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Cytoprotective effect against mercury chloride and bioinsecticidal activity of *Eugenia jambolana* Lam.

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Abstract The fruit fly *Drosophila melanogaster* is often utilized in genetic research, and in the last decades, it has become one of best organisms for studies of human diseases and toxicological research. Mercury chloride ($HgCl_2$), the main representative of mercury compounds, is the target of numerous investigations, not only because of its intrinsic toxicity but also because it accounts for the toxicity of elemental mercury since the latter is converted to Hg^{+2} by oxidation. *Eugenia jambolana* Lam. Myrtaceae, known in Brazil as "jambolão", is of great interest because of its medicinal applications, especially its leaves and fruits. The aim of this work was to characterize, by CG–MS, the chemical constituents of the essential oil of *Eugenia jambolana* and to evaluate its bioinsecticidal action in the *Drosophila melanogaster* model, as well as to determine the cytoprotective and chelating effect of the extract of *E. jambolana*. The results obtained here point to the

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potential of essential oils as a source in biological prospecting for bioinsecticides. Because of their biodegradability, essential oils can be important tools in the biological control of pests. The results demonstrated that the extract has an allelopathic effect on lettuce seeds and that its interaction with mercury chloride allows a greater growth of the radicle and plumule of *Lactuta sativa* seedlings, showing that this plant can provide an alternative solution to the problem of contamination by heavy metals, besides having cytoprotective potential and moderate chelating activity.

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1. Introduction

The family Myrtaceae has a wide distribution, occurring preferentially in tropical and subtropical regions, and it is considered one of the most important families of Brazilian flora due to the extensive occurrence of species that are eaten and/or used in traditional medicine. *Eugenia jambolana* Lam., Myrtaceae, known in Brazil as ''jambolão'', is of great interest for medicinal applications, where its leaves and fruits are especially used in the treatment of diabetes (Pepato et al., 2005).

The fruit fly *Drosophila melanogaster* is often utilized in genetic studies, and in the last decades, it has become one of the best organisms for studies of human diseases and toxicological research (Siddique et al., 2005). Another advantage is the absence of mitosis in flies in the adult phase. Therefore, the adult fly displays synchronized aging of its cells, except gonad cells and some cells of the intestine (Jimenez-Del-Rio et al., 2009). This makes it possible to determine the damage caused by a xenobiotic over time and effect on cell viability. Therefore, the *Drosophila* model for testing bioinsecticidal activity can be an important alternative in the screening of plants of the ''Caatinga'' biome.

According to WHO (1991), mercury is a heavy metal that is considered a dangerous environmental pollutant. Mercury occurs naturally in the Earth's crust (Lee et al., 2009), in organic and inorganic forms, in the solid state, dissolved form and gaseous phase. Consequently, its biogeochemical cycle involves processes that occur in the soil, water and atmosphere (Tinôco et al., 2010). Mercury chloride ($HgCl_2$), the main representative of mercury compounds, is the target of numerous investigations, not only because of its intrinsic toxicity but also because it accounts for the toxicity of elemental mercury since the latter is oxidized to Hg^{+2} (Patrick, 2002). Our knowledge of the toxic levels of heavy metals in plants is still lacking (Kabata-Pendias and Pendias, 1984).

The objective of this work was to characterize, by CG–MS, the chemical constituents of the essential oil of *E. jambolana* and to evaluate its bioinsecticidal action, in the *D. melanogaster* model, as well as to determine the cytoprotective and chelating effect of the ethanol extract of *E. jambolana*.

2. Experimental

2.1. Plant material

The plant material of *Eugenia jambolona* was collected in the Horto Botânico de Plantas Medicinais do Laboratório de Pesquisa de Produtos Naturais (LPPN) of Universidade Regional do Cariri (URCA). Coordinates: 07° 14° 18.2° S and 39° 24° 53° W. The plant material was identified, and a voucher specimen was deposited in the Herbarium Dardano Andrade Lima of URCA, under #3107.

