Evaluation of phytochemical composition, toxicity in *Drosophila melanogaster* and effects on antibiotics modulation of *Plathymenia reticulata* Benth extract

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**ABSTRACT**

Bacterial resistance is interfering with the action of antibiotics for clinical use in treating pathologies. The search for new substances capable of combating this resistance is necessary. An alternative to the search for these substances is in the extract of medicinal plants. *Plathymenia reticulata*, plant of the Fabaceae family, is a common tree species from the Brazilian cerrado, and is commonly used in areas of environmental degradation. This species is rich in phenolic compounds, such as flavonoids and tannins, compounds that are associated with various biological effects. A hydroethanolic extract from the bark of *Plathymenia reticulata* (HEPrB) was produced and then tests were carried out to verify the direct antibacterial activity, the modulatory effect of antibiotics for clinical use and their toxicity in *Drosophila melanogaster* flies. Through the analysis with UPLC, a wide variety of flavonoids contained in the HEPrB was observed. Direct antibacterial activity was observed for the standard strain of *Staphylococcus aureus*, however, the extract showed antagonistic activity or no significance in relation to the antibiotics tested in this study. As for toxicity, the HEPrB did not show significant damage in the proposed model. The results emphasize care when associating the consumption of teas with treatments with antibiotics for clinical use.

**1. Introduction**

The antibiotic therapy brings na effective effect against bacterial infections, controlling bacterial diseases previously considered without cure. However, due the uncontrolled usage of these drugs, the surveillance of drug resistant bacteria becomes a worldwide problem of public health [1].

These resistance can be due diferente mechanisms as target modified structures, inactivation of the antibiotic action and others, and all of these mechanisms can be transmitted to other bacteria by horizontal gene transference [2].

Bacterial resistance prevents many antibiotics for clinical use from being effective in treating infections, and the search for new alternatives for antibacterial treatments is necessary [3]. By this fact, the search for new antibiotics and ajuvants for these drugs is very importante to face the bacterial resistance to drugs [4].

Based in this situation, the studies seeking secondary metabolites from plants to combat the bacterial resistance to antibiotics as na adjuvante or as na antibacterial agente with direct action are more and more importante [5–10]. Therefore, the use of medicinal plants as a...
source of these new alternatives is emphasized, which may be a promising approach [11].

Plathymenia reticulata Bentham, from the Fabaceae family, is popularly known as vinhaticana, has high economic value due to the quality of the wood, as well as it is used for the recovery of degraded areas [12]. This species is common in the cerrado and is rich in phenolic compounds, such as tannins and flavonoids [13]. Studies point out extracts of Plathymenia reticulata associated with glycemic control and cholesterol [13], protection against mercury toxicity [14] and against local inflammations caused by the Jararaca (Bothrops atrox) poison [15].

This study tested the hydroethanolic extract of the Plathymenia reticulata bark (HEPRB) for direct antibacterial activity, in standard strains (Staphylococcus aureus 25923, Pseudomonas aeruginosa 9027 and Escherichia coli 25922) and in multidrug-resistant strains (Staphylococcus aureus 10, Pseudomonas aeruginosa 03 and Escherichia coli 06), its modulatory activity associated with antibiotics for clinical use and toxicity in tests with Drosophila melanogaster flies.

2. Material and methods

2.1. Collection and preparation of botanical material

The bark of Plathymenia reticulata were collected in Serra do Boqueirão, in a private property, in an amazing state of preservation, in the municipality of Lavras da Mangabeira-CE, according to the coordinates 6° 72′24″S; 38° 97′73″W, at an elevation that varies between 282 and 401 m above sea level. An extract from this plant was deposited at the Herbarium Caririense Dárdano de Andrade Lima (HCDAL), Universidade Regional do Cariri - URCA, under the number 13,692.

The collected material was cut into pieces of ±10 cm and immersed in 70 % ethyl alcohol for 72 h, in the proportion of 10 mL of alcohol to 1 g of crushed skin [16]. After filtration, the liquid obtained was mixed with water in a 1:1 ratio. This procedure was necessary for lyophilization of the extract in a Mini-spray dryer MSDI 1.0 (Labmaq do Brasil), with a 1.2 mm spray nozzle.

