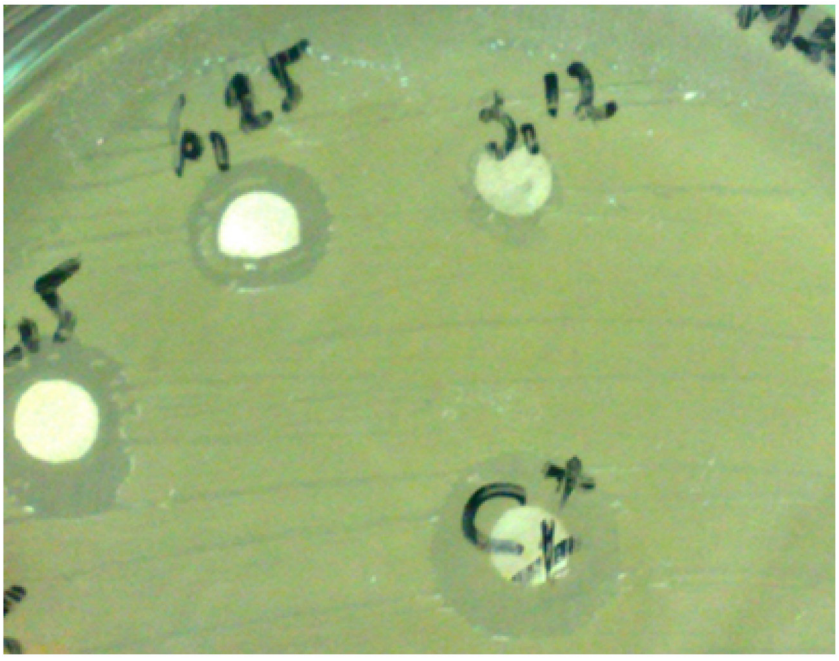

Figure 1. Illustrative photo of *E. coli* forward antimicrobial activity test using 100% to 1.56% copaiba oil, positive (chlorhexidine 0.12%) and negative (dimethylsulfoxide) control - disc diffusion technique.

microbial growth after 24h of contact with the different concentrations of copaiba oil (Figure 3).

It was observed that the minimum inhibitory concentration (MIC) for most of the multidrug resistant isolates of bubaline mastitis evaluated from the states of Pernambuco, Ceará, Bahia and Alagoas was 6.25%, which presented bactericidal activity against multiresistant *S. aureus*, as no microbial growth was observed in the plates after contact with this concentration of copaiba natural oil.

The effects of copaiba oil concentrations on the growth of multiresistant *S. aureus* was evaluated. The highest inhibition occurred at 25% concentration. Declining progressively until it reached 6.25%, which was the Minimum Bactericidal Concentration (MBC) recorded in our study. The Minimum Bactericidal Concentration of this oil was similar for all bubaline mastitis isolates from different states evaluated.

The chromatographic analysis of copaiba oil showed the presence of three compounds, which presented Rf of 0.93, 0.55 and 0.17, however only the last component (Rf 0.17) showed antimicrobial activity against *S. aureus* (Figure 4).

DISCUSSION

Machado et al. (2008) reported that high resistance to various antimicrobials who studied 109 *Staphylococcus* coagulase-negative isolates, and all isolates were resistant to at least one of the antimicrobials tested. High resistance to ampicillin (85%), penicillin (93%), sulfonamides (89%), novobiocin (89%), lincomycin (76%), kanamycin (79%), streptomycin (63%), erythromycin (61%) and oxacillin (81%) have also been reported (Machado et al. 2008). According to Medeiros et al. (2009) who studied the sensitivity and *in vitro* antimicrobial multidrug resistance profile in cows for *Staphylococcus* spp isolates and found a better sensitivity for the association between neomycin, bacitracin and tetracycline and a multidrug profile for eight or more antibiotics including the beta-lactamics group, gentamicin and tobramycin, enrofloxacin, ciprofloxacin and danfloxacin.

Table II. Inhibition halos of copaiba natural oil (*Copaifera multijuga* Hayne) against bubaline mastitis isolates - well diffusion technique.

Oil concentration (%)	States of Brazil			
	Pernambuco	Ceará	Alagoas	Bahia
100.00	NI	NI	NI	NI
50.00	NI	NI	NI	NI
25.00	NI	NI	NI	NI
12.50	17.8 ± 2.08	18.09 ± 7.17	16.46 ± 5.28	18.52 ± 6.97
6.25	18.15 ± 2.64	17.22 ± 5.21	13.23 ± 9.36	18.63 ± 5.67
3.12	19.00 ± 1.5	NI	NI	NI
1.56	NI	NI	NI	NI

Halos determined in mm. NI = No inhibition halo formation. Value represents the mean and standard deviation of the inhibition zone in millimeter.