2.1.1. Collection of essential oil

Leaves of *E. jambolana* were collected at 09:00 h, ±30 min, chopped into pieces of approximately 1 cm^2 and placed in a 5-liter glass flask. The leaves were extracted with a Clevenger apparatus, according to the method described by Matos (2009), giving a yield of 0.08%.

2.1.2. Preparation of ethanol extract of Eugenia jambolana leaves(EEEJ)

The extract was prepared by immersing 650 g leaves in ethanol for 72 h at room temperature, which was filtered and concentrated using a vacuum rotary evaporator (model Q-344B- Quimis, Brazil) and warm water bath (model Q214M2- Quimis Brazil). The yield of crude extract was 6.97 g.

2.2. Microorganisms

The bacterial strain utilized was *Escherichia coli* 11105, donated by the Mycology Laboratory of Universidade Federal da Paraíba (UFPB).

2.3. Culture of Drosophila melanogaster

Fruit flies, *D. melanogaster*, were bred in the Microbiology and Molecular Biology Laboratory (LMBM) of URCA. The flies were kindly donated for breeding by Prof. Dr. Jeferson Franco Laboratory of Universidade Federal do Pampa (UNIPAMPA), São Gabriel, RS.

2.4. GC–MS analysis

Oil analysis was performed using a Shimadzu GC MS – QP2010 series (GC/MS system): Rtx-5MS capillary column (30 m × 0.25 mm, 0.25 μm film thickness); helium carrier gas at 1.5 mL/min; injector temperature 250 °C; detector temperature 290 °C; column temperature 60–180 °C at 5 °C/min, then 180–280 °C at 10 °C/min (10 min). Scanning speed was 0.5 scan/s from m/z 40 to 350. Split ratio (1:200). Injected volume: 1 μL of (25 μL essential oil/5 mL $CHCl_3$) (1:200). Solvent cut time = 2.5 min. The mass spectrometer was operated using 70 eV ionization energy. Identification of individual components was based on their mass spectral fragmentation based on Mass spectral library NIST 08, retention indices, and comparison with published data.

2.5. Total phenols and flavonoids

The quantity of total phenols was determined by adding 200 μL of extract dilution (300, 100, 50 and 25 μg/mL in 99.6% ethanol) to 1 mL of Folin–Ciocalteau reagent (10%, v/v) and mixing for 1 min. Next, 800 μL of 7.5% sodium carbonate was added

and the sample was homogenized for 30 s. After 1 h, absorbance was read in a spectrophotometer with the wavelength set at 765 nm. The blank was determined with all reagents, but the extract was substituted by distilled water. The test was done in triplicate. The mean of three readings was used to determine the total phenols, expressed as mg gallic acid equivalents/g extract, by extrapolating this value on the calibration curve constructed with gallic acid standards. The calibration curve for gallic acid was determined using different concentrations (300, 100, 75, 25 and 10 μg/mL). Flavonoids were quantified by preparing solutions of extract (300, 200, 100 and 50 μg/mL) and adding 1 mL of these to 1 mL of 2% (w/v) aluminum chloride ($AlCl_3$). Aluminum chloride was replaced with distilled water in a blank tube. After 30 min incubation at room temperature, absorbance was read using a 415-nm filter. The test was done in triplicate, and the mean was used for determination of the quantity of total flavonoids and expressed as mg quercetin equivalents/g extract. The calibration curve for quercetin was determined using different concentrations (400, 300, 200, 100, 50, 25 and 10 μg/mL) prepared in 99.6% ethanol.

