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Assessment of Different Pharmacological Activities of *Annona Ambotay* (Aubl.)

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Abbreviations: CLSI: Clinical and Laboratory Standards Institute; CFU: Colony Forming Units; DMSO: Dimethyl Sulfoxide; MIC: Minimum Inhibitory Concentration; TSB: Tryptic Soy Broth; MFC: Minimum Fungicidal Concentration; DMEM: Dubelcco's Modified Eagle's Medium; SD: Standard Deviation; NCIM: National Collection of Industrial Microorganisms

ABSTRACT

Annona ambotay (Aubl.) (Annonaceae) is known to contain alkaloids, sesquiterpenes, and flavonoids, and antimicrobial activity of its bark, and seeds have been investigated. However, there is limited information available regarding biological activities of its barks. To perform a phytochemical screening of the hydroalcoholic extract from *A. ambotay* (Aubl.) barks and evaluate different pharmacological activities. The antioxidant activity was performed by the DPPH free radical scavenging method; the antifungal potential was evaluated by the broth microdilution method; cell cytotoxicity by the MTT assay; and lethality assay in Artemia salina. Phytochemical screening revealed the presence of flavonoids, sterols, pentacyclic triterpenes and was also active for annonaceus acetogenins in *A. ambotay* extract. The results indicated good antioxidant activity with IC50 of 8.30 μg mL-1. Additionally, the antifungal effect of the extract against different strains of Candida sp was observed. About the toxicity in murine fibroblasts (L929), a reduction in cell viability (43% to 84%) was observed; in human keratinocytes (HaCat) there was a reduction in viability (32% to 72%). Cytotoxicity in breast cancer tumor cells evidenced a high antiproliferative effect, with IC_{50} = 116.32 μg mL^{-1} (MDA-MB-231), IC_{50} = 126.87 μg mL^{-1} (MCF7) and IC_{50} = 11.04 μg mL^{-1} (4T1). A toxic effect was evidenced in the Artemia salina assay (LC_{50} of 296.78 μg mL^{-1}). The extract presented promising biological activities, because of the good antioxidant activity and antiproliferative effect on human cancer cell lines.

Keywords: Annona Ambotay; Antifungal Agent; Antioxidants; Antitumor Agent; Toxicity

Introduction

The use of medicinal plants to treat diseases is a common practice among populations worldwide. Due of the great biodiversity that exists, both Brazilian pharmaceutical industry and researchers are interested in native medicinal plants for the development of new therapeutic approaches Dutra, et al. [1]. The Annonaceae family comprises 135 genera and 2,500 species Lúcio, et al. [2], including *Annona ambotay*. This shrub is distributed throughout South America and is popularly known as envira-cajú or envirataia Maas, et al. [3]. The members of this family provide edible fruits Vendramin, et al. [4] and are used in perfumery, as well as in popular medicine for the treatment of diabetes Madaleno [5] and hypertension Battisti, et al. [6]. Moreover, the genus Annona has different pharmacological properties, such as insecticidal Bravo, et al. [7,8] antitumor Santos Pimenta, et al. [9], antibacterial, cytotoxic Rinaldi, et al. [10], and anticholinesterase activities Formagio, et al. [11]. In popular Bolivian medicine, the seed or bark of *A. ambotay*

is used to treat sprains through direct application to the site of lesion Bravo, et al. [7]. Previously, Takahashi, et al. [12] reported the antibacterial activity of a benzene extract obtained from the bark of *A. ambotay* against Gram-positive and -negative bacteria. In terms of chemical composition, the presence of alkaloids Leboeuf, et al. [13-15], sesquiterpenes, and flavonoids Bravo, et al. [7] has been demonstrated in extracts from species of the Annonaceae family. From *A. ambotay*, in addition to the above-mentioned chemical constituents, Oliveira, et al. [14] isolated geovanine and Bravo, et al. [7] isolated argentilactone. The lack of studies on the pharmacological potential of *A. ambotay* is noticeable. Therefore, this study is the first to perform a screening of potential biological activities of a hydroalcoholic extract of the bark of *A. ambotay* through of evaluating its antioxidant and antifungal activities, as well as toxic activities against strains of murine fibroblasts, human keratinocytes, human adenocarcinoma mammary gland/breast, murine tumor mammary gland, and *Artemia salina.*

Materials and Methods

Plant Material

The bark of *A. ambotay* was purchased at Ervas medicinais (CNPJ 02.117.644/0001-90, Belém, Pará, Brazil). The bark was dried at 40 °C in an oven and then reduced to powder using a knife mill (Metvisa, Brazil).