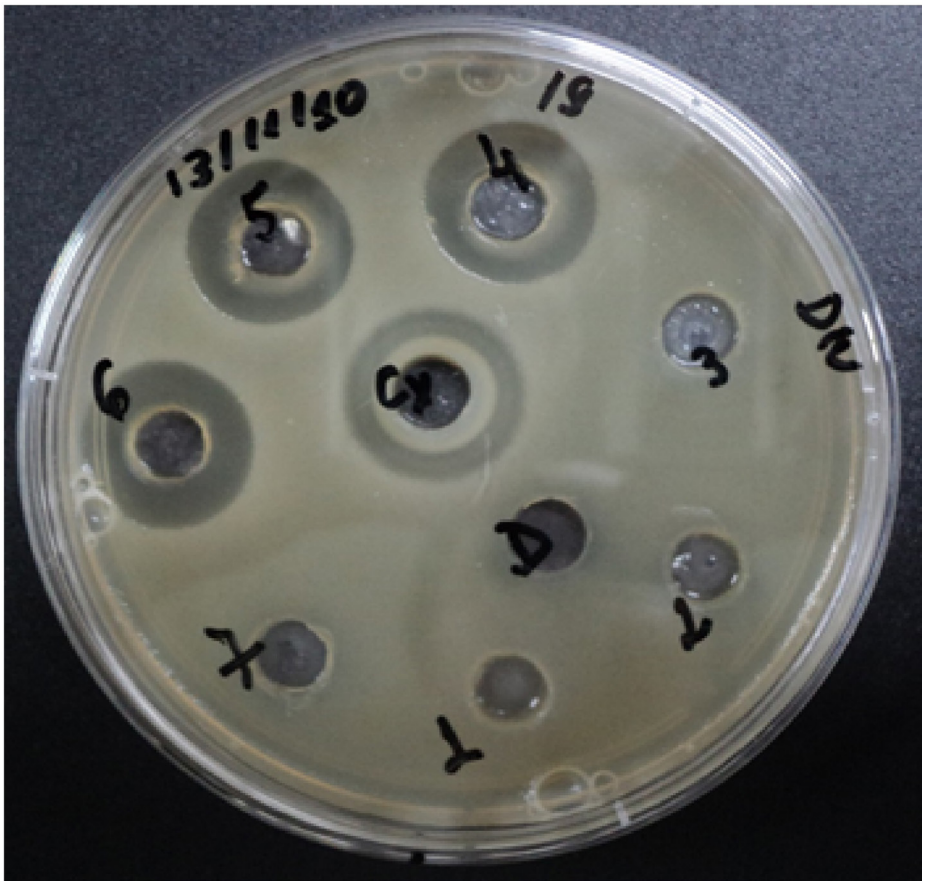

Figure 2. Illustrative picture of the microbial growth inhibition halos against *S.* buffalo mastitis *S.aureus* after diffusion test using the copaiba oil. Wells: 100% 1-oil, 2-50%, 3-25%, 4-12.5%, 5-6.25%, 6-3.12%, 7- 1.56%, 8-chlorhexidine (C +) 0.12% and 9-DMSO (D).

Medeiros et al. (2009) determined the multiresistance profile of bubaline mastitis isolates using in vitro antimicrobial susceptibility testing of isolated using the diffusion technique in Mueller Hinton agar according to Bauer et al. (1966). Using antibiotic-impregnated disks: amoxicillin (10mcg), ampicillin (10mcg), azithromycin (15mcg), cefquinome (30mcg), cephalexin (30mcg), ciprofloxacin (5mcg), cloxacillin (25mcg), danofloxacin (10mcg), enrofloxacin (5mcg), erythromycin (15mcg), florfenicol (30mcg), gentamicin (10mcg), penicillin + novobiocin (10mcg), sulfa (25mcg) + trimethoprim (5mcg), tobramycin (10mcg) and tetracycline (30mcg) + neomycin (30mcg) + bacitracin (10mcg) and also used PCR (Polymerase Chain Reaction) technique, which proved resistance to four or more different groups of antimicrobial drugs, simultaneously. The production of beta-lactamase enzyme by bubaline mastitis isolates by the nitrocefin method was biochemically demonstrated. The production of beta-lactamase enzyme by bubaline mastitis isolates by the nitrocefin method was biochemically demonstrated.

Mendonça & Onofre (2009) reported that copaiba oil had antimicrobial activity and that it varied according to the oil dilution in DMSO, demonstrating that inhibition halos with pathogens were observed at 100% to 1.56 % concentration. Halos at intervals of 8 ± 1.34, 9 ± 1.65, 7 ± 1.23 mm, respectively for *E. coli*, *P. aeruginosa* and *S. aureus*. The use of DMSO as a solvent is due to its well-known pharmacological and therapeutic properties, which result from its ability to interact or combine with nucleic acids,

carbohydrates, lipids and proteins and many drugs without irreversibly changing molecular configuration.