2.6. Chelating effect

The method of Benzie and Strain (1996), Benzie and Szeto (1999) with modifications was adopted for the assay. The principle is based on the formation of O-phenanthroline–Fe^{2+} complex and its disruption in the presence of chelating agents. A volume of 100 μL of extract, from 64 to 2048 μg, was added to 50 μL of 2.0 mM aqueous $FeSO_4$. The controls contained all the reaction reagents except the extract or positive control substance. After 10 min incubation, the reaction was initiated by 200 μL of 6.0 mM O-phenanthroline. After a 10-min equilibrium period, the absorbance at 510 nm was read. Iron chelation activity was calculated from the absorbance of the control (Ac) and of the sample (As) and expressed as Na_2EDTA equivalents (mg Na_2EDTA/g extract). The values are presented as the means of triplicate analyses.

2.7. Evaluation of cytoprotective potential of EEEJ against mercury chloride

The minimum inhibitory concentration (MIC) of EEEJ was determined by the microdilution assay according to Coutinho et al. (2008), with modifications, using suspensions of *E. coli* ATTC 11105 in saline at 10^5 CFU/mL and extracts at a starting concentration of 1024 μg/mL. MIC was defined as the lowest concentration at which no growth was observed. The protective effect of EEFEJ against heavy metals was evaluated by using subinhibitory concentrations of extracts, bacterial suspensions of 10^5 CFU/mL in M9 Tris medium with 2% glucose and dilutions of mercury chloride from 10 to 0.004883 mM. The microdilution plates were incubated for 48 h at 37 °C. The minimum bactericidal concentration (MBC) was determined as the lowest concentration capable of inhibiting the growth of the microorganisms, utilizing Petri plates with heart infusion agar (HIA) for transfer of solutions incubated in microdilution plates.

2.8. Test for cytoprotective effect in lettuce seeds (germination)

The experiments were performed in clean, dry, sterile Petri plates lined with two filter paper disks, on which lettuce seeds were placed. In each plate, 3 mL of solution was added. The concentration of extract was 256 μg/mL, and mercury chloride solutions were at concentrations of 1.25, 0.5, 0.1 and 0.05 mM. A control plate was moistened with 3 mL of distilled water. The experiments were conducted in a BOD germination chamber at a temperature of approximately 25 °C with a 12-h photoperiod for seven days. The treatments were arranged in a randomized complete block design (RCBD), with three repetitions of 20 seeds per plate. The parameters analyzed at the end of seven days were: number of seeds germinated, germination speed index (GSI), biometry of plumule and radicle, and number of necrotic radicles and seedling abnormalities, according to the guidelines for analysis of seeds (Brasil, 2009). Seeds were considered germinated if their radicle reached 1 mm in length. GSI was evaluated every 24 h, which was determined by determining the ratio between the number of seeds germinated on day i (ni) and number of days (i) (Fernandes et al., 2007).

2.9. Mortality tests

Adult flies (males and females) were placed in flasks of 330 mL, containing at the bottom 1 g filter paper impregnated with 20% sucrose in distilled water. A counter-lid of polyethylene teraphthalate (PET) was introduced on the screw cap of the flask, to which parchment paper was taped for application of the different concentrations of essential oil. The flasks received the following treatments: 20% sucrose (control) and 2.5, 5 and 7.5 mg essential oil, with a 12-h light/dark cycle and controlled temperature at 25 ± 5 °C. Readings were taken at 6, 12, 24 and 48 h.

2.10. Statistical analysis

The microbiological tests were done in triplicate and the results expressed as geometric mean. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post-tests using GraphPad Prism 5.0 software. The assays for cytoprotective and biocide effects in plants and in animals were done in triplicate and results expressed as the mean ± standard error of mean (SEM). The comparison between groups was performed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. $P < 0.05$ was considered statistically significant.

3. Results and discussions

3.1. Chemical composition of essential oil

The chemical composition of the essential oil studied revealed the presence of 14 phytoconstituents, namely α-pinene, camphene, β-pinene, myrcene, p-cymene, L-limonene, trans-β-ocimene, β-ocimene, α-terpinolene, fenchol, β-fenchyl alcohol, bornyl acetate, β-caryophyllene, α-humulene. The phytoconstituents that occurred at levels above 10% were the following: α-pinene, 30.04%; trans-β-ocimene, 26.85%; and β-ocimene, 11.13%. The chemical composition of the essential oil is shown in Table 1 and the chromatogram in Fig. 1.