Preparation of the Extract

Ten grams of dried bark were macerated and extracted with 500 mL of ethanol-water (70:30, v/v) for 72 hours at room temperature. Then, the residue was removed by filtration, and the extract was evaporated to dryness at a lower temperature (<40 °C) under reduced pressure in a rotary evaporator (Buchi, Switzerland), followed by lyophilization (Christ, Germany) under 1.8 mbar pressure and −14 °C. The yield of the extract was 5.9% w/w. The material was stored protected from light at-20 °C until use.

Phytochemical Assay

The phytochemical screening was performed with the dried extract for flavonoids using a 1% aluminum chloride solution in methanol and concentrated hydrochloric acid 36% Kapoor, et al. [16] and for alkaloids using the reactive of Dragendorf Wagner, et al. [17]. The presence of annonaceus acetogenins was achieved by a comparison between results obtained after spraying Dragendorff and Kedde reactives. Samples containing positive spots in both tests were considered active for acetogenins. Tests for sterols and triterpenes were carried out, according to Rizk [18] using Liebermann–Burchard reaction.

Antioxidant Activity

The scavenging activity of *A. ambotay* bark was measured according to the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) method, as described previously by Sreejayan and Rao [19], with minor modifications. Briefly, the sample (50 µL) at different extract concentrations (0.97–250 µg mL^{-1}) was added to each well of a 96-well microplate and mixed with 150 µL of 50 µM DPPH in ethanol solution. The reaction mixture was kept for 30 minutes in the dark at room temperature. Then, the absorbance was measured in a spectrophotometer at 510 nm against the negative control (ethanol). Resveratrol was used as a positive control at the same concentrations. Inhibition of DPPH radical was calculated using Equation 1: IC_{50} (%) = 100 x (A0 – As) / Ao (Eq. 1), being A0 negative control absorbance and A_s test-sample absorbance. The IC_{50} value was calculated from the straight-line equation of the linear dispersion graph and represents the extract concentration that inhibits 50% of DPPH radical. All tests were performed in triplicate.

Antifungal Activity

The standard strains used in this study were as follows: Candida. albicans, American Type Culture Collection (ATCC) 10231; *C. glabrata* (Taniwaki, M.H.), Collection of Tropical Cultures (CCT) 0728; *C. krusei*, (FTI) CCT 1517; and *C. guilliermondii* (CCT) 1890 from the Foundation André Tosello (Campinas, São Paulo, Brazil). The procedures were performed according to the M27-A2 protocol from the Clinical and Laboratory Standards Institute (CLSI) [20]. The fungal suspension was prepared in sterile saline (0.85% NaCl w/v) and then it was diluted in RPMI 1640 culture medium, buffered with 3-(N-morpholino)-propanesulphonic acid (MOPS) and the pH was adjusted to pH 7.0 ± 0.1, to obtain from 5 x 10^2 to 2.5 x 10^3 colony forming units (CFU) per mL. The dried extract was diluted in RPMI 1640 medium buffered with MOPS and tween-80/dimethyl sulfoxide (DMSO) (1:1, v/v). The final DMSO concentration was maintained as less than 1%. Concentrations ranged from 39 to 5,000 µg mL^{-1} for extract. The assay was performed in 96-well sterile microplates to which 100 µL of analogs dilutions and 100 µL RPMI 1640 were added, buffered with MOPS and inoculated with a suitable number of the microorganism's colony forming units. The growth control consisted of 100 µL of the same inoculated culture medium and 20 µL mL^{-1} tween 80/DMSO (1:1, v/v) and a sufficient quantity of the uninoculated medium to make up 200 µL. The negative control was prepared by adding 200 µL of the uninoculated medium. Amphotericin B (Cristália, Brazil) was used as a reference drug at concentrations from 0.0313 to 16.0 µg mL^{-1}. The microplates were incubated at 35 °C for 48 hours. The Minimum Inhibitory Concentration (MIC) was established as the lowest concentration at which no turbidity was observed in the culture medium. After checking the MIC, an aliquot of 20 µL was retained from those wells which showed no visible growth and re-incubated with 4 mL of Tryptic Soy Broth (TSB) without the addition of an antifungal agent, for another 48 hours at 35 °C. The lowest concentration at which no turbidity was noticed after this period was considered to be the Minimum Fungicidal Concentration (MFC).