2.2. Plant extract and antibiotics for clinical use

The concentration used in all products in this clinical trial was equivalent to 1,024 μg / mL. To obtain these solutions, 10 mg of each sample was weighed and diluted in 0.5 mL of Dimethylsulfoxide (DMSO) to guarantee complete dilution. Subsequently, the resulting solutions were diluted in distilled water until the desired concentration was obtained.

2.3. UPLC-QTOF-MS / MS analysis

The instrumental analysis of the filtered samples of the Plathymenia reticulata extract was performed on an ACQUITY UPLC BEH column (150 × 2.1 mm, 1.7 hm, Waters Co.) in a Waters Acuity UPLC system coupled to a Q-TOF Premier mass spectrometer (Waters MS Technologies, Manchester, United Kingdom). MS data were collected for m/z values in the range of 110–1180 Da. The exact mass and molecular formula assignments were obtained using Masslynx 4.1 software (Waters MS Technologies).

2.4. Bacterial strains

In the tests, multi-resistant strains of Staphylococcus aureus 10, Pseudomonas aeruginosa 03 and Escherichia coli 06 were used; and standard strains of Staphylococcus aureus 25923, Pseudomonas aeruginosa 9027 and Escherichia coli 25922, all cultivated at the Microbiology and Molecular Biology Laboratory (LMMB) of the Regional University of Cariri (URCA).

According to the resistance profile, all multiresistant strains used in this test are resistant to ciprofloxacin and levofloxacin. The strain of Staphylococcus aureus 10 is also resistant to cephalixin.

2.5. Direct antibacterial activity test

In this test, the methodology proposed by Javadpour et al. [17] was adapted to be used in broth microdilution tests. The bacterial inoculants were grown on HIA (Heart Infusion Agar), growing for 24 h. A strain drag was diluted in 0.9 % Saline (SF), contained in a test tube, with turbidity measured according to the McFarland 0.5 scale (1.5 × 10^9 UFC/mL). A BHI (Brain Heart Infusion Broth) solution and 10 % of the bacterial inoculum was distributed in eppendorfs microtubes, in a total volume of 1 mL, to be used in the test. This procedure was performed in triplicate for each strain tested.

The BHI solution and inoculum were distributed in 96-well plates, and subsequently microdiluted in HEPRB at a ratio of 1:1. HEPRB concentrations in the wells decline by half the previous well, ranging from 512 μg/mL to 8 μg/mL. After 24 h of incubation in a bacteriology oven at a temperature of 37 °C, the reading of the Minimum Inhibitory Concentration (MIC) was performed using the colorimetric method, using Resazurin (400 μg/mL solution for use (5.33 μg/mL by well).

2.6. Antibiotic action modification test

For this test, eppendorfs microtubes, in a total volume of 1.5 mL, were prepared as described in the previous subchapter. However, the HEPRB was added to the final solution at a sub-inhibitory concentration (CIM/8), according to the methodology described by Coutinho et al. [18]. Serial microdilution was performed using antibiotics for clinical use, and the concentration dropped from 512 μg/mL to 0.5 μg/mL.

In order to try to infer a mechanism of action for the HEPRB, a control was added, in sub-inhibitory concentrations, with the Efflux Pump Standard Inhibitor (EPSI) Clopomazine (CPZ).

2.7. Toxicity test on Drosophila melanogaster

The toxicity test was carried out by exposing flies to the HEPRB in two tests: Mortality test, according to the model proposed by Cunha et al. [19], and the negative geotaxis test, proposed by Coulom & Birman (2004).

In the mortality test, twenty adult flies, four days old, were isolated in pots containing filter paper soaked in a solution of sucrose, extract and distilled water. Concentrations of 50, 25, 10 μg / mL and a 20 % sucrose control were used. This test was repeated in six times for each concentration. Then, mortality readings were taken at time intervals of 3, 6, 9, 12, 24, 36 and 48 h. 12 h light / dark cycle and temperature controlled at 25 °C were respected during the tests.

For the negative geotaxis test, the glass tube in which the flies were contained was tapped so that the flies were positioned at the bottom of the tube. Then the number of flies that were able to climb up to the edges to a height of 4 cm was counted. The reading was performed twice for each tube, following the same time intervals as the mortality test.

