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ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANTS AGAINST MULTIDRUG-RESISTANT BACTERIA

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ABSTRACT

The main cause of increasing of infectious diseases cases is due to multidrug-resistant microorganisms emergence, particularly *Pseudomonas aeruginosa* and *Staphylococcus aureus*, responsible for most of hospital-acquired infections and millions deaths related. Despite development of new antibiotics, control of these microorganisms is not always successful. Several plant extracts have demonstrated antimicrobial effects and may be used as an alternative therapy for these infections. Aiming to evaluate antibacterial activity of extracts from *Eleutherine plicata* (marupazinho), *Geissospermum vellosii* (pau-pereira) and *Portulaca pilosa* (amor crescido) against multidrug-resistant bacteria, samples of Oxacillin-Resistant *Staphylococcus aureus* (ORSA) and multidrug-resistant *P. aeruginosa* (MDR *P. aeruginosa*) isolated from human clinical processes were tested. The antibacterial activity was determined by disk diffusion method and minimum inhibitory concentration (MIC) by microdilution method. Extracts and fractions were tested at concentrations of 500, 250, 125, 62.5, 31.2 and 16.2 µg/mL dissolved in DMSO 10%. *E. plicata* and *G. vellossi* have shown activity against ORSA at MIC of 125 µg/L, whilst *P. pilosa* have shown action on MDR *P. aeruginosa* at MIC of 250 µg/mL. Results suggest the extracts of *E. plicata*, *G. velossi* and *P. pilosa* have antimicrobial activity with potential use as phytoterapic drugs or for further research on new antimicrobial drugs.

INTRODUCTION: Medicinal plants have been used in treatment of diseases as a common strategy virtually by all populations around the world. In Brazil it is common in the poorest regions as well as in big cities, to find medicinal plants in free markets, supermarkets and even in backyards of houses [1].

In Brazil, over 55 thousand plants species have been described, about 20% of plants on the planet, the largest biodiversity in the world. There is a well stablished acceptance of medicinal plants use associated to traditional knowledge [2].

Infectious diseases affect millions of people around the world and they are one of the main causes of death in History. This problem is exacerbated by emergence of multidrug-resistant microorganisms, especially *Staphylococcus aureus* and *Pseudomonas aeruginosa*, present in hospital and community acquired infections, decreasing antibiotic therapy options [3, 4].

Due to increasing of resistance against several antimicrobial drugs, searching for new therapeutic alternatives using medicinal plants play an important role for obtaining new drugs.

Aiming to evaluate antibacterial activity of medicinal plants extracts against multidrug-resistant bacteria, we have selected plants *Eleutherine plicata* ("marupazinho"), *Portulaca pilosa* ("amor crescido") and *Geissospermum vellosii* ("pau-pereira") usually used in popular medicine in Brazil for treatment of infectious diseases [5, 6, 7].

MATERIAL AND METHODS:

Sources and parts of plants:

- ***Eleutherine plicata*** **-** bulbs collected at Fátima Village, Traquateua-PA, Brazil in September 2010.
- ***Portulaca pilosa*** - aerial parts of plants collected in February 2011 at community of St. Helena, Acará, Pará, Brazil.
- ***Geissospermum vellosii*** - bark collected in July 2010, at Ramal Madereiro-Moju-PA, Brazil.

Botanical identification of plants was held at Emílio Goeldi Museum, Belém-PA, Brazil.

Preparation of Plant Material: *E. plicata* was stored in crude ethanol extract (CEE) and dichloromethane fraction (DF), *G. vellosii* in CEE and alkaloids fraction (AF), and *P. pilosa* in CEE, ethyl-acetate fraction (EF) and hydroalcoholic fraction (HF). All extracs were lyophilized and packaged in sealed glass containers and kept at refrigeration. These materials were obtained by protocol used in Phytochemistry Laboratory of Pharmacy Faculty of Federal University of Pará [8].

