

# Chemical Characterization and Cytoprotective Effect of the Hydroethanol Extract from *Annona coriacea* Mart. (Araticum)

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## ABSTRACT

**Introduction:** *Annona coriacea* Mart. (araticum) is a widely distributed tree in the cerrado. Its value is attributed principally to the consumption of its fruit which possesses a large nutritive potential. The objective was to identify the chemical profile and evaluate the antimicrobial and cytoprotective activity of the hydroethanol extract of *A. coriacea* Mart. (HEAC) leaves against the toxicity of mercury chloride. **Materials and Methods:** The characterization of components was carried out using high-performance liquid chromatography (HPLC). The minimum inhibitory concentration (MIC) was determined by microdilution method in broth with strains of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. For evaluation of the modulatory and cytoprotective activity of aminoglycoside antibiotics (gentamicin and amikacin) and mercury chloride (HgCl<sub>2</sub>), the substances were associated with the HEAC at subinhibitory concentrations (MIC/8). **Results and Discussion:** The HPLC analysis revealed the presence of flavonoids such as Luteolin (1.84%) and Quercetin (1.19%) in elevated concentrations. The HEAC presented an MIC  $\geq 512 \mu\text{g/mL}$  and significant antagonistic action in aminoglycosides modulation, and it also showed cytoprotective activity to *S. aureus* (significance  $P < 0.0001$ ) and *E. coli* (significance  $P < 0.05$ ) bacteria against the mercury chloride heavy metal with significance, this action being attributed to the chelating properties of the flavonoids found in the chemical identification. **Conclusions:** The results acquired in this study show that the HEAC presents cytoprotective activity over the tested strains *in vitro* and can also present antagonistic effect when associated with aminoglycosides, reinforcing the necessity of taking caution when combining natural and pharmaceutical products.

**Key words:** Aminoglycosides, *Annona coriacea* Mart., antimicrobial, flavonoids, mercury chloride

## SUMMARY

- The hydroalcoholic extract of *A. coriacea* Mart. presents *in vitro* cytoprotective activity against the toxic effect of Hg.



**Abbreviations Used:** HPLC-DAD: High-performance liquid chromatography with a diode array detector; MIC: Minimum inhibitory concentration; DMSO: Dimethyl sulfoxide

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## INTRODUCTION

Research for the production of new pharmaceuticals from natural products involve diverse fields of knowledge and various methods of analysis, from the collection and identification of materials, to biological triage and pharmacological tests, determination of the active principle and mechanism of action by which the pharmacological effect is exerted.<sup>[1]</sup>

*Annonaceae* is a large family of plants in the cerrado region of Brazil, containing approximately 27 genera and 290 species, and including a great variety of exotic fruits.<sup>[2]</sup> *Annona coriacea* Mart. popularly known as “araticum” is a widely distributed tree in the cerrado. Its value is attributed principally to the consumption of its fruit which possesses a large nutritive potential and are appreciated for its pulp commonly consumed *in natura* or in the form of sweets, jams, juices, liqueurs

and pies, in addition to its pharmacological properties associated with different parts of the plant.<sup>[3]</sup>

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The necessity of research with natural products motivates the substitution of synthetic chemical substances for natural materials of low cost and of easy access, once the benefits of the research can contribute to the conservation of natural vegetation and sustainability of semi-natural production systems.<sup>[4]</sup>

The anthropic aggression to the environment has been considered in many forms, with the improper use of mercury normally being shown as one of the more representative examples of what man can do to natural cycles, due to population growth and intensification of human activities involving these elements, the concentration of heavy metals has principally increased in bodies of water, where these levels threaten biotic aquatic and terrestrial organisms, for example, man himself.<sup>[5]</sup> Mercury is present in the environment in the chemically oxidized form or as metallic mercury, in its elementary state it emerges from volcanoes of the Earth's crust degradation; however, the artificial forms are more diversified than those considered natural.<sup>[6]</sup>

