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# Larvicidal Evaluation of *Aedes aegypti* and Antioxidant, Cytotoxic and Antimicrobial Potential of the Aqueous Acid Extract of *Pseudoxandra cuspidata* (ANNOACEAE).

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**Abstract:** The Anonnaceae family has 2,500 species and 135 genera, which has about 14 representatives of species in Brazil, including *Pseudoxandra cuspidata*. The objective of this research was to study the aqueous-acid extract of *P. cuspidata*, evaluating its antioxidant, larvicidal, antimicrobial and cytotoxic potential. In the preliminary phytochemical analysis, there were positivity to organicacids and alkaloids. Inhibitory Activity of EAA was 65% against DPPH. The AAE presented to *A. Salina* nauplii at LC50% of 600.79 µg/mL for a periodof 24 hours. In relation to larvicidal activity to *Aedes aegypti* intermediates, AAE presented the LC50% in 24 Hours of 475.91 ppm and 290.73 ppm in 48 Hours. Regarding the antimicrobial action, the EAA presented 50 mg/mL MIC for *P. Aeruginosa* and MBC at 100 mg/mL, and for *S. aureus* it presented MIC of 50 mg/mL and did not present MBC. This research characterized the presence of alkaloids and organicacids present in the aqueousacid extract of *P. cuspidata*. The extract presented lowtoxicity in relation to the microcrustacean *A. salina*. It also had a relevant antioxidant potential (65% of Inhibition), larvicidalaction considered effective, antimicrobialaction for both *P. Aeruginosa* and *S. Aureus* even did not present bacterici dalaction.

**Key words:** *Pseudoxandra*, DPPH, *Artemia salina*, *Aedes aegypti*.

## 1. Introduction

Nowadays, a large part of the world's population uses medicinal plants as an alternative medical resource to treat various diseases [1]. The World Health Organization (WHO) estimates that between 65% and 80% of the populations of developing countries currently use medicinal plants as medicines (WHO, 2011). In addition, the development of new products from natural sources should also be encouraged, because of about 300,000 species of plants that exist in the world only 15% have been pharmacologically studied. Researches to demonstrate the efficacy and importance of the use of medicinal plants are being carried out around the world [2, 3].

The Annonaceae family has approximately 2,500 species and 135 genera and can be divided into four subfamilies: Ambavioideae, Anaxagoreoideae, Annonoideae and Malmeoideae [4]. Most of these species have pharmacological action, such as antinociceptive activity due to their greater interaction with the central nervous system, demonstrated in rodents [5]. They are usually found in tropical areas of the American, African and Asian continents. About 900 species can be found in the neotropics (South America, Central America, part of Mexico and the

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Caribbean) [6, 7].

The genus *Pseudoxandra* belongs to the subfamily Malmeoideae, represented in Brazil by the Malmeeae tribe characterized by flowers with imbricated sepals, fruits with free carpids and an egg by carpel [4]. It has about 14 species in Brazil, most of them in the Amazon. They are trees with leaves with a prominent primary rib in the upper part, small articulated pedicel, besides two or more several bracts below the joint and none above. They also have globular buds, imbricated, rounded and concave petals, as well as pointed carpels with a marginal ovule and free and globular carpids, their seeds are flattened and have an equatorial groove [8]. There are few reports about its chemical constituents. Cortes et al. (1985-1986) [9, 10] isolated isoquinolinic alkaloids from the stem bark of two species of *Pseudoxandra*, *P. lucida* and *P. sclerocarpa*, both found in Colombia.

The species *Pseudoxandra cuspidata* Maas (1983) is commonly found in the state of Amapá [11]. It has a small number of reports on its chemical constituents and biological potential. In their essential oils, 39 constituents were identified, 53% are monoterpenes, 42% are sesquiterpenes, 1 alcohol and 1 phenylpropanoid [11]. Roumy (2008) [12] isolated and determined 4 constituents of *P. cuspidata* stem and tested its anti-plasmodic activity.

Researchers are looking for plant extracts with antioxidant substances that are justified by the fact that they fight free radicals, organic or inorganic molecules and atoms that contain one or more unpaired, highly unstable and reactive electrons, precursors of diseases such as arthritis, cataracts, cancer, diabetes, cerebral dysfunction, atherosclerosis, cardiac and neurological diseases [13, 14].