Bacterial samples: Samples of ORSA (02) and MDR *P. aeruginosa* (3) from human clinical samples were used. All bacteria were isolated and identified in Public Health Laboratory of Pará (LACEN-PA). Strains were kept in freezer at 20°C in BHI broth added of glycerol 15% until use. *P. aeruginosa* strains were initially spread on EMB (eosin-methylene blue agar), and *S. aureus* strains on mannitol salt agar incubated at 35^{o}C for 24 hours. After growth, bacteria were spread in nutrient agar and stored at room temperature until use.

Preparation of Inoculum: Inoculum was prepared by direct suspension in saline solution, from 3-4 colonies of bacteria selected from a Muller Hinton agar plate after 18-24 hours incubation at 35^{o}C. Bacterial suspension was adjusted for McFarland standard turbidity 0.5 (about 1 to 2 x 10^8 UFC/mL) [9].

Contamination extracts evaluation: Extracts and fractions were was evaluated for contamination, prior to antimicrobial tests by inoculation in TSA (Tryptic Soy Agar) and incubation at 35°C for 24 hours for bacteria, and in Sabouraud agar medium at room temperature for 5 days for fungi.

Preliminary evaluation of Antimicrobial Activity of Plant Extracts: Only CEE plants have been tested as screening for the following steps. For evaluation of antimicrobial activity was used the method of disc diffusion on agar [9,10]. Each microorganism suspension was spread (in duplicate), using a disposable swab all over the surface of Muller Hinton agar medium plates. Afterward, 6 mm sterile white discs for antibiotics (INTERLAB) were impregnated with 10 µL of each plant extract in a concentration of 500 µg/mL and 250 µg/mL dissolved in DMSO 10%. A disk impregnated with 10 µL DMSO 10% was used as control of solvent toxicity. After incubation at 36°C for 24 hours reading of results was carried out by measuring the zone of susceptibility around the disks containing plant extracts. Average of two measures was stablished as the final result for each extract and it was considered susceptible a zone equal to or greater than 8 mm diameter.

Determination of the minimum inhibitory concentration (MIC): The method used was the microdilution method according to Eloff (1998) [11] with adjustments [12,13].

The inoculum was a bacterial suspension in saline, with turbity corresponding to 0.5 McFarland scale (1 x 10^8 CFU/mL) as previously described. This suspension was diluted up to 1 x 10^6 CFU/mL n Muller Hinton broth and 100 µL were then homogenized into sterile 96 wells microplate containing 100 µL of different concentrations of plants extracts dissolved in DMSO 10% and Muller Hinton broth. Final volume in each well was 200 µL and final concentration in each well was 500, 250, 125, 62.5, 31.2 and 16.2. Tests were performed in duplicate.

Microplates were incubated at 36°C for 18-24 hours. For revelation of the results a solution of 1% sodium resazurin was prepared. After incubation, 15 µL of this solution was added to each well and incubated for

three hours at room temperature. Results of MIC was considered positive for those wells with blue coloring indicating absence of visible microbial growth, and negative for those wells in red due the presence of viable cells [13,14].

RESULTS AND DISCUSSION:

Preliminary Evalution of antimicrobial activity of plant extracts: Evaluation of contamination of extracts and fractions held before the antimicrobial tests indicated these no contamination by bacteria or fungi because there were no development of colonies in Sabouraud agar and TSA after incubation. These results have shown that the products had good microbiological conditions to be used in this research.

Preliminary evalution of antibacterial activity was carried out by disk diffusion method in solid medium. Between the several sreanning protocols, this method is best suited for using plant extracts colored and/or organic solvents, because it is possible to evaporate the solvent from the disk before the placement in culture medium [15] and the color does not interfere in the reading of results. In this method, however, presence of suspended particulate matter in the sample can interfere with diffusion of antimicrobial substance in agar, but the small volume and the possibility of testing various compounds against to a single microorganism were advantages of this method [16].

