ARTIGO ORIGINAL

Antimicrobial activity of extracts of *Piper peltatum* and *Piper marginatum* on *Klebsiella* spp., *Staphylococcus aureus, Salmonella* spp., *Escherichia coli* and *Bacillus subtilis*

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ABSTRACT: The increase in resistance that bacteria develop against antibiotics is making it increasingly difficult to control them. Therefore, further development of studies focused on the use of natural antimicrobials is necessary. This study aimed to evaluate the antimicrobial activity in extracts of *Piper marginatum* and *Piper peltatum* species on *Klebsiella* spp., *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Bacillus subtilis*. By means of microdilutions, the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of the extracts were evaluated. Subsequently, the bacteria were treated with the extract at the Minimum Inhibitory Concentration (MICs) found and analyzed under a scanning electron microscope. The *Piper peltatum* extract showed activity against *Bacillus subtilis* and *Staphylococcus aureus* species, MIC of 31.25 and 125 µl/ml. On the other hand, the extract of *Piper marginatum* did not show antibacterial activity, MIC above 16,000 µl/ml. The activity of the extract of *P. peltatum* was considered good against *S. aureus* and *B. subtilis*.
Key-words: Antimicrobials, *Piper* sp., extracts, bioactivity.

RESUMO: Atividade antimicrobiana de extratos de *Piper peltatum* e *Piper marginatum* sobre *Klebsiella* spp., *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* e *Bacillus subtilis*. O aumento da resistência que as bactérias desenvolvem contra os antibióticos está tornando cada vez mais difícil controlá-las. Portanto, é necessário um maior desenvolvimento de estudos focados no uso de antimicrobianos naturais. Este trabalho teve como objetivo avaliar a atividade antimicrobiana de extratos das espécies *Piper marginatum* e *Piper peltatum* sobre *Klebsiella* spp., *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* e *Bacillus subtilis*. Por meio de microdiluições, avaliou-se a Concentração Inibitória Mínima (CIM) e a Concentração Bactericida Mínima (CBM) dos extratos. Posteriormente, as bactérias foram tratadas com o extrato na Concentração Inibitória Mínima (CIMs) encontrada e analisada em microscópio eletrônico de varredura. O extrato de *Piper peltatum* apresentou atividade contra as espécies *Bacillus subtilis* e *Staphylococcus aureus*, CIM de 31,25 e 125 µl/ml. Por outro lado, o extrato de *Piper marginatum* não apresentou atividade antibacteriana, CIM acima de 16.000 µl/ml. A atividade do extrato de *P. peltatum* foi considerada boa contra *S. aureus* e *B. subtilis*.
Palavras chave: Antimicrobianos, *Piper* sp., extratos vegetais, bioatividade.

INTRODUCTION

The severity of bacterial infections rose even after the discovery of several antibiotics, mainly due to strains of bacteria resistant to them (Ahmad et. 2001; Hall-Stodley et al. 2004). Furthermore, the use for antibiotic treatment in order to control bacterial infections is hampered by multidrug-resistant bacteria (Feng et al. 2021). As a result, the search for new ways to obtain antimicrobials is increasing. One of these means is through the use

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of plant extracts with antimicrobial properties. Some studies have observed that plants are effective as antimicrobials, even against bacteria of strains resistant to some antibiotics (May et al. 2000; Bozin et al. 2006). Plant extracts have also shown broad-spectrum inhibitory activity against various Gram-positive and Gram-negative pathogens (Edris 2007; Lang et al. 2012; Teixeira et al. 2013).

Piper peltatum L. species, popularly known as *mão-de-macaco*, has several uses. In French Guiana, for example, it is used to fight worms. In Africa, it is used for swelling, boils and burns. A decoction of leaves or roots is performed, with use aimed at jaundice, malaria, urinary problems, syphilis, gonorrhea, constipation and stomach pain, in addition to its antibacterial activity (Pinto 2006; Pascoli et al. 2018). *Piper marginatum* Jacq. species also belonging to the Piperaceae family is known as *malvaísco*, especially in northeastern Brazil. The essential oil obtained from its leaves shows several biological activities, being them in the treatment of liver and bile duct diseases, inflammation and even snake bites and antimicrobial action (D'angelo et al. 1997; Pascoli et al. 2018).

Thus, the use of extracts from plants that have an antimicrobial effect can help to reduce or inhibit the growth of microorganisms, which can be deteriorating or pathogenic, worrying the food chain and human health. Therefore, the present study aimed to evaluate the antimicrobial activity of extracts of *Piper marginatum* and *Piper peltatum* against strains of *Klebsiella* spp., *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Bacillus subtilis*.

MATERIAL AND METHODS

Plants species and strains

The species used, *P. marginatum* and *P. peltatum*, were identified and collected at Fazenda Florentino, Novo Progresso, Pará, Brazil, coordinates: 7°06'43.82"S 55°23'42.54"W. After collection, they were weighed and dried in a circulating air oven at 40 °C. After 10 days, the plant sample was ground in a knife mill, packaged and stored in a dry place and protected from light, as described in Pascoli et al. (2018).