Metals act as selective agents of resistant bacteria to antibiotics, in contrast to antibiotics, metals suffer a greater difficulty in being naturally degraded; however, microorganisms have developed diverse resistance mechanisms in response to toxic metals, bacterial resistance to toxic metals such as mercury is one of the most studied resistance mechanisms. In Gram-negative bacteria, especially those belonging to the *Escherichia coli* species, the presence of genetic resistance determinants to mercury have been described, which makes a promising alternative to bioremediation processes.<sup>[7]</sup>

Various genera from the Enterobacteriaceae family, including the *E. coli* (Theodor Escherich) and *Pseudomonas aeruginosa* (Schroeter) species, nonfermenting Gram-negative bacilli have been considered problematic due to the increase in resistance rates to antimicrobials.<sup>[8,9]</sup> The genus *Staphylococcus aureus* (Rosenbach) is also greatly associated with human infections and are classified as coagulase-positive strains.

This study had its objective to identify the chemical profile and evaluate the antimicrobial and cytoprotective activity of the hydroalcoholic extract from *A. coriacea* Mart. (Araticum) leaves against mercury chloride.

## MATERIALS AND METHODS

### Plant material *Annona coriacea* Mart.

The botanical material was collected in the Cariri in Crato, CE, Brazil. After collected a voucher, specimen was compared with voucher Herbarium Caririense Dárdano de Andrade-Lima, Regional University of Cariri-URCA and identified as belonging to *A. coriacea* Mart. species.

### Concentration of the hydroethanol extract

For preparation of the hydroethanol extract, 500 g of leaves were collected and cut in pieces which were immersed in equal parts of distilled water and ethanol for 72 h at room temperature. After this period, it was filtered and concentrated on rotaevaporator (Q-344B - Quimis model, Brazil), to evaporate the ethanol. Dehydration was performed in Bath Mary (model Q214M2 - Quimis, Brazil), then the material was placed in freezer for freezing and freeze-dried in (Liotop equipment, Model L101). The extract was packed in amber glass and stored at -20°C temperature. The yield of the extract was 3.53%.

### Bacterial material

Three microbial standard lineages from the American Type Culture Collection were used: *S. aureus* ATCC 25923; *P. aeruginosa* ATCC 25853; *E. coli* ATCC 9027. To evaluate the modulatory activity of the natural products, the following multiresistant bacterial isolates were used:

*S. aureus* SA 358; *P. aeruginosa* PA 03; *E. coli* EC 06 [Table 1]. The strains were maintained using Heart Infusion Agar (HIA, Difco Laboratories Ltda.). Before the tests, the cells were cultivated for 24 h in Brain Heart Infusion (BHI, Difco Laboratories Ltda.).

## Quantification of compounds by means of high-performance liquid chromatography-diode array detector

High-performance liquid chromatography-diode array detector (HPLC-DAD) was carried out with a Shimadzu Prominence Auto Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20AT reciprocating pumps connected to a DGU 20A5 degasser with a CBM 20A integrator, SPD-M20A DAD and LC solution 1.22 SP1 software.

Reverse phase chromatographic analyses were carried out under gradient conditions utilizing a C18 column (4.6 mm × 150 mm) loaded with 5 µm diameter particles; the mobile phase was water containing 2% formic acid (A) and acetonitrile (B), and the composition gradient was the following: 17% of B until 10 min and altered to obtain 20%, 30%, 50%, 60%, 70%, 20%, and 10% of B at 20, 30, 40, 50, 60, 70, and 80 min, respectively, following the method described by Kamdem *et al.* (2013) with slight modifications. The Araticum (*A. coriacea* Mart.) hydroalcoholic extract was analyzed to a concentration of 15 mg/mL. The presence of antioxidant compounds was investigated, that is, gallic acid, chlorogenic acid, caffeic acid, coumaric acid, catechin, epicatechin, quercetin, quercitrin, rutin, and luteolin. The identification of these compounds was made by comparison of their retention times and ultraviolet absorbance spectrum with commercial standards.

