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ANTIBACTERIAL ACTIVITY OF THE HYDROALCOHOLIC EXTRACT OF THE RED PROPOLIS OF THE PARAIBA SEMIARID ON *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Introduction: The red propolis is a resinous product consumed in the Brazil, of botanical origin of *Dalbergia ecastophyllum.* The *Pseudomonas aeruginosa* is a non-fermentative bacillus, negative gram. **Objective:** To evaluate the antibacterial activity of the hydro alcoholic extract of red propolis over strains of *Pseudomonas aeruginosa* (ATCC27853). **Method:** Exploratory research, *in vitro*. The sample of red propolis was obtained in the apiary in João Pessoa – PB, Northeast, Brazil. The hydroalcoholic extract of propolis was dissolved in Twin to 80 per cent and sterile distilled water; the Minimum Inhibitory Concentration (MIC) was determined by the 96-well plate micro dilution method. The sub inhibitory concentration of the extract and the antibiotics in decreasing concentrations, from 1024µg/ml, diluted sequentially up to 16µg / ml were used to evaluate the modulating activity of the antibiotic action. The statistical analysis was done using ANOVA. **Results:** The extract of red propolis did not present antimicrobial activity on *P. aeruginosa* (ATCC27853), because the MIC value was less than or equal to 1024µg/ml. It is suggested the identification of the chemical components of the red propolis of Paraiba, as it may present variation due to seasonality, collection period, different geographical origins, among others.

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INTRODUCTION

The *Pseudomonas aeruginosa* is a is a non-fermentative bacillus, that belongs to *pseudomonadaceae* family, It's present in water, soil, fruits and vegetables and it is capable of infecting plants, insects, animals and humans (Gooderham, 2009).It acts as an opportunistic pathogen, causing infections in several organs and tissues, as well as being one of the main agents that cause hospital infections (WILLIAMS *et al*., 2012). Hospital infections are known as Health Care-Related Infections and they are a serious public health problem, which is demanded by the control, prevention and treatment of HCRI. Therefore, it is a biological, historical and social episode that has a direct impact on health care security and, therefore, one of the main global challenges for the quality of health care (SILVA, 2008; Oliveira, *et al*., 2016). About HCRI, there is a growing search for new alternatives in Brazil, due to a series of problems related to multiple resistance, most often caused by the indiscriminate and prolonged use of synthetic chemical antimicrobials, which lead to the selection of microorganisms pathogenic mutants resistant to these compounds, making the use of natural antimicrobials an effective and economical alternative (SILVA, 2008). In this context, the increase in the resistance of *Pseudomonas aeruginosa* to antimicrobials has restricted the alternatives of treatment of infections related to health care in Brazilian hospitals, making measures to solve such problems, as the search for new therapeutics, preventive actions and eradication, developed to control the use of antimicrobials.

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Another proposal is the development of researches for a better understanding of the genetic mechanisms of resistance of *Pseudomonas aeruginosa* and continuity of the studies concerning the development of new synthetic and natural drugs (Nascimento *et al*., 2008). Among these new natural products studied, propolis stands out because it presents a wide diversity of active compounds (BARBOSA *et al*., 2010). The use of propolis is ample, and can be applied in the closing of small holes, to embalm dead insects and protect the beehive from invaders. Its composition is a direct reflection of the flora where the bees are located (CABRAL, 2008). Due to the diversity of the Brazilian flora, the Brazilian propolis were grouped into 12 distinct groups, according to chemical composition and biological activities (Silva, 2008).

A new type of propolis from the mangrove region of the State of Alagoas had its botanical origin identified as Dalbergia ecastaphyllum, L. Taub, known as the rabo-de-bugio, a legume species native of the mangroves of Alagoas, in which it produces and exhibits reddish sap (Portilho *et al*., 2013). The "red propolis" was named because of its intense red color and was classified as the 13th type of Brazilian propolis, demonstrating several biological activities in *in vitro* experiments (Alencar *et al*., 2007). The biological properties of red propolis are attributed to flavonoids and phenolic acids, with emphasis on anti-inflammatory, cytotoxic, antiatherogenic, healing, regenerating cartilage and dental pulp, antioxidant and antimicrobial activities (Cabral *et al*., 2009; Oliveira *et al*., 2014). Besides red propolis to be little known and disseminated among health professionals and, considering that it has a low cost, because it is easily accessible to the population and there aren't contraindications to its therapeutic use and, supposing that it may present antibacterial activity on the *Pseudonomas aeruginosa* was what motivated this research. Therefore, the general objective was to evaluate the antibacterial activity of the hydroalcoholic extract of the red propolis, on strains of Pseudonomas aeruginosa. The specifics were: to determine the Minimum Inhibitory Concentration (MIC) of the red propolis extract against the *P. aeruginosa* strain; to verify the action of the red propolis extract (RPE), isolated and in combination with the antibiotics amikacin, benzylpenicillin, ciprofloxacin and oxacillin.