3.2. Total phenols and flavonoids

The results in Table 2 show the quantities of phenolic compounds and flavonoids of EEEJ. An allelopathic effect on

Table 1 Chemical composition (%) of the essential oil of leaves of *E. Jambolana*.

Rt	Phytocompound	(%)	Ki lit.	Ki calc.
3.721	α-Pinene	30.04	934	930
3.939	Camphene	1.17		780
4.401	β-Pinene	8.26		975
4.703	Myrcene	2.78	991	991
5.332	p-Cymene	0.38		1024
5.408	L-Limonene	6.52		1028
5.644	Trans-β-ocimene	26.85		1040
5.857	β-Ocimene	11.13		1050
6.707	α-Terpinolene	0.56		1087
7.268	Fenchol	0.46		1111
9.178	β-Fenchyl alcohol	7.27		1189
11.536	Bornyl acetate	1.31		1283
14.737	β-Caryophyllene	2.10		1412
15.547	α-Humulene	1.17	1454	1447
TOTAL		100		

Rt – retention time; Ki lit. – Kovalts índex from literature; Ki Calc – Kovalts índex calculated.

lettuce seeds can be observed, since the presence of *E. jambolana* extract, at a low concentration, inhibited the germination of the seeds (Fig. 3). The allelopathic effects observed in plants can be due to the different chemical groups (such as phenolic acids, cumarins, terpenoids, flavonoids, alkaloids, tannins, complex quinones) (Einhellig, 1986; Medeiros, 1990).

On the other hand, one of the principal mechanisms in which high concentrations of heavy metals cause damage in plant tissues is oxidative stress, which can be caused by the stimulation of free radical production (Fernandes, 2006).

Compounds that possess antioxidant activity include the class of phenols, phenolic acids and their derivatives, flavonoids, tocopherols, phospholipids, amino acids, phytic acid, ascorbic acid, pigments and sterols. Phenolic antioxidants are primary antioxidants that act as scavengers of free radicals (Xing and White, 1996).

3.3. Chelating effect

Table 2 shows that 567 μg/mL EEEJ chelated 50% of the metal, in comparison with an IC_{50} of 136 μg/mL for EDTA, indicating moderate metal-chelating activity for the extract. Lim et al. (1982) studied the effects of the chelating agents 1,10-phenanthroline in the autoxidation reactions of S(IV) catalyzed by Fe(II) and Mn(II). EDTA has a greater inhibitory effect on the catalyst Fe(II). The Fe(II)/EDTA complex is reactive and in the presence of oxygen, it is rapidly oxidized to Fe(III)/EDTA, which is very stable. The inhibitory effect of these chelating agents is related to the occupation of coordination sites of the catalyst and not only to the change in redox potential of the metal ion due to complexation (Dellert-Ritter and van Eldik, 1992).

3.4. Evaluation of cytoprotective potential of EEEJ against mercury chloride

The data shown in Fig. 2 indicate a slight protective effect of the extract against mercury chloride in relation to control, since the bacteria were able to survive at higher concentrations of the metal. According to Silver and Hobman (2007), microorganisms are resistant to the majority of naturally occurring metals through efflux mechanisms that remove metals from the cytoplasm for extracellular sequestering of the contaminant. Microorganisms utilize the *mer* operon system for mercury.