The rate of flux was 0.6 mL/min, injection volume was 50 µL and wavelength was 271 nm for gallic acid, 276 nm for coumaric acid, 280 nm for catechin and epicatechin, 327 nm for caffeic and chlorogenic acid, and 365 nm for quercetin, quercitrin, rutin, and luteolin. The samples and mobile phases were filtered through the 0.45 µm membrane filter (Millipore) and it was then degassed by ultrasonic bath before use. The standard reference stock solutions were prepared in the mobile phase of the HPLC in a range of concentrations of 0.025–0.300 mg/ml for catechin, epicatechin, quercetin, quercitrin, rutin, and luteolin; 0.030–0.350 mg/ml for caffeic, coumaric, chlorogenic, and gallic acid. The chromatography peaks were confirmed by comparison of the retention time with standard references and by DAD spectrum (200–500 nm). Calibration curve for catechin:

**Table 1:** Bacterial antibiotic resistance profile

Bacterium	Source	Resistance profile
<i>S. aureus</i> ATCC 25923	-	-
<i>P. aeruginosa</i> ATCC 25853	-	-
<i>E. coli</i> ATCC 9027	-	-
<i>S. aureus</i> SA 358	Surgical wound	Oxa, Gen, Tob, Ami, Neo, Para, But, Sis, Net
<i>P. aeruginosa</i> PA 03	Nasal discharge	Cf, Cfp, Cfd, Caz, Lv, Im Mero, Pip
<i>E. coli</i> EC 06	Urine	Cf, Ca, Clx, Amp, Nor, Lm, Cip, Lv, Of, Ampisul

Amp: Ampicilina; Ampisul: Ampicilina-sulbactam; Ami: Amicacina; Amox: Amoxicilina; Ca: Cefadroxil; Cfc: Cefaclor; Cf: Cefalotina; Clx: Cefalexina; Caz: Cefazidimida; Cfp: Cefepimo; Cfd: Ceftaridimida; Cip: Ciprofloxacina; Im: Imipenem; Can: Canamicina; Lm: Lomefloxacina; Lv: Levofloxacina; Tob: Tobramicina; Of: Ofloxacina; Oxa: Oxacilina; Gen: Gentamicina; Mero: Meropenem; Nor: Normofloxacina; Neo: Neomicina; Para: Paramomicina; Pip: Piperacilina; But: Butirosina; Sis: Sisomicina; Net: Netilmicina; *S. aureus*: *Staphylococcus aureus*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. coli*: *Escherichia coli*

$Y = 12509x + 1,263.8$  ( $r = 0.9995$ ); epicatechin:  $Y = 11958x + 1,309.1$  ( $r = 0.9997$ ); gallic acid:  $Y = 11875x + 1,253.4$  ( $r = 0.9999$ ); caffeic acid:  $Y = 13165x + 1,185.3$  ( $r = 0.9996$ ); chlorogenic acid:  $Y = 12603x + 1,274.9$  ( $r = 0.9998$ ); rutin:  $Y = 12657x + 1,378.9$  ( $r = 0.9999$ ); quercitrin:  $Y = 13591x + 1,183.7$  ( $r = 0.9995$ ); quercitrin:  $Y = 11783x + 1,263.8$  ( $r = 0.9999$ ); coumaric acid:  $Y = 12574x + 1,261.8$  ( $r = 0.9993$ ); and luteolin  $Y = 13509x + 1,267.5$  ( $r = 0.9997$ ). All the chromatography operations were carried out in room temperature and in triplicates. The limit of detection (LOD) and limit of quantification (LOQ) were calculated based on the standard deviation of the responses and the slope from three independent analysis curves.<sup>[10]</sup> The LOD and LOQ were calculated as  $3.3$  and  $10 \sigma/S$ , respectively, where  $\sigma$  is the standard deviation of the response and  $S$  is the slope of the calibration curve.

