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Assessment Of Different Biological Capacities Of Aspidosperma Excelsum Benth

Nathália Araújo Rocha[a], Débora Brandão Barbosa[a], Francisco José Raposo[a], Alexandre Augusto Barros Lataliza[a], Cristine Bastos do Amarante[b], Nádia Rezende Barbosa Raposo[a]*

[a]Núcleo de Pesquisa e Inovação em Ciências da Saúde (NUPICS), Faculdade de Farmácia, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n, 36036-330 Juiz de Fora-MG, Brazil

[b]Museu Paraense Emílio Goeldi – Centro de Pesquisa, Avenida Perimetral, 1901, 66077-830 Belém-PA, Brazil

*Tel.: +55 32 2102 3809 E-mail address: nadiacritt@gmail.com

***Abstract*—Species of the genus Aspidosperma is popularly used as a medicinal plant in the treatment of various conditions, such as asthma, diabetes, and malaria, considering they have antimicrobial, anti-inflammatory and wound healing activity. Studies relate their biological activities to the production of alkaloids in their secondary metabolism. The objective of this study was to analyze the antioxidant, antimicrobial and toxicity of the hydroethanolic extract of Aspidosperma excelsum, in view of the biological importance of the species of this genus. The antioxidant activity was analyzed by the reduction of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). The antifungal action in vitro was evaluated against four Candida strains (Candida albicans ATCC 10231, C. glabrata CCT 0728, C. krusei CCT 1517 and C. guilliermondii CCT 1890). Finally, the toxicity potential of the extract of A. excelsum was tested by the MTT method 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide in murine fibroblasts (L929), and through the bioassay using Artemia salina Leach. The results indicated a good antioxidant activity for the tested extract, resulting in IC_{50} of 5.89 µg/mL. The extract was not effective against any Candida sp. strain, showing MIC > 5,000 µg/mL. Regarding the toxicity, the extract showed cytotoxic activity against the L929 cell line, resulting in 40% to 85% reduction of cell viability in the concentrations tested and, it was also considered toxic to Artemia salina, LD_{50} of 273.52 µg/mL. The species studied has important biological activities. This fact may serve as an incentive for future studies, in the search for new properties not yet explored.**

***Keywords*—Aspidosperma excelsum, antimicrobial, antioxidant, toxicity.**

1. INTRODUCTION

The use of plants for a medicinal purpose can be observed from the earliest civilizations. In recent years, there has been growing interest in the development of drugs from elements of nature [1,2] Such interest may be related to the fact that 30% of medicine on the market are derived from natural products, mostly plants and microorganisms [3].

Brazil has approximately 20% of plants and microorganisms present on the planet [4]. Due to this country great biodiversity, Brazilian researchers have shown an increasing interest in the study of native plants, aiming the discovery of its active principles [5,6]. Despite the crescent number of researches, only 5% of the Brazilian pharmaceutical market is represented by medications developed from medicinal plants [3,7].

The genus *Aspidosperma* Mart. is one of the most important of the family Apocynaceae, and it is used for healing purpose in Brazil. There are high concentrations of their species in the Amazon, and they are popularly known as carapanaúba, pau-pereiro, guatambus, amargoso and quina [8–10]. *Aspidosperma excelsum* stands out amid the other species of *Aspidosperma*. It is widely used in traditional medicine, and generally known as carapanaúba [4]. In Peru, it is used by Shipibo-Conibo Indians for the treatment of hepatitis and malaria, and by other natives in order to treat bronchitis as well as a vasodilator drug, antiseptic, antimicrobial, and wound healing [9]. In Brazil, tea made from the stem bark and leaves of *A. excelsum* is utilized in the treatment diabetes and malaria. It is also used in blood pressure control, likewise anti-inflammatory, wound healing and contraceptive [11,12]. Its biological activities are principally attributed to the presence of indole alkaloids in its structure, of which 18 compounds have already been isolated [13].