Cell Viability Assay

The immortalized cell lines [murine fibroblasts (L929), human keratinocytes (HaCaT) human adenocarcinoma mammary gland/ breast (MDA-MB-231 and MCF7) and murine tumor mammary gland (4T1)] were grown in Dubelcco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 10 mM HEPES and maintained at 37 °C in a 5% CO2 humidified atmosphere at pH 7.2. The cell viability study was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay Mosmann [21]. Briefly, all cell types tested were seeded in 96-well plates at a density of 5×10^3 cells in 100 µL of medium per well. After 24 hours of incubation, the culture medium was replaced by fresh medium with the treatments. Quintuplicate wells were treated with *A. ambotay* extract at concentrations ranging from 7.81 to 1,000 μg mL^{-1}. The plates were incubated at 37 °C in 5% CO_2. A control experiment was performed under the same conditions but without cell treatment. After 48 hours, the medium was removed and 90 µL of DMEM with 10 µL of MTT (5 mg mL^{-1}) dye solution was added, followed by incubation for 3 hours at 37 °C. The precipitated formazan was dissolved in DMSO, and the absorbance was measured at 540 nm using a microplate reader. All experiments were performed in a single experiment, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells. The IC_{50} value is the concentration of the sample required to inhibit 50% of the cell proliferation and was calculated by plotting the percentage survival vs. the concentrations, using the Microsoft Excel Program.

Brine Shrimp Lethality Assay

The brine shrimp lethality assay was carried out according to Meyer, et al. [22], with some modifications. Encysted eggs of the brine shrimp Artemia salina Leach were obtained from Maramar Aquacultura (Cabo Frio, Rio de Janeiro, Brazil) and incubated in artificial seawater at pH 8–9. After 48 hours of incubation at room temperature, the active nauplii free from eggshells (n=10 units) were collected and added to each set of wells containing dried extract dissolved in 2.5% DMSO and made up to 5 mL total volume using artificial saltwater. The extracts were tested in triplicate at 10 to 1,000 μg mL^{-1}. Thymol and 2.5% DMSO were used as positive and negative controls, respectively (and artificial seawater as negative control too). After 24 hours, the number of survivors was counted, and the percentage of death was calculated. The lethal concentration 50% (LC_{50} value) and the standard error were calculated by Probit analysis Finney [23].

Statistical Analysis

The results were calculated as a mean ± Standard Deviation (SD). Statistical comparisons were made using the Student t-test, one-way analysis of variance (ANOVA) and Bonferroni's post-hoc test, using the software PRISM 6 (GraphPad, USA). The limit of statistical significance was set at $p < 0.05$.

Results and Discussion

Phytochemical screening revealed the presence of flavonoids, sterols (blue color) and pentacyclic triterpenes (pink color) in *A. ambotay* extract. The dried extract was active for annonaceus acetogenins (positive both for Kedde and Dragendorf tests). These compounds are in agreement with the typical chemical profile of plants of the Annonaceae family. The antioxidant activity was evaluated for hydroalcoholic extract bark from *A. ambotay* and the results are depicted in Table 1. The production of oxygen reactive species causes health damages and are involved in the growth of different diseases such as atherosclerosis, rheumatoid arthritis, cancer and neurodegenerative diseases Chen, et al. [24]. Different studies including species of Annona gender describe the antioxidant activity for the extracts of different parts of the plant with the same analytical method used in this study (DPPH). Roesler, et al. [25] found IC_{50} higher than of this study for the bark extract (IC_{50}= 48.82 μg $mL^{-1)}$ and seeds extract (IC_{50}= 31.14 μg mL^{-1}) from *A. crassiflora*, as well as Kalidindi, et al. [26] for the chloroform extract of leaves (IC_{50}= 308.3 μg mL^{-1}) from *A. squamosa* Linn. Moreover, Formagio, et al. [27] found comparable results with this study for the fractions ethyl acetate (IC_{50} = 8.53 μg mL^{-1}) and hydromethanolic (IC_{50} = 10.57 μg mL^{-1}) from leaves of A. dioica St. Hill.

Table 1: Antioxidant activity of Annona ambotay extract and resveratrol.