2.8. Statistical analysis

In microbiological tests, the triplicate values were used to determine the central values, using geometric means. The central data for the survival test in the Drosophila melanogaster model were obtained as an arithmetic mean of the values in each glass tube (n), by concentration and measured hours. For the negative geotaxis tests, an arithmetic average of the two consecutive observations was performed in each glass tube, this being the value of “n”. As the tests were performed in sextuplicate, an average with all "n" was obtained with their respective standard deviations, per hour and measured concentrations. These were the central data.

The central data were submitted to a two-way ANOVA with post hoc Bonferroni test (p < 0.05) and analyzed using the statistical program...
GraphPad Prisma 5.0.

3. Results and discussion

3.1. UPLC-QTOF-MS/MS analysis

In the identification by UPLC–ESI-QTOF-MS/MS of the HEPrB, sixteen chemical substances were found, as shown in Fig. 1 and Table 1.

Of the sixteen substances found, eight are flavonoids (1,2,5,7,8,14,15,16) and an anthocyanin (3). Study by Wang et al. [23] points out that the extract of Moringa oleifera, rich in flavonoid glycosides of quercetin and kaempferol, has high antioxidant activity, while Zhang et al. [24] observed the correlation between antioxidant activity and concentrations of anthocyanins, flavonoids and polyphenols found in three colors of onions (Allium cepa L.). Such properties are described in the literature for the flavonoid Kaempferol (14), which besides the mentioned therapeutic property, still demonstrates anti-cancer and anti-inflammatory activities [25].

In the composition of the HEPrB, two fatty acids (9,10) and another phenolic compound from the family of stilbenes (11) were also identified. Adili et al. [26] point out the importance of omega-3 and omega-6 fatty acids in platelet regulation and combating cardiovascular diseases. The octadecadienoic acid (linoleic) found in the composition of this extract is implicated in chronic inflammatory processes and obesity [27], however, salts of this acid (Linoleic) are related to the decrease in the skin microbiota, producing a protective effect against several species of the genus Staphylococcus [28].

Reinheimer et al. [29], when investigating the action of synthetic isopropylstilbene derivatives on strains of M. tuberculosis ATCC 27294 and clinical isolates of multidrug-resistant M. tuberculosis, pointed out the potential of synthetic stilbene derivatives as drugs for the treatment of tuberculosis. Regarding the stilbene derivative, Resveratrol (11), the literature points out the antioxidant [30], anticancer [31], cardioprotective [32] and its viability for treatment of Alzheimer’s disease [33]. In another study, Resveratrol was able to cause damage to the Salmonella typhimurium cell membrane, showing an antibacterial effect [34].

Procyanidin dimers produced from the rhizome of Bistorta officinalis showed pro-inflammatory effects and reasonable antibacterial activity, with MICs ranging from 31.25 μg/mL to >500 μg/mL on Gram-positive strains of Staphylococcus aureus and Staphylococcus epidermidis [35].

3.2. Direct antibacterial activity

The Minimum Inhibitory concentration values (MIC) for the antibacterial activity of HEPrB are shown in Table 2.

The HEPrB showed antibacterial activity for the standard strain of Staphylococcus aureus, with an MIC equal to 256 μg/mL. In the other
strains, MIC was ≥ 1024 μg/mL, being therefore considered insignificant for in vivo effects, that is, irrelevant for clinical use [36]. Although the result for the *Staphylococcus aureus* strain has a value within a clinically acceptable MIC, for the extract to have a promising clinical importance, this value should be below 100 μg / mL [37].

Studies by Fernandes et al. [38] point out the antibacterial activity of *Plathymenia reticulata* for most Gram-positive bacteria using concentrations of 625 μg/mL, as well as the inability to inhibit some gram-negative bacteria tested by the authors. Ramos et al. [39] observed the activity of *Plathymenia foliolosa* against *Mycobacterium tuberculosis* and *Mycobacterium kansasii*, with MICs between 0.78 μg/mL and 100 μg / mL, respectively.

The anti-*Staphylococcus* effect of natural products is known in the literature, corresponding with the interaction of the natural products with the cell membrane, altering the electric potential and the structure of this membrane [40,41].

Decoction, infusion and hydroalcoholic extract rich in flavonoids, some also present in the composition of HEPb (Quercitin hexose, Kaempferol and Luteolin-O-hexoside), produced from leaves of *Thymus vulgaris* L., were tested for their antioxidant and antibacterial capacity, being verified a high antioxidant power from the DPPH test, as well as a direct antibacterial action on Gram-positive and Gram-negative strains [42]. Partially similar results were obtained by Bitsi et al. [43], when testing different extracts of Rosa sempervirens L. in strains of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. In the study in question, the extracts had a high content of flavonoids and other phenolic compounds, exhibiting potent antioxidant activity, in addition to not showing antibacterial action against any strain of the study.