The toxicological bioassay with *Artemia saline* Leach is frequently used for the toxicity test of plant extracts. O microcrustaceo utilizado é cosmopolita de água salgada, possui reprodução simples, favorecendo seu uso em ensaios toxicológicos, serve como ensaio de triagem para outras ações biológicas como anticancerígena, inseticida, moluscicida e antifúngica [15].

Population control of *A. aegypti* is a challenge in developing countries, such as Brazil [16]. The mosquito *A. aegypti* (Linnaeus 1762) is the vector of arboviruses, such as dengue, chikungunya and zika [17].

*A. aegypti* develops through complete metamorphosis (holometabolia) through the egg, larvae (4 stages), pupa and adult stages [16, 18]. Mosquito control occurs mainly in the larval phases, when it is more vulnerable [16, 19].

The medicinal plants, such as *P. cuspidata*, are of scientific interest as a therapeutic alternative, especially against the framework of resistant microorganisms, due to the possibility of being used as phytopharmaceuticals [20]. The uncontrolled and increased use of antimicrobials leads to a process of artificial selection of mutant pathogenic microorganisms that are resistant to commonly used chemical compounds, making the use of natural antimicrobials an economical and effective alternative. Therefore, antibacterial properties of extracts of plants and/or substances isolated from plants are of relevant importance from the scientific and public health point of view [20].

The objective of this research was larvicidal evaluation against *A. aegypti* and the antioxidant, cytotoxic and antimicrobial potential of the aqueous-acid extract of *Pseudoxandra cuspidata*.

## 2. Materials and Methods

The vegetal species was collected in the municipality of PedraBranca, in the interior of the state of Amapá - Brazil (Latitude: 0.769468, Longitude: -51.9537). The exsicata of the vegetal species was cataloged by a specialist of the area, in the Herbarium of the Federal University of Amapá, registry number: 458.

The stem bark was conditioned in an oven at 45 ℃ for four days, then milled in a knife mill (model

TE-625). Then the total alkaloids were extracted in an acidic environment (EAA) following the methodology proposed by Simões (2010) [21]. During this method, the plant drug was sprayed and extracted directly using an acidic aqueous solution (1 mol/L HCl, 0.1 mol/L $H_3PO_4$). The alkaloids were precipitated as free bases by alkalinization of the ammonia solution and extracted with an immiscible organic solvent (petroleum ether).

Preliminary Phytochemical Prospecting:

The preliminary phytochemical prospecting was performed with the purpose of determining which organic groups were present in the EAA. It was based on chemical and physicochemical reactions resulting in color change, a formation of precipitates, formation of phase, among other characteristics. The tests were carried out according to the methodology proposed by Simões (2010) [21].

*2.1 Antioxidant Activity of EAA against DPPH Radical*

This analysis was performed according to the methodology proposed by Souza et al. (2007) [22] and Pitaro et al. (2012) [23] with modifications. A solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) 40 µg/mL and solutions with EAA at concentrations of 5; 2.5; 1.0; 0.75; 0.5 and 0.25 mg/mL in methanol. After 30 minutes of contact with the activity-inducing agent, the concentration of the monitored radical is observed by spectrophotometry in the visible region at $\lambda = 517$ nm. The absorbance measurements were taken in spectrophotometer Biospectro SP-22. The experiment was performed in triplicate and the mean absorption was analyzed for each concentration and for the positive control. The percentage of antioxidant activity was calculated according to Sousa et al. (2007) [22].

$(AA\%) = 100 - \{[(Abs_{sample} - Abs_{white}) \times 100] / Abs_{control}\}$

% AA = percentage of antioxidant activity

$Abs_{sample}$ = Sample Absorbance

$Abs_{white}$ = Absorbance of white

$Abs_{control}$ = Control Absorbance

*2.2 Preliminary Toxicological Test of EAA in A. salina*

For the evaluation of the cytotoxicity of AAE against *A. salina* Leach the methodology was based on the research of [24-26]. A solution of synthetic sea salt at a concentration of 3.5 g/L was used to incubate 25 mg of *A. saline* Leach cysts. Subsequently, the solution was exposed to artificial light for 24 hours for hatching and methane migration, then the larvae were separated into a dark environment (dark phase) for another 24 hours to reach the nauplii stage. The nauplii were divided into 6 groups with 10 individuals in each test tube. In each group, there were added aliquots of 2500, 1900, 1250, 625, 250, 125 µL of the AAE stock solution composed of 62.5 mg of the sample, 28 mL of synthetic sea salt and 2 mL of Tween 80 (5%), the volume was then added to 5 mL with artificial marine solution. The test was performed in triplicate, counting the number of survivors and considering dead individuals totally immobilized for a period of 10 seconds, to determine the LC50% (lethal concentration in 50%). The results were submitted to Probit analysis, with 95% confidence intervals.