Despite the various therapeutic indications and its application as a potent antimalarial agent [14], few biological researches have been conducted toward this species [15].

Aware of its potential, this paper intended to study its antioxidant and antifungal actions, in addition to its toxic potential *in vitro*.

2. MATERIAL AND METHODS

A. Plant material

The bark of *Aspidosperma excelsum* Benth. was purchased at Bio Ervas Comércio market in Castanhal, Pará, Brazil. The dried bark was macerated and extracted with 400 mL of ethanol-water (70:30, v/v) for 72 hours at room temperature.

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 (Rotavapor® R-210, Buchi, Switzerland), followed by lyophilisation (ALPHA 1-4 LD plus, Christ, Germany) under 1.8 mbar pressure and −14 °C. The material was stored protected from light in air-tight containers with cap at −20 °C until use.

B. Antioxidant activity

The scavenging activity of *Aspidosperma excelsum* dry extract was measured according to the 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) method, as previously described by Sreejayan e Rao [16], with minor modifications. Briefly, the sample (50 µL) at different extract concentrations (0.97–250 µg/mL) was added to each well of a 96-well microplate and mixed with 150 µL of 0.05 mM DPPH (Sigma-Aldrich, USA) 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 (Sigma-Aldrich, USA) was used as a positive control at the same concentrations. Inhibition of DPPH radical was calculated using (1):

$$IC_{50}\ (\%) = 100 \times (A_0 - A_s) / A_0 \qquad (1)$$

being A_0 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. The results were submitted to Student's t test for independent samples and the level of significance considered was 95%.

C. 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 and the Laboratory of Reference Microorganisms of the National Institute for Quality Control in Health of the Oswaldo Cruz Foundation.

The procedures for determination of the Minimum Inhibitory Concentration (MIC) were performed according to the M27-A3 and M27-S4 protocols of the Clinical and Laboratory and Standards Institute (CLSI)[17,18], adapted according to Almeida et al. [19]. All analyzes were done in triplicate. The fungal suspension was prepared in sterile saline (0.9% NaCl w/v), then it was diluted in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich, USA), buffered with 3-(*N*-morpholine)-propanesulphonic acid (MOPS) (JTBaker, Germany). The pH was adjusted to 7.0 ± 0.2 in order to obtain final concentration of 5 x 10^2 to 2.5 x 10^3 colony-forming units (CFU) per mL.

The dry extract of *A. excelsum* was diluted in RPMI-1640 medium buffered with MOPS and 20 µL/mL Tween-80/dimethyl sulfoxide (DMSO) (1:1, v/v), in the concentration range of 9.76 to 5.000 µg/mL. Amphotericin B (Cristália, Brazil) and nystatin (Sigma-Aldrich, USA) were used as reference drug, at concentrations of 0.0313 to 16 µg/mL to amphotericin B, and 0.08 to 10.6 µg/mL to nystatin.

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 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 uninoculated medium. The microplates were incubated at 37 ± 2 ºC during 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 37 ± 2 ºC. The lowest concentration at which no turbidity was noticed after this period was considered the Minimum Fungicidal Concentration (MFC).

D. Cell viability assay

The immortalized murine fibroblasts (L929) cell line from *Mus musculus* murine was grown in Dulbecco's modified Medium Minimum Media (DMEM-Gibco, USA) high glucose supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA), 1% (w/v) antibiotic penicillin/streptomycin, and maintained at 37 ± 2 ° C in a 5% CO_2 humidified atmosphere.