### Preparation of the original solution and test solutions

In preparing the initial solution, the extract was solubilized in dimethyl sulfoxide (DMSO), BEING as observed following proportions: 10 mg of extract solubilized in 1 ml of DMSO, obtain an initial concentration of 10 mg/ml. Then, this solution was diluted in distilled water reaching extract concentration of 1024  $\mu\text{g}/\text{mL}$  (pH 6.76; 2,138 mOsm/kg  $\text{H}_2\text{O}$ ) and reducing DMSO concentration paragraph 10%, and from this, effected If serial dilutions 1:1 during the microdilution test, yielding to extract concentrations ranging 512-8  $\mu\text{g}/\text{mL}$  DMSO and paragraph 5% concentration.

### Determination of the minimum inhibitory concentration and modulation of aminoglycosides

The minimum inhibitory concentrations (MIC) of the *A. coriacea* Mart. hydroalcoholic extract (HEAC) and fractions, were determined by microdilution tests using suspensions of  $10^5$  CFU/mL of standard strains, and the HEAC in the initial concentration of 1.024  $\mu\text{g}/\text{mL}$ .<sup>[11,12]</sup> For evaluation of aminoglycoside antibiotic modulatory activity (gentamicin and amikacin), the solutions were prepared with the addition of sterile distilled water to obtain a concentration corresponding to 1.024  $\mu\text{g}/\text{mL}$ . A volume of 100  $\mu\text{L}$  of each antibiotic solution was serially diluted in wells containing BHI Broth culture medium – BHI at 10% (pH 6,85; 404 mOsm/kg  $\text{H}_2\text{O}$ ) and the multiresistant inoculum suspension, with the HEAC at subinhibitory concentration (MIC/8).<sup>[13]</sup> The culture medium final concentration of antibiotics were of 512-0.5  $\mu\text{g}/\text{mL}$ . The plates were incubated for 24 h at  $35^\circ\text{C} \pm 2^\circ\text{C}$  and the activity was evidenced by the use of Resazurin sodium.

### Cytoprotective activity against mercury chloride test

For evaluation of the protective effect of HEAC and fractions to mercury chloride ( $\text{HgCl}_2$ ), a modulation using sub-inhibitory concentrations of the products were carried out,  $10^5$  CFU/mL suspensions of *S. aureus* ATCC 25923 and *E. coli* ATCC 9027 in BHI Broth medium – BHI and one concentration of mercury chloride (271.52 g/mol) varying from 10 mM to 0.0048 mM. The microdilution plates were incubated for 24 h at  $37^\circ\text{C}$ . The minimum bactericidal concentration was determined using the lowest concentration capable of inhibiting the growth of microorganisms, using petri dishes with BHI Agar – BHI for transferring solutions incubated for 24 h at  $37^\circ\text{C}$  in microdilution wells. The natural product was evaluated in relation to the control experiment with only mercury. The petri dishes were incubated in an incubator at approximately  $37^\circ\text{C}$ , and the reading was taken after 24 h of incubation.<sup>[14]</sup>

### Statistical analysis

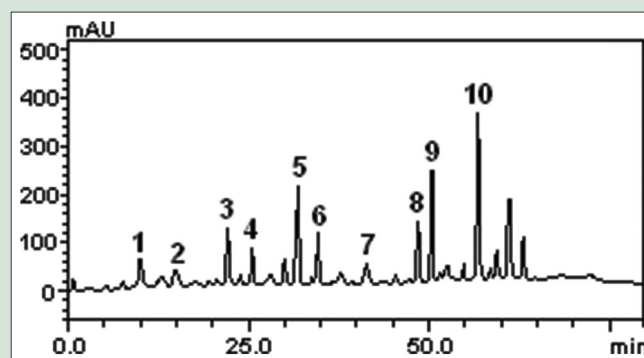
The graphs were produced by the GraphPad Prism 5 software. Statistical analysis was done using a two-way ANOVA followed by the Bonferroni test.<sup>[15]</sup>