There is no consensus about the acceptable concentration for natural products when compared to known antibiotics. Aligianis *et al*., (2001) [17] proposed a classification for plant materials based on the results of MIC considering strong inhibition - MIC until 500 µg/mL; moderate inhibition MIC between 600 and 1500 µg/mL; and weak inhibition MIC above 1500 µg/mL.

For Sartoratto *et al*., (2004) [18], a strong activity of plant extracts would be for MIC values between 50 and 500 µg/mL; MIC with moderate activity between 600 and 1500 µg/mL; and weak activity above 1500 µg/mL. Holetz *et al*., (2002) [19] have suggested MIC below 100 µg/mL as strong antimicrobial activity; moderate activity for MIC between 100 and 500 µg/mL; MIC between 500 and 1000 µg/mL as weak activity; and above 1000 µg/mL as absence of activity.

In this work, was carried out a screening of ethanolic extracts of plants and their antibacterial activity starting concentrations of 500 and 250 µg/mL whereas these products are crude extracts. These values according to criteria suggested by Holetz *et al*., (2002) [19] would be moderate antimicrobial activity. But considering the less stringent criteria of Aligianis *et al.,* (2001) [17] and Sartoratto *et al*., (2004) [18], the extracts at concentrations studied would have a strong antimicrobial activity. In this way, we point out the need for more advanced studies, aiming to standardize the acceptable concentration of antimicrobial activity of plant extracts with potential for herbal medicines or for research of new antimicrobial drugs.

All extracts and fractions in study demonstrated activity against for at least one species of multi-drug resistant bacteria tested, suggesting the medicinal plants as an important alternative in control of bacterial resistance.

The *Eleutherine plicata* have shown antibacterial activity against ORSA at concentrations of 500µg/mL of CEE (Table 1). This result is promising, because one of the few therapeutic alternatives to ORSA is the drug vancomycin [20]. Moreover, strains of vancomycin-resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA) have already been isolated and they are a great concern in the control of bacterial resistance [21, 22, 23].

TABLE 1: EVALUATION OF ANTIBACTERIAL ACTIVITY OF CRUDE ETHANOLIC EXTRACT (CEE) OF *ELEUTHERINE PLICATA* BY DISK DIFFUSION ON AGAR

Bacteria	Concentration CEE 500 µg/mL	Concentration CEE 250 µg/mL
S. aureus strain 1	S (13 mm)	S (10 mm)
S. aureus strain 2	S (13 mm)	S (10 mm)
P. aeruginosa strain 1	R (0)	R (0)
P. aeruginosa strain 2	R (0)	R (0)
P. aeruginosa strain 3	R (0)	R (0)

R- resistant; S- susceptible (Zone of bacterial inhibition in mm)

Despite having action on Gram-positive bacteria *S. aureus*, the *E. plicata* extract has not shown activity to the bacteria Gram negative *P. aeruginosa*. Urzua *et al*., (1998) [24] suggested that the outer membrane of gram-negative bacteria could act as a barrier against the active substances present in extracts of plants. Ribeiro *et al*., (2009) [25] proved the CEE of *E. plicata* had greater activity against Gram positive bacteria *S. aureus*.

Presence of tannins in the extract was cited as being responsible for the antimicrobial activity.

Malheiros (2008) [26] reported the CEE and chloroform fraction of *E. plicata* had activity against *S. aureus* and the yeast *Candida albicans*, but with no action against *Escherichia coli* and *P. aeruginosa*. By determining of MIC the author found that the chloroform fraction had more active than the ethanolic extract.

Preliminary study on CEE of *G. vellosii* (**Table 2**) have shown activity against ORSA in concentrations of 500 and 250 µg/mL, with no activity against MDR *P. aeruginosa*. The zones of bacterial inhibition formed were smaller than those developed by *E. plicata*.