MATERIALS AND METHODS

The research has done was exploratory, *in vitro*, procedure with a quantitative approach, carried out in partnership with the Campina Grande Federal University (CGFU), Pombal-PB and the Microbiology and Parasitology's Laboratory of the Center for Teacher Training of the CGUF-Campus of Cajazeiras-PB, in addition to the Microbiology's Laboratory of the Regional University of Cariri - CE. The red propolis sample was obtained from the apiary EDIMEL, in João Pessoa - PB, Northeast, Brazil. The apicultural material, 50g of red propolis, was weighed, macerated, and then, in a transparent glass vessel, soaked in 250mL of absolute ethanol and 250mL of distilled water for a period of 72h. Then the solution was filtered to retain the solid part and sent to the Regional University of Cariri (RUCA). The drying of the extract was performed by means of the spray drying technique using the MSDi 1.0 Mini-spray dryer (Labmaq do Brasil) using 1.2 mm sprinkler nozzle under the following operating conditions: a) flow control: 500 mL / h; b) inlet temperature: 120 ± 2 ° C; c) outlet temperature: 74 ± 2 ° C; d) atomization air flow rate: 45 L / min; e) blower flow: 1.4 m3 / min. The spray-drying process consists of the change from a product that is in the fluid state to the solid state in the form of powder, through its passage in a heated medium, in a continuous operation (MASTERS, 1991). The experiments were done with the standard and multi resistant bacterial lineage strains of *Pseudomonas aeruginosa* (ATCC 27853), obtained from the collection of microorganisms from the Laboratory of Microbiology of the CFP / CGFU. Bacterial strains were maintained and activated prior to experiment in bacteriological oven for 24 hours at 37 ° C in Agar Heart Infusion (HIA). The classes of antibiotics (amikacin, benzylpenicillin, ciprofloxacin, oxacillin) obtained at the University Hospital of the CGFU were used to evaluate the modulating activity of the antibiotic action of the red propolis extract. With the exception of ciprofloxacin, all drugs were dissolved in sterile water.

The minimum inhibitory concentration (MIC) was determined in Brain Heart Infusion (BHI 100%) by the microdilution method using a suspension of 105 Colony Forming Units (CFU / mL - for Mcfarland 0.5 scale) and a concentration of the red propolis extract 1024µg / ml diluted sequentially by the title 1:2 (JAVADPOUR *et al*., 1996). Following experiments, the delivery mean was prepared in sterile tubes using 100 µL of the inoculum in 900 µL of the BHI liquid culture mean. Subsequently, the contents of the tube were transferred to a 96-well micro dilution plate and serial dilutions of the red propolis extract were performed at concentrations ranging from 1024µg / mL to 16µg / mL (1: 2).The plates were incubated in a growth oven at 37 ° C for 24h and bacterial growth was evaluated by the use of resazurin. The reading of this experiment had, as a characteristic, the color change of its, from blue to red, indicating to the presence of bacterial growth and, the permanence in blue, the absence of growth.

Antibacterial experiments were performed in triplicates. In order to evaluate the action of the red propolis extract associated with the antibiotics, in each well of the microdilution plate the red propolis extract in subinhibitory concentration (MIC/8) was used, being constantly mixed in 100% BHI broth prepared with sterile distilled water, while the antibiotics were diluted in decreasing concentrations in the wells, starting from the concentration of 1024 µg / mL in the first well, with a sequential dilution of 1: 2 to 16 µg / mL, which was the last well to be diluted with the antibiotic. In each well was added 100µL of 100% BHI broth, 128µg/ml of the red propolis extract and bacterial inoculum (105 UFC / mL). Plates were incubated at 37 ° C for 24h and bacterial growth was assessed by the use of resazurin. Trials were performed in triplicates. The data were expressed as average and ± standard error of the average. Statistical analysis was performed using ANOVA and two-way with multiple comparison test (Bonferroni), with the help of GraphPad Prism version 7.0. When the F value was significant, post hoc comparisons were made by the Newman-Keuls test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Yield of the red propolis extract: The initial mass of the propolis extract was 50g. Finally, as powder, 4.926 g was obtained, generating a yield of 9.852%. To dilute the extract, 0.5µg, 1mL of Twin to 80% and 4mL of distilled water were used in a sterile container.