*2.3 Larvicidal Activity of EAA of P. cuspidata*

The larvae of *Aedes aegypti* used for analysis are from the insectary Laboratory of Arthropods of the Federal University of Amapá, all generation F12, in the 3rd young stadium. Biological assays were performed under controlled conditions of luminosity, temperature, and humidity. The methodology used followed the Standard Protocol of the World Health Organization (2009) [27] with adaptations. The tests were performed at concentrations: 500, 400, 300, 200 and 100 ppm of the AAE of *P. cuspidata*. A stock solution of 465 mg of AAE was solubilized in Tween 80 and dissolved in 93 mL of water, to obtain the concentration of 5000 ppm. The solution was diluted to obtain the concentrations of 500, 400, 300, 200 and 100 ppm. Ten larvae of *A. aegypti* were used in each aliquot. After 24 and 48 hours, the dead larvae were counted, being considered dead all those that did not

move after agitation during a period of 1 minute. Data obtained from mortality (%) x concentration (ppm) were analyzed by the SPSS ® program in Probit analysis, to determine the CL50%. The variation between the means in the biological assays was evaluated through the tests of variance (ANOVA), with 95% confidence intervals.

*2.4 Antimicrobial Activity of EAA of P. cuspidata*

For the evaluation of the antimicrobial activity, the microorganisms *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538P) were used. The results were expressed as Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). MIC was determined by the microplate dilution technique (96 holes) according to the methodology described under the M7-A10 standard of Manual 38 of the CLSI (2015) [28] with adaptations. The analyses were performed in triplicate. In each well was added 100 µL of Mueller-Hinton broth. Then, 50 µL of the respective extract with known concentration was added to the first well, and a serial dilution of well A1 to well A12 was performed successively. Subsequently, 50 µL of the inoculum was added in the wells, the inoculum presented turbidity 0.5, which on the McFarland nephelometric scale is equivalent to 1.5 x $10^8$ CFU.mL$^{-1}$. As a positive control, amoxicillin (50 mg/mL) was used. For the determination of MIC, the extract was diluted in Dimethylsulfoxide (2% DMSO). Each well of the plate was initially filled with 50 µL of 0.9% NaCl, except for the first column, which was filled with 100 µL of the extract at the concentration of 200 mg.mL$^{-1}$. Subsequently, two base serial dilutions were performed in the ratio of 1:2 to 1:128 dilutions in a final volume of 50 µL. After this procedure, 50 µL of cells (1.5 x $10^8$ CFU.mL$^{-1}$) adjusted was added to each well, resulting in a final volume of 100 µL. There were performed the control of the culture environment, the turbidity of extract control, a negative control (2% DMSO) and positive control using amoxicillin (50 µL.mL$^{-1}$).

The reading was performed on an Elisa microplate reader after 24 h with the absorbances measured at 630 nm. For the determination of the MBC, the sample was plated at concentrations that did not show turbidity in the MIC (4 concentrations) with the aid of a sterile bacteriological loop. The plates were incubated at 35 ℃ ± 2 for 24 h, this being considered MBC the lowest concentration in which there was no bacterial growth.

## 3. Results and Discussion

In the preliminary phytochemical analysis, there was positivity for two organic groups: organic acids and Alkaloids. Previous studies have reported the presence of these organic groups in the phytochemical constitution of *P. cuspidate* [10]. The extractive method was selective and specific, so it should be used as a standard for the extraction of alkaloids from the species (table 1).

The DPPH has free radical scavenging activity based on the transfer of electrons, justifying its use during the test as a control for the determination of antioxidant action [11].

On the antioxidant activity, it was observed that in the highest concentration (5 mg/mL) the sequestering activity was inhibited around 65%, it is an acceptable result due to the presence of alkaloids and groups of organic acids known to not demonstrate strong antioxidant action.