TABLE 2: EVALUATION OF ANTIBACTERIAL ACTIVITY OF CRUDE ETHANOLIC EXTRACT (CEE) OF *GEISSOSPERMUM VELLOSII* BY DISK DIFFUSION ON AGAR

Bacteria	Concentration CEE 500 µg/mL	Concentration CEE 250 µg/mL
S. aureus strain 1	S (10 mm)	S (9 mm)
S.aureus strain 2	S (10 mm)	S (8 mm)
P. aeruginosa strain 1	R (0)	R (0)
P. aeruginosa strain 2	R (0)	R (0)
P. aeruginosa strain 3	R (0)	R (0)

R- resistant; S- susceptible (Zone of bacterial inhibition in mm)

It has been shown the CEE of *Gesissospermum argenteum*, belonging to the same family of *G. vellosii*, had antimicrobial activity against against *S. aureus* and *P. aeruginosa*, both multidrug resistant [27]. Mbeunkui et al (2012) [28] showed the alkaloids *geissolosimine*, *geissospermine*, *geissoschizoline* and *vellosiminol,* isolated from crude methanolic extract of *G. vellosii*, had antiplasmodial activity *in vitro* antiplasmodial activity against the chloroquine-sensitive strain of *Plasmodium falciparum*, and showed the highest activity for *geissolosimine* and a weak activity for *vellosiminol*.

Our work is the first one to report antibacterial activity of *Geissospermum vellosii*.

The increasing frequency of ORSA and the possibility of emergence of vancomycin-resistant samples are important finds to enhance development new drugs against *Staphylococcus* and, in this context, extracts of *E. plicata* and *G. vellosii* have shown potential as a therapeutic alternative.

Preliminary evaluation of CEE of *P. pilosa* (**Table 3**), have shown antimicrobial activity only against MDR *P. aeruginosa* in concentrations of 500 and 250 µg/mL. Mendes et al., (2011) [29] in a preliminary evaluation of *P. pilosa* extract by agar diffusion method against *P. aeruginosa* showed zone of 25 mm in diameter at concentration of 500 µg/mL and 20 mm at a concentration of 250 µg/mL, which corroborates our results. The authors suggested the presence of phenols and tannins found in the phytochemical survey of plant extract, would be responsible for antimicrobial activity.

The CEE of *P. pilosa* has not demonstrated activity when tested against bacteria alcohol-acid-resistant *Mycobacterium tuberculosis* [30]. It is important to point out this bacterium has a high content of lipids in cell wall composition, which could prevent action of extract.

TABLE 3: EVALUATION OF ANTIBACTERIAL ACTIVITY OF CRUDE ETHANOLIC EXTRACT (CEE) OF *PORTULACA PILOSA* BY DISK DIFFUSION ON AGAR

Bacteria	Concentration CEE 500 µg/ml	Concentration CEE 250 µg/ml
S. aureus strain 1	S (0)	S (0)
S.aureus strain 2	S (0)	S (0)
P. aeruginosa strain 1	S (10 mm)	S (9 mm)
P. aeruginosa strain 2	S (10 mm)	S (9 mm)
P. aeruginosa strain 3	S (9 mm)	S (8 mm)

R- resistant; S- susceptible (Zone of bacterial inhibition in mm)

Determination of the minimum inhibitory concentration (MIC): After preliminary determination of antimicrobial activity of CEE against the microorganisms tested, we selected extracts and fractions from plants with positive result (zone ≥ 8 mm) against each of the microorganisms in study. To determine MIC, plant products (extracts and fractions) were tested at concentrations of 500, 250, 125, 62.5, 31.25, and 15.62 µgmL by microdilution plate method. MIC is the lowest concentration of the product able to inhibit the multiplication of a bacterial isolate. The DMSO 10% did not stop bacterial growth in the tests carried out.

To determine MIC was chosen method stablished by Eloff (1998) [11], a methodology widely used for plant extracts due their sensitivity and minimum amount of reactants, allowing a greater number of replicas, increasing the reliability of the results [31].