Direct antibacterial activity of extracts with a high amount of phenolic compounds was correlated to the content of total phenolic compounds, as well as to the content of their non-flavonoid fraction. Greater effectiveness of these extracts on Gram-positive strains has also been reported [44]. These facts corroborate with the results presented so far, with the divergences possibly resulting from the total phenolic composition, the flavonoid composition and the non-flavonoid content.

### 3.3. Antibiotic action modifying effect

The modulating effect of the antibiotic activity of HEPb with Ciprolfloxacin and Levofloxacin can be seen in Fig. 2.

Chlorpromazine is a standard efflux pump (EP) inhibitor, widely used in similar experiments to detect this resistance mechanism [45,46]. The results demonstrate an inhibition of EP when the standard inhibitor is associated with the antibiotic, resulting in a MIC less than that of the control, thus proving the presence of EP in the three strains tested for ciprofloxacin, as can be seen in Fig. 2A. When the associated antibiotic is replaced by Levofloxacin (Fig. 2B), we find an absence of EP. The values are informed in the Table 3.

In both situations, the HEPb presents either antagonistic action or no interaction on the effect of the antibiotic, visualized through a MIC higher than the control or without statistical significance. We can infer that the extract is not able to harm EP and act as a mechanism for altering antibiotic resistance.

HEPb also impaired the action of antibiotics even though there is no specified mechanism of resistance. Thus, the antagonisms presented lead us to suspect that the extract was not only unable to inhibit the resistance mechanisms, but also impaired some mechanism of action of the antibiotic.

Studies suggest that in addition to the specific mechanism of each antibiotic, its pro-oxidant effect is capable of increasing its effectiveness against microorganisms [47,48] and that the presence of antioxidant substances in the environment interferes in this pro-oxidative effect of the antibiotic, its pro-oxidant effect is capable of increasing its effectiveness against microorganisms [47,48].
A high rate of phenolic compounds in an extract is indicative of its high antioxidant capacity [49]. In this context, the numerous presence of phenolic compounds and flavonoids in HEPrB may have impaired the action of antibiotics during the modulation test, as it affects their pro-oxidative effect, thus explaining antagonisms.

Another hypothesis for antagonisms would be the large number of interactions between the various compounds of the extract with each other and with the antibiotic, disturbing the structure of the antibiotic, and with it, its action.

Bark extract of *Ziziphus joazeiro* Mart., Which composition had numerous flavonoid compounds, was tested against 3 g-positive strains (*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis*), presenting 8 antagonistic results out of 9 possible [8]. On the other hand, a study with isolated flavonoids derived from plants demonstrated a high modulatory capacity on the *S. aureus* strain. Some flavonoids were unable to effectively modulate the antibiotic used due to structural differences [51].

The fact that there is a difference between the flavonoids found in the HEPrB and the studies in question may explain the different results found, as well as the interaction of numerous compounds in the extract may end to interfere in a promising action of a compound that alone would have an interesting action, explaining the lack of bioactivity of the extract on the strains.

### 3.4. HEPrB toxicity test

The toxicity test on *Drosophila melanogaster* flies is a viable model for the verification of toxic activities of substances [52,53]. Toxicity is verified through mortality and the effect of the extract on the flies’ locomotor system. Mortality is verified according to the number of fly deaths when they are in contact with different concentrations of the extract, while the deficiency under the locomotor system is indicative of a possible disabling injury.

![Fig. 2. Effect of the antibiotic modifying action of HEPrB on Gram-positive and Gram-negative strains.](image-url)

Combined action of the extract with the antibiotic Ciprofloxacin (A); Combined action of the extract with the antibiotic Levofloxacin (B); CPMZ = Chlorpromazine; HEPrB = Hydroethanolic Extract of *Plathymenia reticulata* Bark; * = Represents the statistical significance of a bar when compared to antibiotic control.