The *A. salina* nauplii toxicity test is based on the number of deaths (mortality rate) tested at different

**Table 1 Shows the percentages of antioxidant activity for *P. cuspidate* AAE.**

| Concentration (mg/mL) | Antioxidant activity (%) |
|---|---|
| 5 | 64.269 ±0.9 [a] |
| 2.5 | 51.994 ±3.03 [b] |
| 1 | 37.975 ±1.85 [c] |
| 0.75 | 34.623 ±1.08 [d] |
| 0.5 | 30.751 ±2.05 [d] |
| 0.25 | 25.147 ±2.19 [e] |

Vertically, values of (% AA) followed by the same letter do not present significant differences for Anova ($p < 0.05$) and tukey.

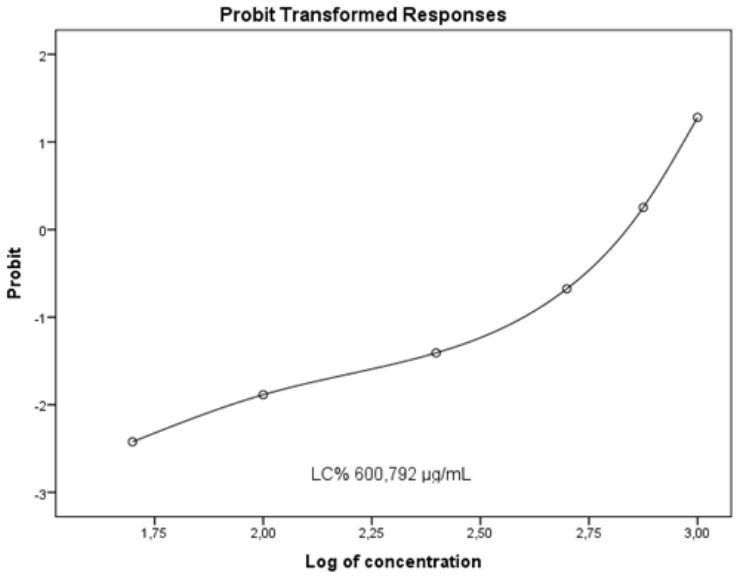



**Fig. 1 Probit responses for% EAA mortality against *A. salina*.**

concentrations. The cytotoxicity of *P. cuspidate* AAE in *A. salina* obtained LC50% of 600.79 µg/mL over a 24 hour period under controlled conditions. Mortality averages were analyzed in Probit in the SPSS® program, and the means submitted to analysis by variance (ANOVA) showed significant differences, where p-value < 0.05. In Figure1 Probit, it is possible to observe the growth of mortality due to concentration.

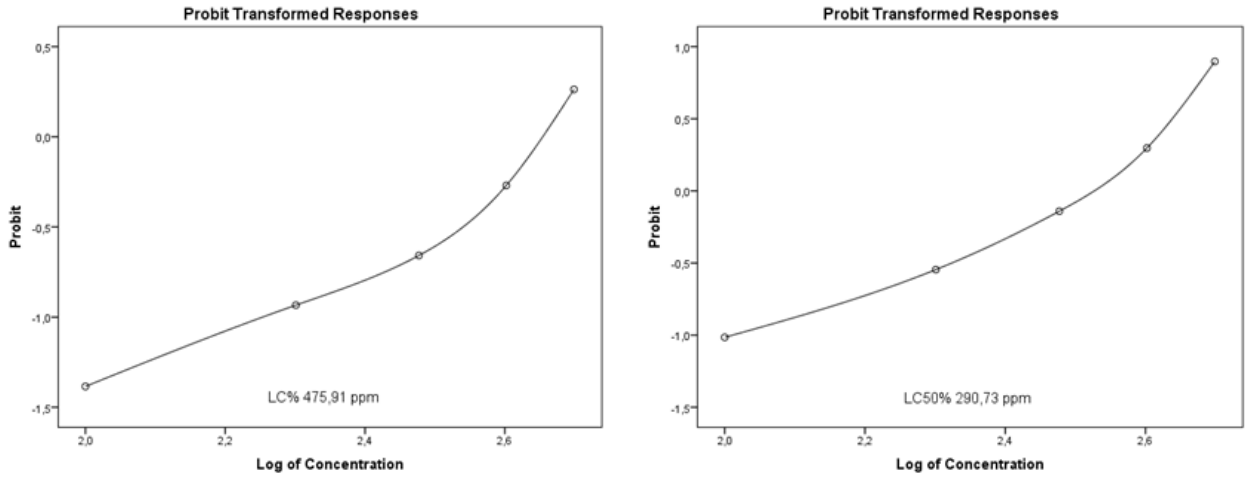
Based on the parameters established by Amarante et al. (2011) [28], where a relation of a degree of toxicity with the LC50% is made. The LC50% of low toxicity value is greater than or equal to 500 µg/mL; moderate toxicity the value should be between 500 µg/mL and 100 µg/mL and high toxicity is attributed to values less than 100 µg/mL.