The microorganisms used for this experiment were: *Klebsiella* spp., *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Bacillus subtilis*. All were maintained and stored in glycerol at -20 ºC in the Laboratory of Microbiology of Water, Environment and Food, State University of Maringá, Maringá, Paraná, Brazil.

To prepare the bacterial cell suspension, the microorganisms were cultured in tubes containing Tryptic Soy Broth broth (TSB) and incubated at 35 °C for 24 h. An aliquot of the cultures was standardized with saline solution with the McFarland Scale at 10^8 CFU/ml for the tests.

Preparation of extracts

To prepare the hydroalcoholic extract of *Piper peltatum* and *Piper marginatum*, the dried (700 g) and crushed leaves were subjected to the maceration process in an amber glass container, at room temperature, for a period of 7 days, with occasional agitation and renewal. of the extractant liquid ethanol:water 9:1 (v/v). For every 100 g of plant material, 1000 ml of extractor liquid was used. Then, the obtained extract was filtered and concentrated under reduced pressure in a rotary evaporator at a temperature of 40 °C for the total elimination of the solvent (Pascoli et al. 2018, with modifications).

Antibacterial activity

Minimum inhibitory concentration (MIC)

Antibacterial activity was performed by serial microdilutions, according to the Clinical and Laboratory Standards Institute (CLSI) protocol M7-A9 (2012), using the crude extracts at concentrations ranging from 3.91 to 16,000 µl/ml. Each well had a final volume of 100 µl (Tryptic Soy Broth culture medium (TSB), plant extract and solubilizer (dimethyl – sulfoxide – DMSO)). After serial dilution, a different concentration of each evaluated substance remained in each well. The microbial suspension was normalized according to the standard McFarland scale of 10^8 CFU/ml, to obtain a final concentration of 5×10^4 CFU/ml. A total of 5 µl was added from the microbial suspension and incubated at 35 °C for 24 h. All tests were performed in triplicate. With the result of this assay, the Minimum Inhibitory Concentration (MIC) of each microorganism was obtained, which was considered the lowest concentration capable of visibly inhibiting bacterial growth in the microplate (dos Anjos et al. 2016, with modifications).

Minimum bactericidal concentration (MBC)

From the MIC previously determined, a microculture of 10 µl (in triplicate) of the concentration of -4xMIC, -2xMIC, MIC, 2x MIC, 4 xMIC, 8xMIC and 10xMIC was carried out, in addition to the control (without extracts) in a plate with medium Tryptic Soy Agar (TSA) to evaluate the Minimum Bactericidal Concentration (MBC). For CBM, the lowest concentration capable of inhibiting the growth of bacteria after inoculation (incubation at 35 ºC for 24 h) was considered. The absence of colonies indicated that the concentration was effective as a bactericidal agent against the microorganism evaluated.

Scanning electron microscopy (SEM)

The bacteria were treated with the selected antimicrobial agents at the concentrations of the MICs and MBCs assays performed previously and incubated at 35 ºC for 24 h. After incubation, the bacterial solutions treated with the extracts were washed in phosphate buffered saline solution (PBS) pH 7.2 at room temperature, three times and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and left at room temperature for at least 1 h or in the refrigerator for up to 48 h. After this period, they were removed from the fixative solution and washed twice with 0.1 M cacodylate buffer.

Subsequently, the bacteria were dehydrated in 50%, 70%, 80%, 90% and 100% alcohol (10-15 min in each alcohol and twice in 100% alcohol). Finally, treated with CO_2 and metallized with gold, for reading in a Shimadzu SS-550 scanning electron microscope (Haddad et al. 2007).

RESULTS AND DISCUSSION

Antibacterial activity

The evaluation of the minimum inhibitory effect of the extracts was evaluated and it can be observed that the plant extract of the *P. peltatum* species showed an inhibitory effect against the bacteria *B. subtilis* and *S. aureus*. The concentration in the inhibitory was more efficient against the strain of *B. subtilis*, as shown in Table 1. However, the extract of *P. marginatum* did not show activity against the strains of microorganisms tested.

For microorganisms that did not demonstrate activity, tests were performed up to a maximum concentration of 16,000 µl/ml of extracts. Thus, microorganisms that may present activity higher than 16,000 µl/ml were considered in this study as having no activity.

The analysis of the MBC was performed in triplicate and only for the microorganisms that had their growth inhibited, that is, for *B. subtilis* and *S. aureus*. MBC was 500 µl/ml for both microorganisms.

The results of this study differ from the results obtained by Figueira et al. (2011), where it was found that both the plant extract and the oil of *P. marginatum* were able to inhibit the growth of *S. aureus*, *Salmonella sp*., *E. coli* and *B. subtilis*. However, in this study, an antibacterial effect of *Piper marginatum* extract was not found against the same bacteria tested. According to Pascoli et al. (2018), strains of *Alycyclobacillus acidoterrestris* had an MIC of 100 µg/ml compared to the extracts tested, *P. marginatum* and *P. peltatum*, indicating strong antibacterial activity.