## RESULTS AND DISCUSSION

The *A. coriacea* Mart. (Araticum) hydroalcoholic extract in the HPLC analysis revealed the presence of gallic acid (tR=9.97 min; peak 1), catechin (tR = 15.03 min; peak 2), chlorogenic acid (tR = 21.54 min; peak 3), caffeic acid (tR = 24.98 min; peak 4), coumaric acid (tR = 31.49 min; peak 5), epicatechin (tR = 34.67 min; peak 6), rutin (tR = 42.15 min; peak 7), quercitrin (tR = 49.16 min; peak 8), quercetin (tR = 50.02 min; peak 9), and luteolin (tR = 56.73 min; peak 10) [Figure 1 and Table 2].

Was reported in the phytochemical identification of the genus *Annonaceae* the predominance of the alporphine and oxoalporphine alkaloids, besides these, constituents such as polyphenols, essential oils, terpenes and aromatic substances are also found in the family's representatives.<sup>[16]</sup>

The alporphine alkaloids possess great representation in the isoquinolines groups and were only found in plants of the *Annonaceae*, *Berberidaceae*, *Lauraceae*, *Magnoliaceae*, *Menispermaceae*, *Monimiaceae*, *Ranunculaceae* (Ranales order), *Papaveraceae* (Rhoadales order), and *Rhamnaceae* (Rhamnales order)<sup>[17]</sup> families. The presence of the alporphine alkaloids in the *Annonaceae* family is constant in species of the genus *Annona* with



**Figure 1:** Representative high-performance liquid chromatography profile of Araticum (*Annona coriacea* Mart.) hydroethanolic extract. Gallic acid (peak 1), catechin (peak 2), chlorogenic acid (peak 3), caffeic acid (peak 4), coumarin (peak 5), epicatechin (peak 6), rutin (peak 7), quercitrin (peak 8), quercetin (peak 9), and luteolin (peak 10)

**Table 2:** Components of araticum (*Annona coriacea* Mart.) hydroethanolic extract

Compounds	<i>A. coriacea</i> Mart.		$\mu\text{g}/\text{mL}$	
	mg/g	Percentage	LOD	LOQ
Gallic acid	2.97±0.02	0.29	0.009	0.030
Catechin	2.64±0.01	0.26	0.014	0.047
Chlorogenic acid	5.85±0.01	0.58	0.021	0.070
Caffeic acid	3.09±0.03	0.30	0.025	0.083
Coumarin acid	10.72±0.02	1.07	0.017	0.056
Epicatechin	5.81±0.02	0.58	0.008	0.028
Rutin	2.59±0.01	0.25	0.029	0.096
Quercitrin	6.11±0.03	0.61	0.007	0.024
Quercetin	11.93±0.01	1.19	0.011	0.037
Luteolin	18.47±0.03	1.84	0.031	0.102

Results are expressed as mean±SD of three determinations. Averages followed by different letters differ by Tukey test at  $P < 0.05$ . SD: Standard deviations; LOD: Limit of detection; LOQ: Limit of quantification; *A. coriacea*: *Annona coriacea*

reports of the isolation and identification in leaves.<sup>[18]</sup> In this way, the following compounds were found in the chemical identification of the *A. coriacea* Mart. hydroalcoholic extract [Table 2].

Natural products of vegetable and animal origin can alter the effect of antibiotics, be it increasing or decreasing the antibiotic activity, and can be denominated modulators of antibiotic activity.<sup>[12,19]</sup>

Alkaloids between the already isolated alkaloids of the *Annona* genus, anonaine and estefarina presented antimicrobial activity against *S. aureus* and *Bacillus cereus*, glazioline presented activity over *E. coli*. Anolobina, norantenina, and lanuginosina are also found and presented antimicrobial activity over the *Salmonella typhimurium* bacteria.<sup>[20]</sup>

The presented results for the MIC of the HEAC for all the standard and multiresistant species were  $\geq 512 \mu\text{g/mL}$ . The graphs below show the evaluation of the modulatory activity of bacterial resistance to aminoglycosides utilizing amikacin and gentamicin combined with the HEAC, determined after the MIC test by microdilution against the multiresistant and standard lineages of *E. coli* [Figure 2], *S. aureus* [Figure 3] and *P. aeruginosa* [Figure 4].