The LC50% for the AAE characterized the extract with the fraction of total alkaloids with low toxicity (greater than 500 µg/mL), and this demonstrated that it does not represent an imminent health risk, and should only have specialized management (by professionals) for their preparation in "garrafadas" or medicinal teas. In the long term, they should be performed to control the dose-response of the AAE avoiding cases of intoxication or misuse.

The chemical control of the vectors is as important as the vaccines and drug treatments, acting mainly in the prevention of endemic arboviruses in Brazil [16]. Insecticides of organic or inorganic origin are used as vectors control in Public Health [29, 30]. Substances with larvicidal action in immature *A. aegypti* become economically important, as they may be a low-cost alternative for the population, or they may be benefited and/or incorporated into pharmaceutical forms leading to the generation of new products [16].

In relation to the larvicidal action against *A. aegypti* L3 larvae, it was carried out under controlled conditions. The AAE of *P. cuspidata* obtained an LC50% in 24 Hours of 475.91 ppm and 290.73 ppm in 48 Hours, characterizing this extract as a potential biocide. The behavior of the percentage of mortality of the larvae in relation to the concentrations used in the test can be observed in Figure 2A and 2B.

Following the methodology, the mortality of the extract was analyzed after 24 hours, precisely to determine if the action could be considered acute or residual. It is possible that with the increase of time there is higher mortality, which was observed during the test (Figure 2B) because the LC50% was lower (290.73 ppm).




(a) (b)

**Fig. 2 Respostas em Probit para % mortalidade do AAE frente *A. aegypti* após 24 Horas.**

According to Komalamisra et al. (2005) [31], they classified the larvicidal activity of medicinal plant extracts as effective, moderate or high, depending on the value of LC50% of the extract. Larvicidal activity is considered effective when LC50% of the extract is less than 750 ppm, moderate when between 50-100 ppm and high activity when a value less than 50 ppm. In this sense, the AAE was effective in periods of 24 and 48 hours of contact with the immature ones of *A. aegypti*.

Monitoring and management of resistance and the use of substances with modes of action distinct from conventional chemical insecticides are of paramount importance for any vector control program. Therefore, the greater the number of plant species or synthetic substances with insecticidal action and great variability of them, the greater and better the vector combat. Characterizing the rational use of insecticides, considering the different components of the integrated control [32].

On the antimicrobial action performed to define the MIC and MBC of the *P. cuspidata* AAE against the *S. aureus* and *P. aeruginosa* microorganisms, it was observed that the dilutions of the AAE presented significant differences in relation to the standard antibiotic positive) inhibition of *S. aureus* growth, with the Minimum Inhibitory Concentration being 25 mg/mL. In Figure 3, inhibition of the major concentrations of AAE in the microtiter plate against *S. aureus* is demonstrated.

Regarding the antimicrobial test of EAA against *P. aeruginosa*, it was observed that there was a variation of inhibition (Figure 4); it is considered the lowest inhibitory concentration of the EAA against *P. aeruginosa* at 3.125 $mg.mL^{-1}$.

On the Minimum Bactericidal Concentration (MBC), it was observed that the *S. aureus* AAE did not present MBC at any of the concentrations tested. However, there is a bacterial effect against *P. aeruginosa* at 100 $mg.mL^{-1}$ concentration.