3.5. Test for cytoprotective effect in lettuce seeds (germination)

The seeds were subjected to germination tests with treatments of water, extract and different concentrations of mercury chloride, as well as extract and metal combined. Toxic effects are only manifested in organisms if the toxic agent reaches specific sites in the organism, at concentrations and for sufficient time to produce some type of effect (Barros and Davino, 2008). In analyzing the treatments with the metal, abnormal seedlings were observed, with necrosis of the radicle, which

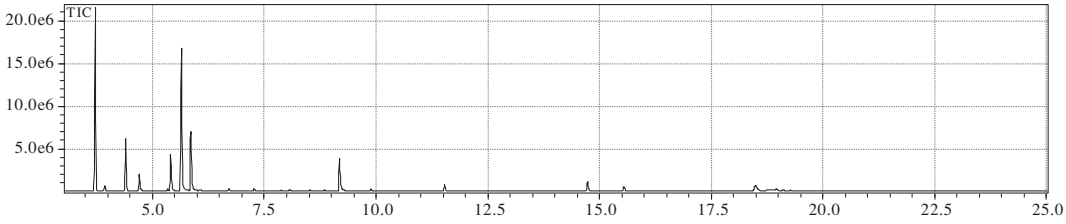

Figure 1 Chromatogram of the essential oil of *E. jambolana*.

Table 2 Total of phenols, flavonoids and chelating effect of EEEJ.

	Gallic acid	Quercetine	EEEJ	EDTA
Total phenols	1079,06 mg/g	–	322,96 mg/g	–
Flavonoids	–	946,94 mg/g	25,16 mg/g	–
IC_{50}	–	–	566.98 μg/ml	–
IC_{50}	–	–	–	136 μg/ml

EEEJ – Ethanol extract of leaves from *Eugenia jambolona* Lam.; IC_{50} – Chelating concentration of 50% of Hg.

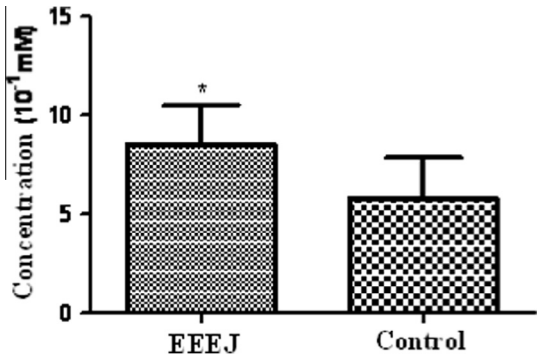

Figure 2 Cytoprotective effect of *Eugenia jambolana* against *Escherichia coli*. EEEJ – Ethanol extract of leaves from *Eugenia jambolana*. * Value statistically significant with $P < 0.05$.

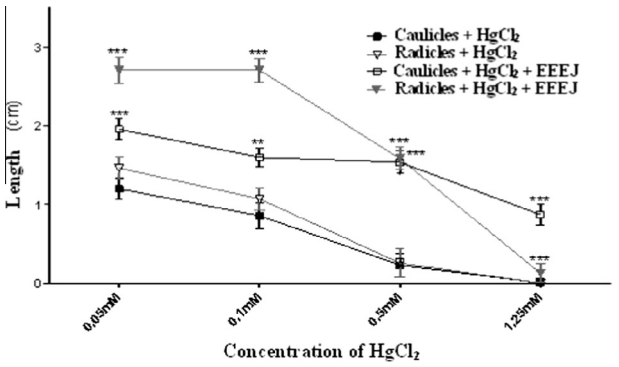

Figure 4 Cytoprotective effect of *Eugenia jambolana* associated with $HgCl_2$. *** Value statistically significant with $P < 0.05$.

was accompanied by discrepancies in the growth of the radicle and plumule.

From Fig. 3, we see that despite the allelopathic effect of EEEJ, seeds grew when mercury chloride was combined with the extract, where this effect was evident at all metal concentrations tested. Fig. 4 shows an increase in the length of the plumule and radicles in the treatment combining different concentrations of mercury with the plant extract.

Contamination with heavy metals affects the growth, distribution and biological cycle of plant species (Barceló and Poschenrieder, 1992), but vegetation can be an alternative for the recovery of soils degraded by excesses of these elements (Salt et al., 1995).