Sample	Antioxidant Activity (IC_{50} = μg mL^{-1})
A. ambotay extract	8.30 ± 0.12$^{n.s.}$
Resveratrol	8.60 ± 0.40

Note: The superscript (n.s.) indicates a statistically non-significant difference between resveratrol and *A. ambotay* extract at $p < 0.05$, as analyzed by Student's t-test (mean ± SD, n=3).

Ruiz-Terán, et al. [28-30] demonstrated a relation between phenolic compounds and antioxidant activity from *A. squamosa*, *A. coriacea*, and *A. cuneata* Oliv, respectively. On the other hand, Lima, et al. [31] isolated twelve acetogenins from *A. cornifolia* and found an antioxidant activity for this compound with IC_{50} between 1.95 ± 0.34 μg mL-1 to 0.99 ± 0.18 μg mL^{-1}. Considering the above exposed and the positive phytochemical results for flavonoids and annonaceus acetogenins, it is tempting to suggest that the antioxidant activity of *A. ambotay* extract can be explained by the presence of these compounds, however, additional experiments are necessary to elucidate this hypothesis. The antifungal activity of *A. ambotay* bark extract is shown in Table 2. The results show that only reference drug was active against Candida species with MIC value of 0.0312 to 2 μg mL^{-1}, whereas the antimicrobial activity of *A. ambotay* was >5,000 μg mL^{-1}, which did not demonstrate clinical relevance of the possible use of *A. ambotay* as an antifungal drug. Padmaja, et al. [32-34] revealed antifungal activity of chemical compounds isolated from Annonaceae species. Okechukwu, et al. [35] analysed

the methanol extract leaves from *Cleistopholis patens* (Annonaceae) and found antifungal potential against clinical strains of Candida albicans (MIC = 9.0 μg mL^{-1}) and Candida krusei (MIC = 9.8 μg mL^{-1}), both isolated from HIV patients in stage II. Additionally, Jamkhande, et al. [36] found the antifungal activity of methanolic extract roots from *A. reticulata* Linn. against Candida albicans from the National Collection of Industrial Microorganisms (NCIM) 3055. Although of these studies demonstrated antifungal activity of different extracts and different parts of vegetal species from Annonaceae family, in our study, the dried extract of the bark from *A. ambotay* showed weak antifungal activity, according to the classification described by Kuete [37] who classified plant extracts having MICs of more than 625 μg mL^{-1} as weak antimicrobial activity.

Table 2: Minimal inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of Annona ambotay extract and reference drug against Candida species.

Compound	Candida species	MIC (μg mL^{-1})	MFC (μg mL^{-1})
A. ambotay extract	*	>5,000	>5,000
Amphotericin B	*C. albicans* ATCC 10231	0.125	0.5
	C. glabrata CCT 0728	0.25	0.5
	C. krusei CCT 1517	2	2
	C. guilliermondii CCT 1890	0.0312	0.0312

Note: *for all tested *Candida* species.

The results of the cell viability assay are reported in Figure 1. It's shown a reduction of 43% to 84% in cell viability against murine fibroblast (L929) (Figure 1A) shown a cytotoxic effect in all concentrations when compared to the control group. Moreover, in relation to keratinocytes (HaCat), the reduction of cell viability was of 32% to 72% (Figure 1B), when compared to the control group. The cytotoxic effect observed can be associated with the presence of secondary metabolites, among them the acetogenins, important class of compounds present in plants from Annona gender Tundis, et al. [38], whose presence was confirmed in the phytochemical screening. Several activities of acetogenins were reported such as pesticide, antimalarial, antiparasitic and antimicrobial Roham, et al. [39]. Moreover, previously study suggest that the cytotoxicity action mechanism of this compound is related to the capacity to inhibit the complex I of the mitochondrial respiratory chain Bermejo, et al. [40], harming ATP production, necessary to supply energy for cells process. Freiburghaus, et al. [41] analysed the cytotoxicity of ether and dichloromethane extract of bark from A. senegalensis in human fibroblast (WI-38) and concluded that the higher concentration which not influence in the cell growth was 56 μg mL^{-1} and 6 μg mL^{-1}, respectively. George, et al. [42] evaluated the cytotoxicity of butanolic leaf extract from A. muricata Linn. against human keratinocytes (HaCat) and found IC_{50} = 30.1 μg mL^{-1}. Comparatively with the studies above, the dried extract from *A. ambotay* presented higher cytotoxicity effect to murine fibroblast (L929), which had an alteration in the cellular growth with fluctuation of 43% to 84% of cell inhibition in the range concentration tested (7.81 a 1,000 μg mL^{-1}), while the cytotoxicity effect in keratinocytes (HaCat) was smaller (IC_{50} = 60.65 μg mL^{-1}).