On the other hand, Packer & Luz (2007) did not observe antimicrobial activity for copaiba oil. However, several studies prove its bacterial action since copaiba oil has in its constitution beta caryophyllene, an active ingredient that has germicidal action (Neta et al. 2016, Francomano et al. 2019).

Vasconcelos et al. (2008) studied *Copaifera multijuga* Hayne oil-resin and evaluated the antimicrobial activity by the liquid dilution test against standard *Streptococcus mutans* (ATCC 25175) and *S. sanguinis* (ATCC 15300) strains and obtained results compared to the experimental groups analyzed. Copaiba oil-resin presented antimicrobial activity against the microorganisms analyzed, with advantages of being a natural product and of lower cost. Its ability bactericidal demonstrated the antimicrobial activity of this oil-resin, as previously observed by other authors (Veiga & Pinto 2002, Menezes et al. 2004, Gonçalves et al. 2005). Gonçalves et al. (2005) reported that the oil of *Copaifera langsdorffiia* showed antimicrobial activity against *Proteus mirabilis* and *Shigella sonnei*, but did not show antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

These compounds oil of copaiba were identified in our studies as belonging to the terpenoid classes, as they were revealed by Libermann's reagent has been corroborates the studies presented by Biavatti et al. (2006), which characterized copaiba oil by gas chromatography, identifying predominantly sesquiterpenes. and diterpenes.

Veiga & Pinto (2002) suggested that works should be developed to standardize the oils obtained in the consumer market, since studies with *Copaifera multijuga* oil showed variations in the composition of oils collected from the same tree, in different periods of the year (summer/winter) and that the substances detected may be basically the same, but their concentrations varied influencing the nature of the oil. These observations may explain the different sensitivity profiles of copaiba oil (*Copaifera multijuga*).

Mendonça & Onofre (2009) describe the results obtained with copaiba oil as promising, as they would allow the intensification of complementary clinical studies, due to the synergistic and/or antagonistic action of these phytoconstituents when used in combination with other products of plant origin or not, mainly on multiresistant bacteria. They also highlighted the possibility of studying the biological effects of copaiba oil on drug resistance plasmids, mainly related to *S. aureus* strains, thus contributing to the search for antimicrobial resistance. Alencar et al. (2015) demonstrated antimicrobial activity of essential oil from *Copaifera* against *S. aureus* (ATCC 29213), *S. epidermidis* (ATCC 12228) and two clinical strains of each species.

Pieri et al. (2009) described the antimicrobial action of copaiba oil against bacteria *Streptococcus pyogenes*. Pieri et al. (2009) on antimicrobial activity with copaiba oil presented results that suggest the use of copaiba oil in the prevention of periodontal disease and as a possible substitute for chlorhexidine in oral antimicrobial therapy. The use of copaiba oil on dental plaque-forming bacteria has been proven in dogs, and at the end of the experimental period a significant reduction in dental plaque formation was obtained with the use of oil-based solution.

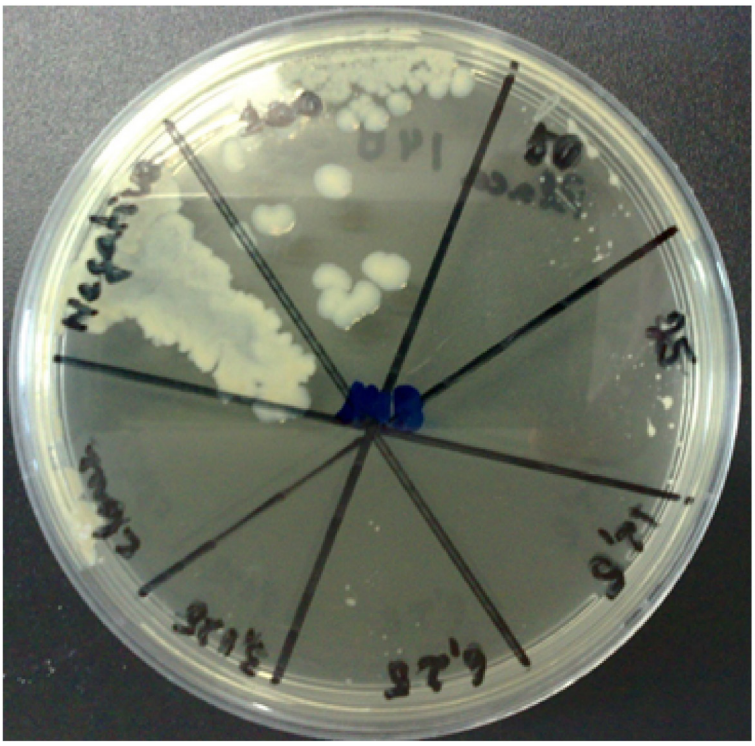

Figure 3. Illustrative photo of microbial growth inhibition against *S. aureus* of bubaline mastitis using copaiba oil after minimum inhibitory concentration test.