3.6. Biocide effect

As shown in Fig. 5, starting at 12 h, mortality increased at concentrations over 2.5 mg/mL: 50% mortality occurred at approximately 24 h at 2.5 and 5 mg/mL and over 50% mortality at the higher concentrations tested within 24 h. Therefore, the time of exposure is also a factor accounting for the increase in mortality. There are various reports on the insecticidal activity of essential oils (Lima, 2006). However, this is the first report of insecticidal activity for the essential oil of *E. jambolana*. Essential oils can act on the digestive and neurological enzymes of insects, as wells as interact with their tegument (Isman, 2006). A study by Kim et al. (2003) demonstrated the importance of the relation between the chemical structure

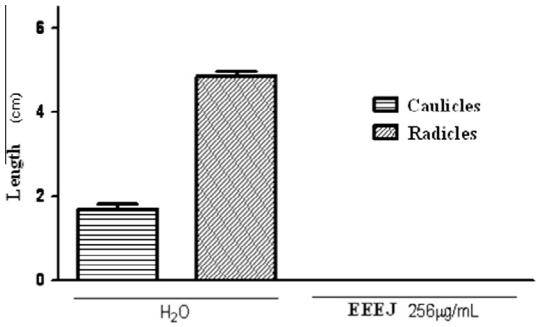

Figure 3 Germination and extension of caulicles and radicles of *Lactuca sativa*.

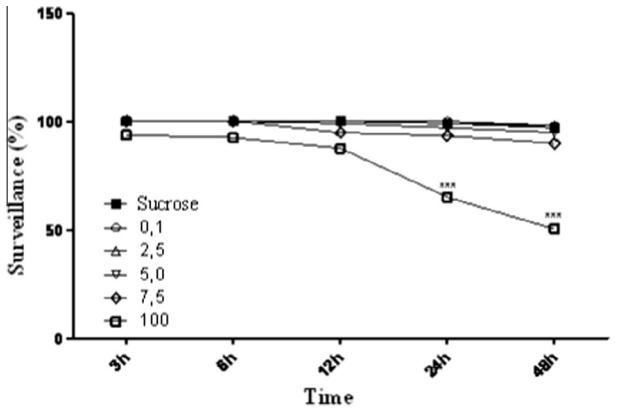

Figure 5 Biocide effect of EEEJ (mg/mL) against *D. melanogaster*. *** Value statistically significant with $P < 0.05$.

and biological activity of compounds; the greater the lipophilicity, the greater the penetration was into the tegument of insects. Essential oils, like their isolated phytoconstituents, have proven to be potential tools for controlling pests, used alone or through the integrated management of pests.

The mechanism of fumigation utilized in this study in view of the tracheal respiration of these flies, is associated with low LC_{50} values in milligrams, demonstrating the level of damage to the locomotor apparatus. This can be associated with damage to dopaminergic neurons, which is encouraging for testing new essential oils, especially those that possess pro-oxidant activity. According to Ennan et al. (1998), some compounds present in essential oils (terpenoids and phenylpropanoids) can block octopamine, a neurotransmitter in insects, which has similar functions as adrenalin in vertebrates. Such compounds are present in the essential oil of *E. jambolana*, which could account for the activity observed.

Analysis of variance (ANOVA) was performed using GraphPad Prism software to determine the difference in the proportion of mortalities between the groups in which EEEJ was administered in relation to the control group (sucrose). ANOVA demonstrated a significant difference in the number of dead flies between the negative control (sucrose) and the groups in which EEEJ was administered ($F = 34{,}1$; $P < 0.05$).

4. Conclusion

The essential oil of *E. jambolana* Lam. appears to be an important alternative in the search for bioinsecticides, since it showed positive results at relevant concentrations against *D. melanogaster* as the target arthropod. On the basis of the results, it is evident that the extract not only has an allelopathic effect on lettuce seeds, but also its interaction with mercury chloride provides better growth of radicles and plumules of *Lactuta sativa*, showing that *E. jambolana* can be an alternative solution to the problem of contamination by heavy metals, besides showing cytoprotective potential and moderate chelating activity.

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