### Table 3

MIC values of the substances and drugs assayed alone and in association.

|                | MIC (μg/mL) |          |          |          |
|----------------|------------|----------|----------|----------|
|                |            | SA 10    | EC 06    | PA 24    |
| Ciprofloxacin  | + CPMZ     | 16 (4)   | 3.17 (2) | 2.51 (1.6) |
|                | + HEPrB    | 101.59 (1.6) | 64 (10.1) | 80.63 (20.2) |
| Levofloxacin   | + CPMZ     | 16 (5)   | 5.04 (2) | 2.52 (1.3) |
|                | + HEPrB    | 5.04 (1.6) | 12.7 (5)  | 4 (1.3)   |

CPMZ: Chlorpromazine; HEPrB: hydroethanolic extract of the *Plathymenia reticulata* bark; SA: *Staphylococcus aureus*; EC: *Escherichia coli*; PA: *Pseudomonas aeruginosa*; Between the parenthesis is demonstrated the the times reduction or enhanced observed.
The results obtained on the mortality and damage to the locomotor apparatus of *D. melanogaster* flies caused by the action of the HEPrB are contained in Fig. 3.

In the mortality test, the extract had no potential to kill flies. It is unlikely that the death that occurred after 24 h was due to the HEPrB. As for the negative geotaxis test, damage to the locomotor system was observed after 36 h of exposure to the extract in the highest concentration produced (50 μg/mL), corroborating the low mortality. From the results presented, we can infer that the tested extract does not present high toxicity.

Tests on the toxicity and analgesic activity of the flavonoids of *Achillea wilhelmsii* and *Teucrium stocksianum* in mice, showed the safety of the action of the flavonoids in doses of up to 100 mg / kg, with prominent lethality in doses of 1,000 mg/kg [54].

Cunha et al. [55], when investigating the cytoprotective effect of *Eugenia uniflora* L. extract against mercury chloride contamination, observed that the extract that had phenols and flavonoids in its composition (1.38 % quercetin, 1.17 % of quecitrin and 1.16 % of ellagic acid) presented low toxicity when tested in *Drosophila melanogaster*.

Extracts of *Cassia fistula* Linn. Demonstrated the presence of phenolic compounds and presented an antibacterial effect against strains of *S. typhosa* and *S. dysenteriae*, with a low in vivo toxicity [56], as demonstrated by our study. Welu et al. [57], working with extracts from banana, demonstrated a correlation between a higher polarity of the solvent and higher toxicity against *Artemia salina* and antibacterial activity. This effect about the polarity of the solvent reinforce our results and putative effect of flavonoids in the observed activities against the antibiotic activity and in the low toxicity using the *Drosophila melanogaster* model.

4. Conclusion

HEPrB presented in its composition numerous phenolic compounds, mostly flavonoids. Some of these flavonoids, in isolation, have numerous biological activities, highlighting the antioxidant activity, characteristic of this group of compounds. Antibacterial activity is also reported in the scientific literature.

HEPrB showed direct antibacterial activity only for the standard strain of *Staphylococcus aureus*, however it did not show any activity to modify bacterial resistance. A probable explanation would be the interference of the extract in some mechanism of action of the antibiotic, notably in the pro-oxidative mechanism, caused by the antioxidant nature of this class of compounds.

The extract also showed low toxicity according to the proposed model.

The result of this research shows the care in relation to the use of teas during treatment with antibiotics, a common activity in popular medicine, since plant extracts when associated with antibiotics for clinical use, can harm the action of the medication.

Author statement

E.M. Luna – Methodology.
T.S. Freitas – Validation.
F.F. Campina – Methodology.
M.S. Costa – Methodology.
J.E. Rocha – Validation.
R.P. Cruz – Validation.
D.L.S. Júnior – Software.
Z.S. Silveira – Software.
N.S. Macedo – Resources.
J.C.P. Andrade – Resources.
F.N. Pereira Júnior – Resources.
M.A.N. Lisboa – First draft of the manuscript.
G.V. Cruz – First draft of the manuscript.
J.T. Calixto Júnior - supervision; Final draft of the manuscript.
A.M.R. Teixeira – supervision; Final draft of the manuscript.
H.D.M. Coutinho – coordination of the project.

Fig. 3. HEPrB toxicity on *Drosophila melanogaster* flies.
Mortality caused by HEPrB on *Drosophila melanogaster* (A) flies; Damage caused by the HEPrB to the locomotor apparatus of the flies (B); * = Represents the statistical significance of a bar when compared to antibiotic control.
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