According to the results obtained on the cytoprotective activity [Figure 5], the HEAC presents significant cytoprotection  $P < 0.0001$  and  $P < 0.05$  in *S. aureus* and *E. coli* strains against mercury chloride ( $\text{HgCl}_2$ ). The data obtained indicates the product as a promising source in the combat to heavy metals, presenting itself as protectors of prokaryotic beings.

Mercury has a large cytotoxic effect over various types of cells, principally those of the immune system, being capable of inducing

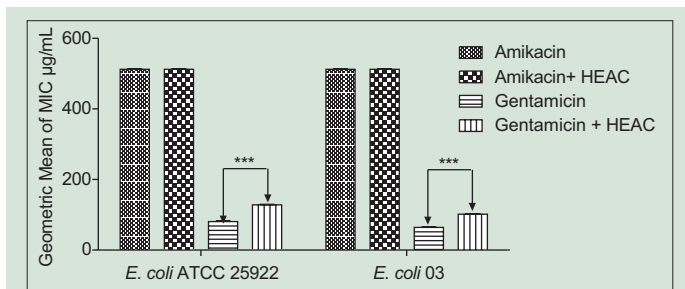
immunosuppression in many animal species.<sup>[21]</sup> In contrast to antibiotics, metals have a great difficulty in suffering natural degradation, thus increasing the importance of their role as bactericidal agents especially in bacteria that are resistant to antibiotics.

Flavonoid compounds can act with an antioxidant activity, reacting with free radicals and also as metal chelators.<sup>[22]</sup> The most abundant flavonoid classes in the *Annona* genus are the flavonols and its glycosylated derivatives, being present both the glycosylated O-glycosides and C-glycosides, within these, it is possible to identify the presence of O-glycosides of quercetin, isorhamnetin, kaempferol, and luteolin in leaves of various *Annona* genera.<sup>[23]</sup>

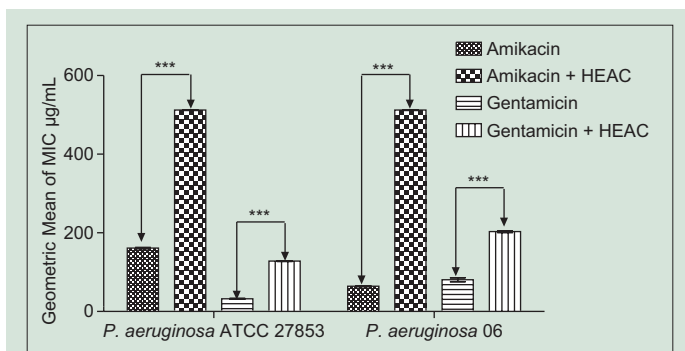
The presence of these may justify the possible cytoprotective effect observed in the tests since the HPLC analysis demonstrated the presence of flavonoids such as luteolin (1.84%) and quercetin (1.19%) in elevated concentrations ( $P < 0.05$ ).