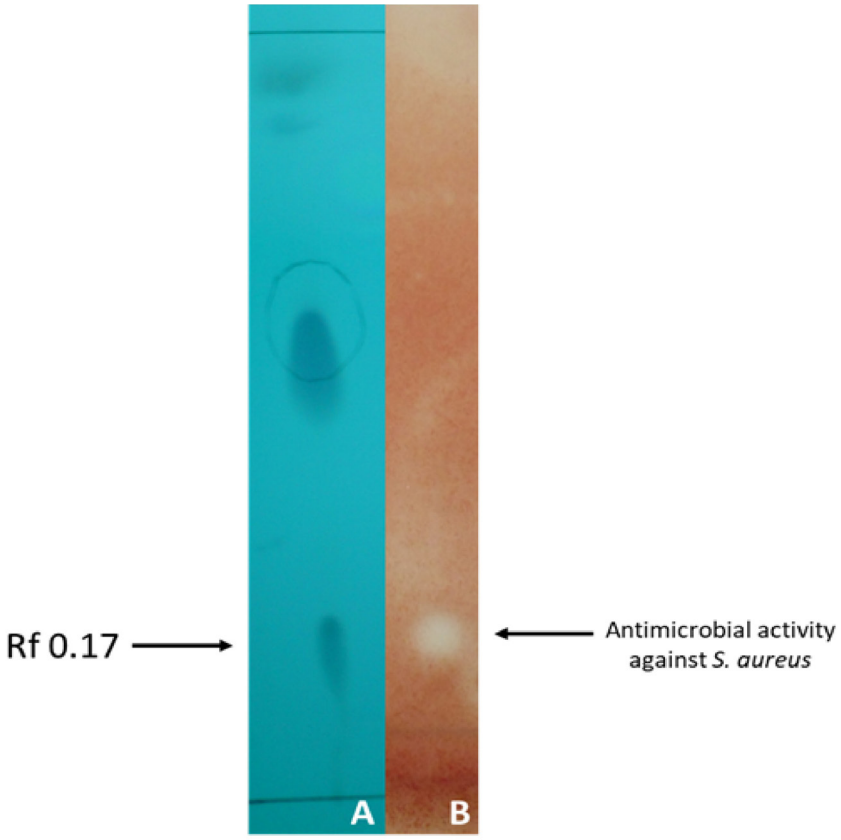

Figure 4. a - Chromatography of copaiba oil eluted with hexane (8): ethyl acetate (2). b - Fraction of copaiba oil with antimicrobial activity after addition of Liebermann reagent.

CONCLUSIONS

The natural oil of Copaiba (*Copaifera multijuga*) showed antimicrobial activity against the multiresistant isolates of bubaline mastitis. The oil concentration of 6.25% was considered minimum bactericidal concentration, which totally inhibited the growth of multiresistant *S. aureus*. Thus, copaiba oil is a promising source for the development of a new antimicrobial drug acting on resistant microorganisms that cause bubaline mastitis.

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All authors contributed to the development of the manuscript: Divânia Fônseca Franco de Oliveira - Activation of buffalo mastitis isolates, Nitrocefin test for detection of beta-lactamase enzyme produced by bubaline mastitis isolates; Thiago Pajeú Nascimento - Activation of microorganisms, analysis of results and correction of the article in English; Cláudio Henrique Rodrigues- Activation of microorganisms and Profile of copaiba oil fractions by Thin Layer Chromatography ; Juanize Matias da Silva Batista - Activation of buffalo mastitis isolates and Nitrocefin test for detection of beta-lactamase enzyme produced by bubaline mastitis isolates; Tatiana Pereira Shiu Lin Liu - Activation of buffalo mastitis isolates and Nitrocefin test for detection of beta-lactamase enzyme produced by bubaline mastitis isolates; Elizabeth Sampaio de Medeiros – Disc and Well Diffusion Antimicrobial Activity Test, Bioautography and Statistical analysis; Rinaldo Aparecido Mota – Disc and Well Diffusion Antimicrobial Activity Test, Bioautography and Statistical analysis; Romero Marcos Pedrosa Brandão Costa - Disc and Well Diffusion Antimicrobial Activity Test; Tatiana Souza Porto - Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC); Camila Souza Porto – Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC), Analysis of results and assistance in writing them; Ana Lúcia Figueiredo Porto- Analysis of results and assistance in writing them.

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