In view of our study, despite not having shown activity on the tested strains, the antimicrobial activity of the same cannot be disregarded, since the period of plant development, location, collection time and several other factors can influence the bioactive characteristics of the plants (Castro et al. 2017). In addition, the Gram-negative bacteria evaluated against the extract of *P. peltatum* did not show growth inhibition, there was only action on the Gram-positive bacteria evaluated. Bacteria classified as Gram-negative are those that have an outer membrane that Gram-positive bacteria do not. Thus, it is suggested that the penetration of the extract in Gram-negative bacteria is more difficult, which may be the reason why they did not inhibit bacterial growth.

Scanning electron microscopy (SEM)

For the scanning microscopy (Figure 1), it is verified that the treatment of the *S. aureus* strain with *P. peltatum* (Figure 1B and C) presents small changes in the cells, such as irregularity in the edges, due to the antimicrobial activity of the extract, compared to the control. For the *B. subtilis* strain, no major changes were observed, but it is noteworthy that the concentration used for scanning microscopy was twice the MIC.

According to Pascoli et al. (2018), *P. peltatum* and *P. marginatum* showed changes in the cell membrane of *A. acidoterristris*, in addition to evidence of cell disruption caused by the treatment.

TABLE 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of extracts of *Piper marginatum* e *Piper peltatum* against *Klebsiella* spp., *Staphylococcus aureus*, *Salmonella* spp., *Escherichia coli* and *Bacillus subtilis*.

Microorganisms	*Piper marginatum*		*Piper peltatu*m	
	MIC (**µl**/ml)	MBC (**µl**/ml)	MIC (**µl**/ml)	MBC (**µl**/ml)
Klebsiella spp.	-	-	-	-
Staphylococcus aureus	-	-	125	500
Salmonella spp.	-	-	-	-
Escherichia coli	-	-	-	-
Bacillus subtilis	-	-	31.25	500

- atividade superior a concentração de 16,000 **µ**l/ml.

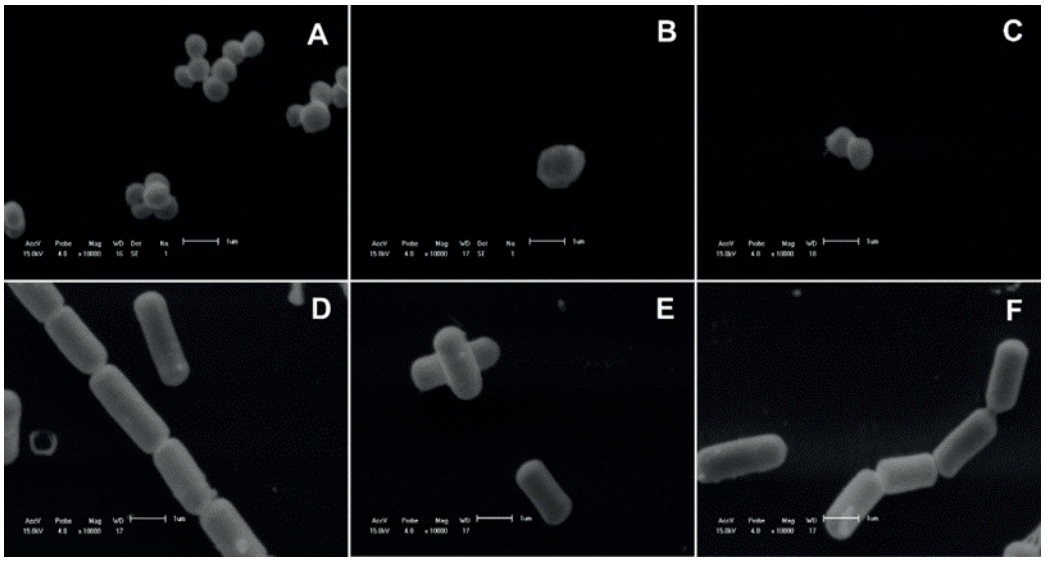

FIGURE 1. Scanning electron microscopy of microorganisms after treatment with *Piper peltatum* extract. A. *Staphylococcus aureus* control; B and C. Treatment of *Staphylococcus aureus* with 250 µl of *Piper peltatum* extract; D. *Bacillus subtilis* control and E and F. Treatment of *Bacillus subtilis* with 62.5 µl of *Piper peltatum* extract. Bar: 1 µm. Magnification: 10,000x.

It is important to emphasize that although changes in membranes have been observed in treatment with *P. peltatum*, it is still important to investigate this activity and action, as well as its mechanism of action.

CONCLUSION

Piper peltatum showed effective bacterial inhibition against *Staphylococcus aureus* and *Bacillus subtilis* strains, compared to the other strains and extract, *Piper marginatum*. Furthermore, scanning electron microscopy showed structural alterations, suggesting this inhibition and alteration of the microbial growth of the strains. However, future studies should be carried out in order to better understand this inhibition, mechanism of action and applications in the food area, as food safety.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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