The antioxidant action of the flavonoids is mainly through the presence of the large number of hydroxyls in the B ring.<sup>[24]</sup> The cytoprotective property against mercury chloride is directed by the chelation which can maintain the metal in solution, making it unavailable through precipitation or the complex formed.<sup>[25]</sup>

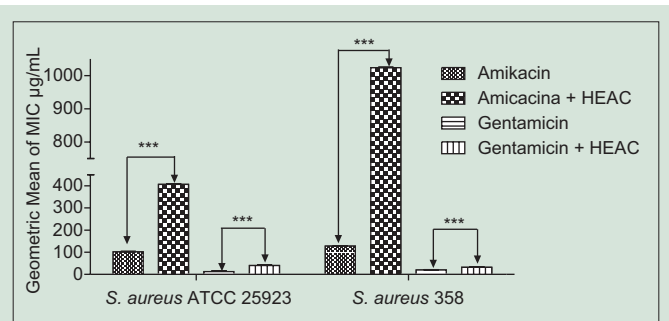
The extremely rapid combinations of metals with free radicals OH and  $\text{O}_2$  through the Fenton or Haber-Weiss reaction, stimulate the presence of hydroxyl radicals.<sup>[26,27]</sup> The hydroxyl group can inactivate various proteins



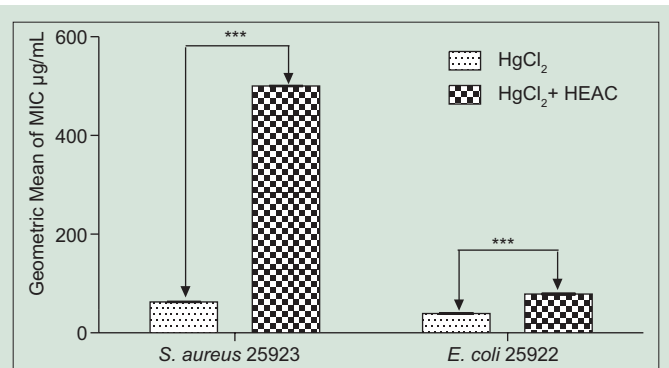
**Figure 2:** Minimum inhibitory concentration of aminoglycosides in the presence and absence of *Annona coriacea* Mart. extract in a concentration CIM/8, against strains of *Escherichia coli*. HEAC: hydroalcoholic extract of *Annona coriacea* Mart. \*\*\*Statistically significant value of  $P < 0.0001$



**Figure 4:** Minimum inhibitory concentration of aminoglycosides in the presence and absence of *Annona coriacea* Mart. extract in a concentration CIM/8, against strains of *Pseudomonas aeruginosa*. HEAC: hydroalcoholic extract of *Annona coriacea* Mart. \*\*\*Statistically significant value of  $P < 0.0001$



**Figure 3:** Minimum inhibitory concentration of aminoglycosides in the presence and absence of *Annona coriacea* Mart. extract in a concentration CIM/8, against strains of *Staphylococcus aureus*. HEAC: hydroalcoholic extract of *Annona coriacea* Mart. \*\*\*Statistically significant value of  $P < 0.0001$



**Figure 5:** Minimum bactericidal concentration of mercuric chloride in the presence and absence of *Annona coriacea* Mart. extract in a concentration minimum inhibitory concentration/8, against strains of *Staphylococcus aureus* and *Escherichia coli*. HEAC: hydroalcoholic extract of *Annona coriacea* Mart.  $\text{HgCl}_2$ : Mercuric chloride. \*\*\*Statistically significant value of  $P < 0.0001$

by oxidizing their sulfhydryl (-SH) groups and disulfide bridges (-SS), they can also initiate lipoperoxidation provoking the disruption of the cellular matrix.<sup>[28]</sup>

Quercetin can inhibit the process of free radical formation through interaction with superoxide ions, the formation of hydroxyl radicals through chelating metal ions and inhibiting lipid peroxidation by reacting with peroxy radicals of lipids, protecting the cell against the cytotoxic action of metals.<sup>[29,30]</sup>

## CONCLUSIONS

The results obtained in this study show that the *A. coriacea* Mart. hydroalcoholic extract can be used as a source of natural products derived from this plant, once cytoprotective activity over tested strains is presented *in vitro*, and can also present an antagonistic effect when associated with aminoglycosides, reinforcing the necessity of caution when combining natural products and pharmaceuticals.

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Nil.

## Conflicts of interest

There are no conflicts of interest.

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