The AAE of *P. cuspidata* inhibited the bacterial growth of *S. aureus* at 6.25 $mg.mL^{-1}$ concentration. This action is characterized as bacteriostasis, from which the host organism itself through phagocytosis or production of antibodies destroys the invasive microorganism [32]. However, AAE had no concentration in which the lysis of *S. aureus* gram-positive bacteria at the concentrations tested could be related to the morphological difference of the bacterial cell wall, changing its specificity to a binding site.

The AAE of *P. cuspidata* versus *P. aeruginosa* presented MIC at 3,125 $mg.mL^{-1}$ and presented MBC at 100 $mg.mL^{-1}$. Then this presented bactericidal and

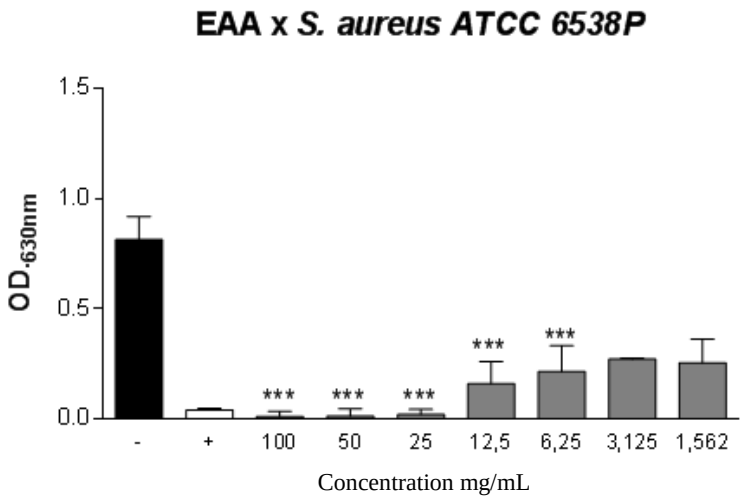



**Fig. 3 AAE sensitivity test against *S. aureus*.**

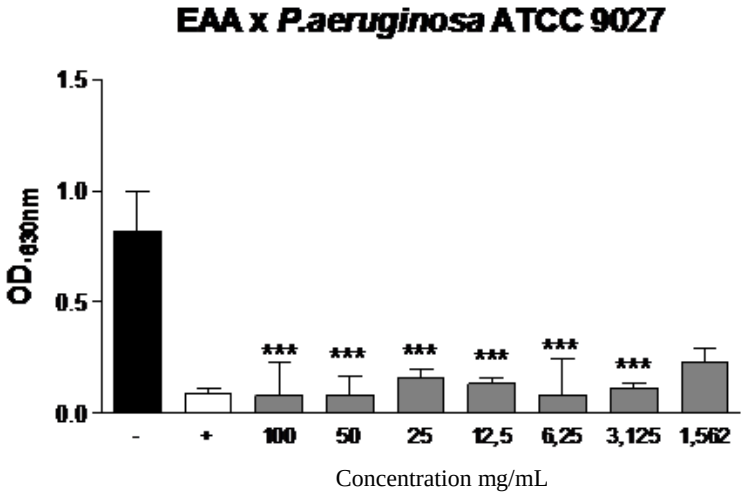



**Fig. 4 AAE sensitivity test against *P. aeruginosa*.**

bacteriostatic action. The bactericidal action can occur through different mechanisms of action, such as inhibition of cell wall synthesis, inhibition of protein production, inhibition of nucleic acid replication (DNA and RNA) and transcription, plasma membrane damage and/or inhibition of synthesis of essential metabolites [33].

Another plant species of the family Annonaceae (*Guatteria elliptica* R. E. Fries) that is rich in alkaloids, demonstrated antimicrobial activity by the turbidimetric method [34]. Therefore, even if this organic group is not known for the antimicrobial action, it is possible that there are species-rich in alkaloids and with this activity. Therefore, in later studies, the alkaloids and organic acids present in the AAE of *P. cuspidata* should be qualified and quantified and new possible biological activities evaluated.

## 4. Conclusions

Through this research, the presence of alkaloids and organic acids present in the aqueous acid extract of *P. cuspidate* was characterized. The extract had low toxicity in relation to the microcrustacean *A. salina*, it also presented a relevant antioxidant potential (65% of Inhibition), larvicidal action considered effective,

bacteriostatic action for both *P. aeruginosa* and *S. aureus*, and bactericidal effect against *P. aeruginosa*.

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