Table 1. MIC found for red propolis extract and antibiotics tested on *Pseudomonas aeruginosa*

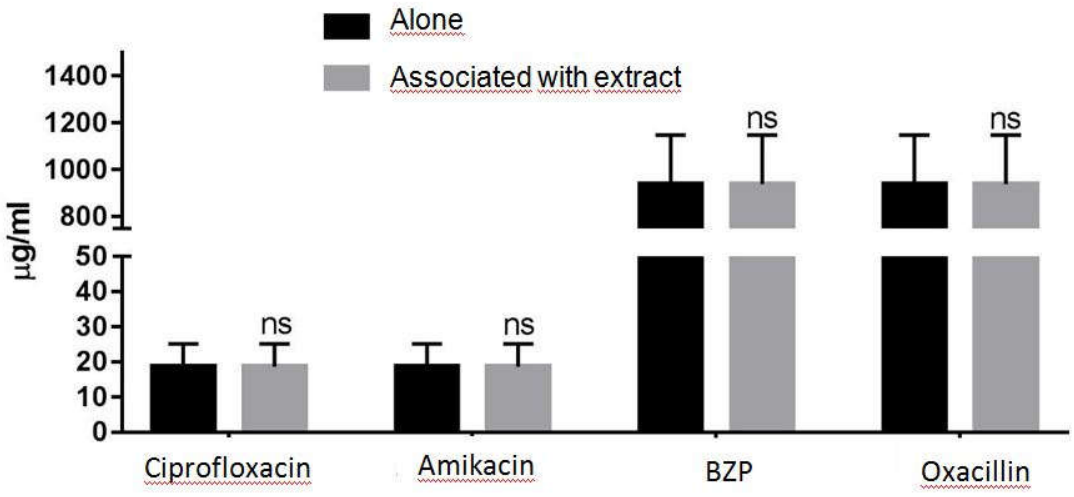
TESTED SUBSTANCE	MIC on *Pseudomonas aeruginosa*
Red Propolis Extract (RPE)	≥ 1024 µg/mL
Antibiotic - Benzylpenicillin	≥ 1024 µg/mL
Antibiotic - Oxacilin	≥ 1024 µg/mL
Antibiotic – Amikacin	16 µg/mL
Antibiotic - Ciprofloxacin	16 µg/mL

Source: From the research itself

Table 2 - MIC found for the antibiotics associated with the red propolis extract on *Pseudomonas* aeruginosa

Antibiotic + RPE	MIC on *Pseudomonas aeruginosa*
Benzylpenicillin	≥ 1024 µg/mL
Oxacilin	≥ 1024 µg/mL
Amikacin	16 µg/mL
Ciprofloxacin	16 µg/mL

Source: From the research itself



Source: research data.

Figure 1 - Comparative graph of the effect of the antibiotics used alone and in association with red propolis extract on *Pseudomonas aeruginosa.*

Minimum Inhibitory Concentration of Red Propolis Extract and Antibiotics on *Pseudomonas aeruginosa* (ATCC 27853): The results of the determination of the minimum inhibitory concentration of Paraíba's red propolis extract showed a MIC value ≥ 1024µg / mL (Table 1), for the bacterium tested - *Pseudomonas aeruginosa*, indicating that there was no clinically relevant antimicrobial activity in isolation. It was noted that the coloration of the contents of all wells, seen with the naked eye, became orange-red, noting the presence of bacterial growth in all wells at all concentrations, inclusive. This effect evidenced the inefficacy of the Paraíba's red propolis extract against the *Pseudomonas aeruginosa* strain. The results of the determination of the minimum inhibitory concentration of the antibiotics Amikacin and Ciprofloxacin revealed a MIC value of 16 µg / mL, respectively. The coloring visualized in all wells with the antibiotic was blue, except for the wells of the negative control, indicating that there was inhibition of bacterial growth or antimicrobial activity of said antibiotics against the strain *Pseudomonas aeruginosa*, (Table 1). However, when the benzylpenicillin and oxacillin antibiotics were tested on *Pseudomonas aeruginosa*, the color of the medium was changed from blue to red, indicating the presence of bacterial growth and absence of antimicrobial activity of the tested substances in all wells and concentrations, which makes it possible to assert that the MIC was ≥ 1024 µg / mL for these antibiotics (Table 1).

Modulation: The results of the modulation test showed that amikacin and ciprofloxacin, associated with the subinhibitory concentration of the red propolis extract - EPV (CIM / 8), didn't present synergism or antagonism to *Pseudomonas aeruginosa* (ATCC 27853). The result was identical to the antibiotics tested alone (Table 1 and 2). In all concentrations of antibiotics: 1) 1024, 2) 512, 3) 258, 4) 128.5) 64.6) 32.7) 16 and 8) CN (Negative Control) there was inhibition of bacterial growth, with the blue color in all concentrations, reinforcing the demonstrated action of the antibiotics on the bacterial strain *Pseudomonas aeruginosa.* The antibiotics benzylpenicillin and oxacillin, associated with the subinhibitory concentration of RPE, didn't show synergism, antagonism nor bactericidal effect, previously demonstrated when the antibiotics were tested alone (Table 1 and 2). The bacterium *Pseudomonas aeruginosa* resistance to these drugs was observed by changing the medium color from blue to red.