The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used for the study of cell viability. Briefly, L929 cells were seeded in a 96-well plate at a density of 5x10^3 cells in 100 µL medium per well. After 24 hours of incubation, the culture medium was replaced by fresh medium containing the treatments. Quintuplicate wells were

treated with *A. excelsum* extract, at concentrations ranging from 7.81 to 1,000 µg/mL. Plates were incubated at 37 ± 2 °C in 5% CO_2. A control experiment was performed under the same conditions, with no addition of treatments. After 24 hours, treatments and control were withdrawn, 90 µL of DMEM with 10 µL of MTT (5 mg/mL) dye solution was added, followed by incubation for 3 hours at 37 °C. The precipitated formazan was then dissolved in DMSO, and the absorbance was measured at 540 nm using a microplate reader. All experiments were performed in quintuplicate in a single experiment, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells, as shown in (2):

$$\text{Cell viability (\%)} = (\text{sample A} / \text{control A}) \times 100 \quad (2)$$

The sample concentration required to inhibit 50% of cell proliferation is denominated $IC_{50,}$ and it was calculated by plotting the survival percentage *vs.* concentrations using Microsoft Excel software. Results were evaluated using analysis of variance (ANOVA), followed by Bonferroni *post-hoc* test. Values of $p < 0.05$ were considered statistically significant. Analyzes were performed using Graphpad Prism 5.0 software (GraphPad Software Inc., CA).

E. Brine shrimp lethality assay

The brine shrimp lethality assay was carried out following an already published procedure described by Meyer et al. [20], with some modifications. Encysted eggs of the brine shrimp *Artemia salina* Leach were incubated in artificial seawater at pH 8–9. After 48 hours of incubation at room temperature, the active nauplii free from egg shells (n=10 units) were collected and added to each set of wells containing dry extract dissolved in 2.5% DMSO and made up to 5 mL total volume using artificial salt water. Extracts were tested in triplicate at 10 to 1,000 µg/mL. Thymol and 2.5% DMSO were used as positive and negative controls, respectively, and artificial seawater was also used as negative control. After 24 hours, the number of survivors was counted, and the percentage of death calculated. The calculation to determine the concentration of *A. excelsum* dry extract responsible for the death of 50% of larvae of *A. salina*, a representative value of LC_{50}, was performed. A graphical analysis method was used to obtain LC_{50}.

3. RESULTS AND DISCUSSION

Twenty (20) grams of *Aspidosperma excelsum* bark were used to obtain the dry extract and the yield of the extract was 5.86% w/w.

The antioxidant activity was evaluated for the dry extract of *Aspidosperma excelsum* and the results are shown in Table 1. According to Martins et al. [21], the antioxidant activity is considered to be optimal when $IC_{50} < 15$ µg/mL, which is the case of the *A. excelsum* extract ($IC_{50} = 5.89 \pm 0.53$ µg/mL). This IC_{50} value was not significantly different ($p > 0.05$) from resveratrol ($IC_{50} = 8.60 \pm 0.42$ µg/mL).

Studies with diverse species of the genus *Aspidosperma* have demonstrated antioxidant activity for the extracts obtained from different parts of the plant, using the same method of the current study (DPPH). The ethanolic extract of the *Aspidosperma pyrifolium* seed showed an IC_{50} of 133.48 µg/mL [22], while the hydroethanolic extract of stem bark from the same species resulted in IC_{50} of 27.13 µg/mL [23]. Martins et al. [21] have found IC_{50} of 99.14 µg/mL for the ethanolic extract of *A. nitidum* bark, and the methanolic extract of the bark of *Aspidoserma dispermum* demonstrated an IC_{50} of 15.12 µg/mL [24] ,being this value similar to that found in the present study. The ethanolic extract of *A. excelsum* bark and its alkaloid fractions at pH 1 and 4, presented IC_{50} of 88.17 µg/mL, 31.74 µg/mL and 34.3 µg/mL, respectively[15].

Excessive reactive species may be associated with various diseases, such as different types of cancer, inflammatory disorders, neurodegenerative diseases, among others [25]. Different studies show that therapies with antioxidant compounds may be beneficial in combating different diseases such cancer [26,27], Inflammatory bowel diseases [28], and chronic obstructive pulmonary disease [29]. Considering the importance of antioxidants in helping to cope with various diseases, maybe the *A. excelsum* extract be a potential adjunct in the treatment of several illnesses.