In relation with the cytotoxic effect against breast cancer cell lines, the viability range of 22% to 83% for MDA (Figure 1C), 38% to 74% for MCF7 (Figure 1D) and 23% to 49% for 4T1 (Figure 1E) when compared with the control group. Gavamukulya, et al. [43] analysed the ethanolic extract of leaves from A. muricata in human breast cancer cell (MDA-MB-231) and found dose-dependency with IC_{50} = 248.77 μg mL^{-1} for exposition time of 72 hours. Najmuddin, et al. [44] demonstrated the antiproliferative effect of 19 crude extracts of the leaves from *A. muricata* from different regions of Malaysa and found variation in the IC_{50} of 221.67 to 799.67 μg mL^{-1} in breast cancer cell line MCF7 and 350 to 769.44 μg mL^{-1} in MDA-MB-231 for exposition time of 72 hours. In comparison with the data above, the cytotoxic effect of hydroalcoholic extract of bark from *A. ambotay* was higher to both cell lines, MDA-MB-231 and MCF7, with IC_{50} = 116.32 μg mL^{-1} and IC_{50} = 126. 87 μg mL^{-1}, respectively. Additionally, the extract showed IC_{50} = 11.04 μg mL^{-1} for murine breast cancer cell line 4T1. From the toxicity results of the extract to breast cancer tumor lines, it is possible to observe that the extract may direct its action to the MDA-MB-231 and 4T1 lines, which are characterized by triple negative, that is, presenting lower expression of estrogen receptors, progesterone and human epidermal growth factor receptor 2 (HER2). Holliday & Speirs [45,46] Additionally, the 4T1 cell represents an animal model for stage IV of human breast cancer, exhibiting high metastatic capacity Associação Técnico Científica Paul Ehrlich [47]. Therefore, due to the high toxicity attributed to this cell line, *A. ambotay* extract represents a possible alternative for the treatment of metastatic breast cancer, usually associated with high mortality.

The results of the lethality assay for Artemia salina are described in Table 3. The hydroalcoholic extract from *A. ambotay* barks showed LC_{50} of 296.78 μg mL^{-1}. According to Meyer, et al. [22], an extract demonstrates toxicity to *A. salina* when LC_{50} <1000 μg mL^{-1}. Therefore, the *A. ambotay* extract can be classified as toxic. In fact, other extracts obtained from species of this genus have already demonstrated an effect similar to that found. Santos Pimenta, et al. [9] evaluated the toxicity of eighteen different extracts obtained from the seeds, leaves, and trunk of the species *A. crassiflora*, *A.*

nutans, *A. hypoglauca* and *A. cherimola* against Artemia salina and demonstrated their biological activity. The same authors correlated their biological activity with the presence of acetogenins. The ethanolic extracts from leaves and stem bark of *A. muricata* also showed a toxic effect with LC_{50} = 324.07 µg mL^{-1} and LC_{50} = 196.04 µg mL^{-1}, respectively Silva, et al. [48]. The lethality assay for Artemia salina has shown a good correlation with antitumor activity *in vitro*, representing an important screening tool for the development of new phytomedicines Arcanjo, et al. [49]. Previous studies on extracts obtained from species belonging to the Annona genus show antitumor action. Suresh, et al. [50] using an ethanolic extract from the roots of *A. reticulata*, demonstrated significant antiproliferative effect against tumor cell lines: human lung carcinoma (A549), human chronic myelogenous leukemia bone marrow (K-562), human cervix (HeLa) and MDA-MB. Moreover, Chen, et al. [51] using an extract of *A. squamosa* seeds, evidenced antitumor effect against human tumor cell lines A549 (human lung carcinoma A549 cell line, IC_{50} 3.2 µg mL^{-1}), HeLa (human cervical cancer HeLa cell line, IC_{50} = 13.0 µg mL^{-1}), MCF-7 (human breast carcinoma MCF-7 cell line, IC_{50} = 0.25 µg mL^{-1}) and HepG2 (human liver carcinoma HepG2 cell line, IC_{50} = 0.36 µg mL^{-1}). Taken together, these data associated with the toxic result against Artemia salina, justify the realization of future studies about the antitumor potential of *A. ambotay*.