Determination of MIC by this method can quantitatively assess the antimicrobial potential of a plant, it is possible to compare responses of different samples as extracts, fractions and pure substances obtained from samples [32].

As bacterial growth developer was used resazurin (7-hydroxy-3-phenoxazin-ona-3-10-oxide) blue color in the presence of viable cells, is oxidized to resofurina, red colouring substance facilitating the verification of the presence of microbial growth and with the blue color indicating the absence of visible growth [33].

Table 4 demonstrates MIC of extracts against microorganisms.

TABLE 4: DETERMINATION THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF PLANT PRODUCTS AGAINST BACTERIA TESTED

Bacteria	*E. plicata*		*G. vellosii*		*P. pilosa*	
	CEE	DF	CCE	AF	EF	HF
S. aureus strain 1	250	125	125	125	-	-
S. aureus strain 2	250	125	125	125	-	-
P. aeruginosa strain 1	-	-	-	-	250	250
P. aeruginosa strain 2	-	-	-	-	250	250
P. aeruginosa strain 3	-	-	-	-	250	250

CEE (Crude Ethanolic Extract); DF (Dichloromethane Fraction); AF (Alkaloid Fraction); EF (Ethyl acetate Fraction); HF (Hydroalcoholic Fraction)

CEE of *E. plicata* have shown MIC of 250 µg/mL, and MIC of 125 µg/mL for DF against *S. aureus*. The plant *G. velosii* have shown MIC of 125 µg/mL against *S. aureus* either for CEE as to AF.

For *E. plicata* dichloromethane fraction was more active fraction that CEE showing MIC of 125 µg/mL, suggesting the active substance is in highest concentration at this fraction. Ifesan (2009) [34] found that the CEE of the plant genus *Eleutherine* had MIC of 62.5 µg/mL to Methicillin-Resistant *S. aureus* (MRSA), while the MIC of sensitive strain was 250 µg/mL.

Both CEE as alkaloid fraction (AF) *G. vellosii* have shown MIC of 125 µg/mL to all isolates of *S. aureus*. It would be expected the AF had a more intense activity. However, it is speculated the effectiveness of antimicrobial activity of CEE may be due to interaction between different chemical compounds found in plants, and not by the activity of isolated compounds, which may explain these results [15, 35].

This MIC characterize a strong antibacterial activity [17, 18], which is particularly interesting as action on multidrug-resistant *S. aureus* strains because spreading of ORSA has frequently been reported around the world, causing an increase in costs associated with infections of this bacteria in hospitals, where arising from prolonged hospitalization, need for antimicrobials more expensive and indirect spending with infection control measures [36, 37, 38].

Fractions EF and HF of *P. pilosa* have shown MIC of 250 µg/mL against MDR *P. aeruginosa* (Table 4). This is a very important result especially because bacteria can express a lot of resistance mechanisms, such as the production of beta-lactamase, permeability greatly reduced to entry of antibiotics and presence of efflux pumps, leading to intrinsic resistance to multiple antibiotics [36]. Fractions had action on strains resistant to all antibiotics tested, proving that the plant is important for herbal studies and new antimicrobial drugs.

MIC of 250 µg/mL to fractions of *P. pilosa* against *P. aeruginosa*, found in this work, was also reported by Mendes et al (2011) [29] for the CEE of the plant. However, these authors did not work with multidrug-resistant strains.

CONCLUSION: Extracts from *E. plicata* and *G. vellosii* have shown antibacterial potential activity against ORSA, and could become a therapeutic alternative for the development of new drugs against these multidrug-resistant strains.

The plant *P. pilosa* have shown a good performance against *P. aeruginosa* multi-drug resistant, a result that should be pointed out, considering that increasingly, the emergence of strains of this bacteria resistant to all antibacterial drugs known. However, further studies are required for verification the toxicity of plants and isolation and identification of active compounds responsible for these activities.

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