Table 1 antioxidant activity of the dried extract from *aspidosperma excelsum* and resveratrol

Sample	Antioxidant activity (IC_{50} = µg/mL) ± SD
A. excelsum	5.89 ± 0.53
Resveratrol	8.60 ± 0.42

IC_{50}: Concentration value of extract that inhibited 50 % of DPPH radicals. SD: Standard deviation.

The antifungal activity of *A. exceslcum* dry extract is shown in Table 2. The results show that reference drug (nystatin) was effective against *Candida* species evaluated presenting MIC values between 0.33 and 1.32 µg/mL, while the hydroethanolic extract of *Aspidosperma excelsum* showed MIC > 5,000 µg/mL, which did not demonstrate activity against the pathogens tested, according to Holetz et al. [30].

Various researches have attested antifungal activity of different species of *Aspidosperma*. Assis [31] showed that the ethanolic extract of the wood of *A. macrocarpon* root was effective against *C. albicans*, with MIC of 62.5 µg/mL, and the hydroalcoholic extract from the stem bark of *A. tormetosum* was active against *C. albicans* and *C. parapsilosis,* with MIC values of 62.5 µg/mL and 31.25 µg/mL, respectively. The alkaloid extract of *A.*

tormetosum presented IC_{50} of 10 µg/mL against *C. parapsilosis* and *C. krusei* [32]. Moderately positive result was found in inhibition of *C. albicans* by the dichloromethane extract from the stem of *A. ramiflorum* [33].

Table 2: *In vitro* antifungal activity of the dried extract from *aspidosperma excelsum* and the reference drugs.

	A. excelsum		**Nystatin**		**Amphotericin B**	
Cepas	**MIC (µg/mL)**	**MFC (µg/mL)**	**MIC (µg /mL)**	**MFC (µg /mL)**	**MIC (µg/mL)**	**MFC (µg/mL)**
Candida albicans ATCC 10231	>5,000	-	0.33	3.31	125	500
Candida glabrata CCT 0728	>5,000	-	0.66	2.65	0.25	0.5
Candida krusei CCT 1517	>5,000	-	2.65	2.65	2	2
Candida guilliermondii CCT 1890	>5,000	-	1.32	10.61	0.0312	0.0312

Aspidosperma excelsum has already been evaluated for its antibacterial activity in different studies. The dichloromethane extract from the bark of this species has shown activity against *Mycobacterium tuberculosis*, with MIC of 50 µg/mL [34]. Alkaloids isolated from *A. excelsum* root bark extract were active against *Bacillus subtillis* and *Staphylococcus aureus*, but not against *C. albicans* [35]. Iombine, which is an alkaloid present in *A. excelsum,* did not show any activity against *C. albicans, C. krusei* and *C. parapsilosis* species [32]. Different species of the genus *Aspidosperma* have shown potential antifungal activity against *Candida* species tested [30-32]. However, the dry extract of *Aspidosperma excelsum* showed no inhibitory effect in opposition to such pathogens (MIC > 5,000 µg/mL).

Results from the cell viability assay are shown in (1). There is reduction in cell viability of murine fibroblasts (L929) from 40% to 85%, when compared to the control group. It demonstrates the toxicity of the extract in all concentrations tested. The species *A. excelsum* was also evaluated by Gomes [14] for its toxicity, using the bark hydroethanolic extract against murine fibroblast cell line (L929), showing cell viability reduction of approximately 40% in concentrations greater than 800 µg/mL.