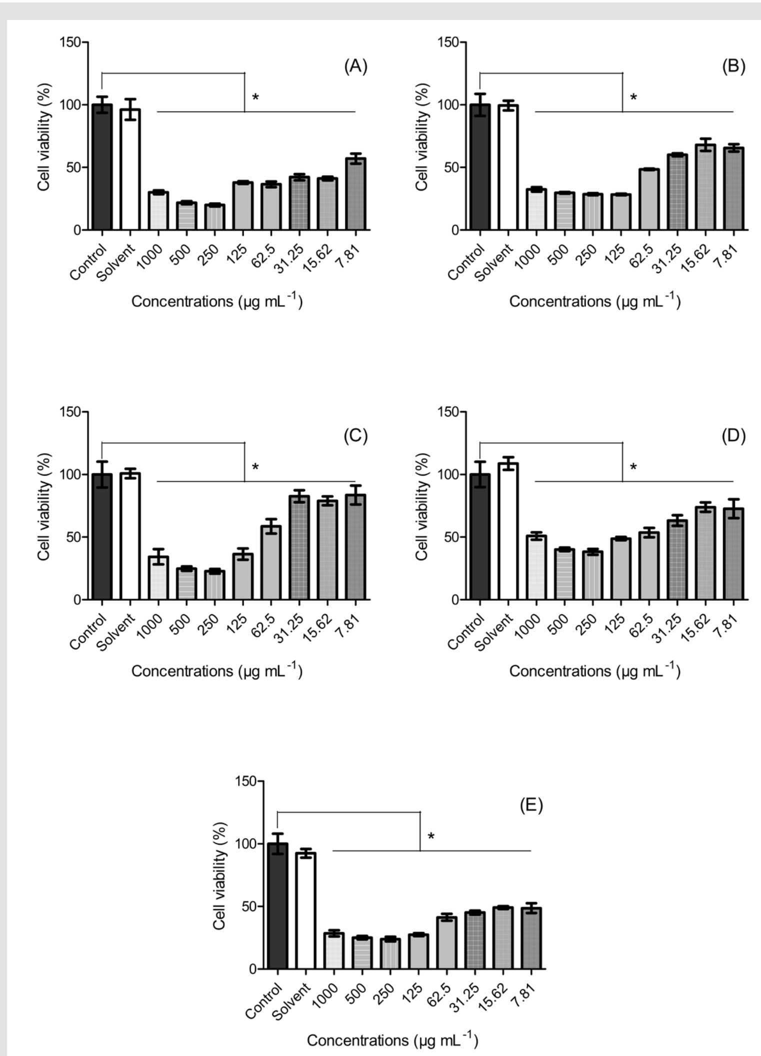

Figure 1:

A. Cell viability of murine fibroblast (L929)

B. Human keratinocyte (HaCat)

C. Breast cancer (MDA-MB-231)

D. (MCF7)

E. And 4T1 with different concentrations of *A. ambotay* extract.

Note: Data was expressed as mean ± SD (n=5). *p<0.05 compared with control group (one-way ANOVA following Bonferroni *post-hoc* test).

Table 3: Lethal concentration 50% (LC_{50}) of the Annona ambotay extract and positive control against the brine shrimp after 24 hours.

Sample	LC_{50} (µg.mL-1)
A. ambotay extract	296.98 ± 5.45*
Thymol	23.0 ± 2.7

Note: The superscript (*) indicates a statistically significant difference between thymol and *A. ambotay* extract at $p < 0.05$ as analyzed by Student's t-test (mean ± SE, n=5).

Conclusion

Altogether, the hydroalcoholic extract bark from *A. ambotay* demonstrated promising pharmacologic activities such as antioxidant activity and possible antitumoral activity, observed by the effect on viability in breast cancer cell lines and by the experimental protocol of Artemia salina. Furthermore, the results of this study indicate cytotoxicity against cell lines murine fibroblasts (L929) and human keratinocytes (HaCat), associated with discrete antifungal action. Hence, this specie can be used to discover bioactive natural products that may serve as leads in the development of new pharmaceuticals research activities in the future.

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Conflict of Interest

There are no conflicts of interest.

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