The results found and compared between the experiment with the antibiotics isolated and also associated with the RPE were relatively equal and there wasn't possibility of synergism or antagonism of the samples tested on the bacterial strain *Pseudomonas aeruginosa*. The results were determined based on the observation of the highest MIC found in both moments (tests with the antibiotic substance isolated and associated with RPE) and by means of a mean, since the tests were performed in triplicates (Figure 1).

DISCUSSION

Previous research showed that the Brazilian red propolis has a large number of phenolic compounds, especially flavonoids, which are associated with anti-bacterial activity in red propolis (CABRAL *et al*., 2009; FREIRES *et al*., 2016). In a study by Righi *et al*. (2011), the Brazilian red propolis extract showed antimicrobial activity against Gram-negative bacteria (*Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium and Escherichia coli*), showing inhibition of the growth of all tested microorganisms. The authors observed minimum inhibitory concentration (MIC) (256 µg / mL) and minimum microbicidal concentration (MMC) (512 µg / mL) against *Pseudomonas aeruginosa*. However, the extract showed the major MMC (1024µg / mL) against *Klebsiella pneumoniae.* Bispo-Junior *et al*. (2012) and Lopes *et al*. (2015), when analyzing the MIC of red propolis from the states of Alagoas, Sergipe and Paraíba, showed MIC values of 512 µg / mL against both *P. aeruginosa* strains (PA03 and PA24). Regueira Neto *et al*. (2017) investigated the effect of the dry and rainy period on the antibacterial activity, chemical composition and antibacterial action of Pernambuco red propolis alone and in association with standard drugs. The extracts were tested alone and in combination with antibiotics against *Escherichia coli and Pseudomonas aeruginosa.* The MIC values against *E. coli* varied from 128 µg / mL to 512 µg / mL (EC 06 and EC ATCC) and showed MIC values of 512 µg / mL against both strains of *P. aeruginosa* (PA03 and PA24). The tests revealed that the red propolis collected in the state of Pernambuco is effective against strains of *Escherichia coli* and *Pseudomonas aeruginosa.*

The studies described above state that the hydroethanolic extract of Brazilian red propolis has antibacterial activity against several microorganisms. However, they differ from the results of the present study, in which the antibacterial activity of the hydroalcoholic extract of Paraíba's red propolis on *Pseudomonas aeruginosa* wasn't demonstrated (ATCC27853). In this study, it wasn't possible to report the reasons why RPE didn't inhibit the growth of the bacterium *Pseudomonas aeruginosa* (ATCC27853). However, Nunes *et al*. (2009) assert that the chemical composition of propolis varies because of seasonality; bees visit shrubs and sub-shrubs during rainy season and woody species during drought. Factors associated with the extraction technique, different geographic origins, time of extraction of the resin, presence of contaminants can lead to variations in microbiological results among different research groups. In this study, tests had done in the antibiotics amikacin, ciprofloxacin, benzylpenicillin and oxacillin. In the first two antibiotics tested was observed inhibition of bacterial growth by blue color in the wells in all concentrations. In the other antibiotics there was bacterial growth evidenced by the alteration of the color of the medium from blue to red. In recent years, several studies have attempted to explain efficient substances or extracts that may overcome or reduce microbial resistance to antibiotics, thus generating a number of important information and several natural products have been shown to be effective for this purpose (JOUNG *et al*., 2012. Nonetheless, research on natural resistance modulators represents a new and important dimension in the problem of microbial resistance (Sibanda, Okoh, 2010). The hydroalcoholic extract of Paraíba's red propolis didn't present antibacterial activity on *Pseudomonas aeruginosa* (ATCC 27853). The inhibitory concentration of the red propolis extract doesn't have clinically relevant activity for *P. aeruginosa* (ATCC27853), because the MIC value ≥ 1024 µg / mL. The antibiotics amikacin and ciprofloxacin demonstrated antibacterial activity against this strain, in compensation the benzylpenicillin and oxacillin demonstrated the opposing. Finally, the extract of the Paraíba's red propolis associated with antibiotics didn't show synergism or antagonism. In this sense, it's necessary to do new research associated with the identification of the chemical composition of propolis that undergoes changes according to seasonal factors and factors associated with extraction technique, different geographic origins, time of resin extraction, presence of contaminants that may lead to variations in microbiological results among different research groups too.

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