Diverse species of the *Aspidosperma* genus have been checked for cytotoxicity, using either their extracts or isolated alkaloids. Lima et al. [23] analyzed the cytotoxicity of *A. pyrifolium* aqueous extract against the mouse fibroblasts cell line (3T3), and the extract demonstrated to be safe up to 1.750 µg/mL. Chierrito et al. [36] investigated the aqueous extract of *A. olivaceum* bark , which presented a 50% lethal dose (LD_{50}) of 20 µg/mL against the hepatoma cell line (HepG2), and the ulein alkaloid showed LD_{50} of 52 µg/mL against HepG2. Aspidospermin, an alkaloid isolated from *A. polyneurom*, was also tested against HepG2 cell line, leading to a 61% decrease in cell viability at the concentration of 100 µM [37]. Mohan et al. [38] analyzed the alkaloid eliptin, a potent anti-

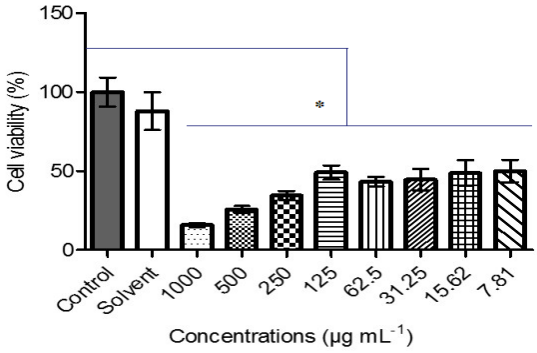

Fig. (1): Cell viability of murine fibroblasts (L929) treated with different concentrations of *A. excelsum* extract. Data are expressed as mean ± SD (n = 5).
*** p <0.05 compared to the control group (one-way ANOVA following Bonferroni *post-hoc* test).**

tumor agent isolated from *A. williamsii*, which demonstrated cytotoxic activity against MCF-7 cells.

The *Aspidosperma excelsum* dry extract, from its hydroethanolic fraction, presented LD_{50} value of 273.52 µg/mL against the shrimp *Artemia salina*. According to Meyer et al. [20], a substance is toxic when the LD_{50} < 1,000 µg/mL. Therefore, the extract tested is considered to be toxic against *A. salina.* In a study conducted by Desmarchelier [39] the methanolic and dichloromethane extracts of *Aspidosperma excelsum* showed LD_{50} values of 756 and 85 µg/mL, respectively, against the *A. salina* larvae. This result is in agreement with the one obtained in this research, reaffirming the toxic potential of the hydroethanolic extract of *A. excelsum* when in contact with *A. salina*.

Diverse studies have shown that different species of *Aspidosperma* are also toxic to microcrustaceans, such as the ethanolic extract from *A. nitidum* bark, which showed 97% lethality against *Artemia franciscana* at 500 µg/mL. The chloroform fraction of the *A. vargasii* ethanolic extract demonstrated high toxicity, with LD_{50} of 118.1 µg/mL, which leaded to a 100% mortality of *Artemia franciscana* at a concentration of 500 µg/mL. The chloroform fraction from *A. desmanthum* ethanolic extract, caused a 95% mortality of the

microcrustacean tested, presenting LD_{50} of 20.01 µg/mL [40]. Bioassay with *A. salina* has various applications. It may indicate toxicity of the substances tested to humans, when there is high lethality of the shrimp against such substances [41]. Furthermore, results obtained from this bioassay generally show good correlation with in vitro toxicity results used for evaluation of antitumor activity, suggesting that this test can be used as a pre-screen for the cytotoxicity of new compounds [42,43]. Therefore, more extensive research on the toxicity of the *A. excelsum* dry extract is required, aiming at the clinical use of this extract in the future.

4. CONCLUSION

The dry extract of *Aspidosperma excelsum* showed antioxidant activity, as well as toxicity against the shrimp *Artemia salina*. Based on these results it can be inferred that the extract has the potential for further studies focused on antitumor activity. In addition, toxicity was verified against the cell line of murine fibroblasts (L929), indicating that the extract may not be suitable for the development of topical medicine. Lastly, weak antifungal action was observed against the tested pathogens.

CONFLICTS OF INTEREST

All authors declare no conflicts of interest.

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