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Antifungal potential of punicalagin against *Cryptococcus neoformans* species complex

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ABSTRACT

This study evaluated the antifungal activity and cytotoxicity profile of the ellagitannin punicalagin, a compound extracted from the *L. pacari* A.St.-Hil (Lythraceae) leaf, against *Cryptococcus neoformans* species complex. Minimum inhibitory concentrations (MIC) were checked using the broth microdilution method. Minimum fungicidal concentrations (MFC) and time of death were used to confirm the antifungal activity of the compound. The *in vitro* cytotoxicity of punicalagin was tested in BALB/c3T3 fibroblasts and A549 human lung cancer cell line, while the hemolytic potential was tested on sheep erythrocytes. The morphological changes induced in yeast strains by the presence of punicalagin were also analyzed. Tested on eight isolates of the *C. neoformans* complex punicalagin showed MIC of 0.5 to 4.0 µg/mL and MFC> 256 µg/mL. Punicalagin also demonstrated a good growth inhibitory activity in time-kill curves, but it was not able to achieve a statistically significant reduction of fungal growth suggesting a fungistatic effect of the compound. *In vitro* cytotoxicity studies using the two cell lines showed that punicalagin has low activity on these cells and no activity on sheep erythrocytes. Morphological changes were seen in the yeasts strains studied when treated with punicalagin. Therefore, punicalagin is a potential antifungal for important pathogenic yeasts and presents a low cytotoxicity profile associated with no hemolytic effects.

KEYWORDS: Punicalagin. *Lafoensia pacari* A.St.-Hil. Cytotoxicity. Antifungal activity. *Cryptococcus neoformans* species Complex

INTRODUCTION

Punicalagin extracted from *Lafoensia pacari* A. St.-Hil (Lythraceae) is a compound that has medicinal properties[1]. *Lafoensia pacari* is a plant known in Brazil as dedaleiro ou pacari. In folk medicine, it is used as wound healing, antipyretic, antidiarrheal, and in the treatment of gastritis, ulcers and cancer[2,3]. The ellagitannin punicalagin has shown activity against *Cryptococcus neoformans* species complex, dermatophytes and some species of *Candida*[4-7].

Cryptococcosis is a severe systemic mycosis, with worldwide distribution[8], and its prevalence is estimated to be more than one million cases with about 650,000 deaths annually[9]. Infections caused by *Cryptococcus gattii* have a protracted course of illness[10], and it is well known that *C. gattii* isolates are significantly less susceptible to azoles than the isolates of *C. neoformans*[11,12]. This fungal infection is mainly associated with resistance to available antifungal drugs[13]. Therefore, the

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development of new products showing broad spectrum of action against cryptococcal species with low toxicity are extremely necessary.

Thus, the present study aims to evaluate the antifungal activity andthe cytotoxicity of punicalagin, a phenolic compound extracted from *L. pacari* leaf against *C. neoformans* species complex, as well morphological change analyses induced in yeast strains by the presence of punicalagin.

MATERIALS AND METHODS

Leaves from *L. pacari* A. St.-Hil (Lythraceae) were submitted to ultrasound extraction with acetone:water (70:30) and punicalagin was characterized by HPLC/UV and ESI-TOF MS, 1D and 2D NMR spectroscopic evaluations as described by Carneiro *et al.*[1].

The yeast strains used in this study were: *C. neoformans* ATCC 28957, *C. gattii* ATCC 24065, six clinical isolates of *C. neoformans* species complex obtained from patients with meningitis in a tertiary hospital of Goiania, Goias State, Brazil (three *C. gattii* - L1, L9, and L20 and three *Cryptococcus neoformans* - L3, L29, and L30). These isolates were collected in a previous study approved by the Bioethics Committee of Hospital de Doenças Tropicais de Goias (protocol 027/07).

Microdilution broth assay

The *in vitro* activity of punicalagin was measured by means of the microdilution broth method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines M27-A3 (CLSI 2012[14]) and M27- S4 (CLSI 2012[15]) for yeasts. Serial twofold dilutions of the pure compound were prepared in 96-well microplates. The minimal inhibitory concentration (MIC) was defined as the lowest concentration that resulted in the total inhibition growth analyzed by visual inspection, giving a numerical score in comparison with the growth present in the control (drug-free) sample.

The minimal fungicidal concentration (MFC) was determined by an inoculum of 10 µL from each well containing the MIC and up to 4 × MIC seeded in petri plates containing Sabouraud dextrose agar (SDA), incubated for 72 h at 35 °C. The MFC was defined as the lowest concentration of the compound that resulted in growth of less than two colonies representing the death of > 99% of the original inoculum. Fluconazole was used as the control.

Time kill assay

Cell growth and death rates of the yeast strains studied were analyzed according to the modified protocols of Klepser *et al.*[16] and Silva *et al.*[17]. Dilutions of 1/10 in RPMI-1640 (Sigma Chemical Co., St. Louis, MO, USA) of broth suspension containing approximately 1 to 5 × 10^6 CFU/mL were prepared in order to obtain the initial concentration of 1 to 5 × 10^5 CFU/mL. Punicalagin was added to this suspension solutions to obtain a final concentration of ½ MIC (2 µg/mL), MIC (4 µg/mL), and 2 × MIC (8 µg/mL) of this compound. Test solutions were placed on a shaker and incubated at 35 °C. At predetermined time points (0, 6, 12, 24, 48 and 72 h), following the introduction of the test isolate, 100 µL of samples were removed from each test solution. Tenfold serial dilutions (10^{-2} to 10^{-5} on RPMI-1640 broth) were performed using samples and aliquots of 10 µL of each dilution, plated in SDA and the number of CFU on each plate was determined. Kill curve assays were run in duplicate.

Cytotoxicity assays

Cell cultures

The BALB/c 3T3 A31 fibroblasts and A549 human lung cells were cultured in DMEM and Ham's F12 nutrient mixture, respectively, supplemented with heat-inactivated FBS (10%, v/v), HEPES (4.5 mM), sodium bicarbonate (0.17 M), L-glutamine (2 mM), in a humidified atmosphere of 5% CO_2 in air at 37 °C. When cells reached approximately 70% confluence, they were harvested with a trypsin (0.025%)/EDTA (0.02%) solution. Cell viability was tested using the TC20™ automated cell counter (Hercules, CA, USA), according to manufacturer's instructions, and a value > 90% was considered satisfactory to conduct the assays.

3T3 neutral red uptake (NRU) assay

The 3T3 NRU assay was performed according to the standard protocol of Borenfreund and Puerner[18], modified by ICCVAM[19]. In brief, 3T3 fibroblasts cells containing 3 × 10^4 cells/well were treated with nine different concentrations of punicalagin ranging of 0.78-200 µg/mL in complete medium for 48 h. Amphotericin B in concentrations ranging from 78 to 200 µg/mL) was used as reference drug. After incubation, the supernatant was removed and the cells were washed with PBS followed by addition of neutral red (0.25 mg/mL) and incubated for 3 h. After that, 100 µL of a developing solution (50 ethanol: 1 acetic acid: 49 ultrapure water) were added to all wells, and shaken for 20 min at 45 g. Absorbance was measured at 550 nm in a spectrophotometer (Thermo Scientific Multiskans Spectrum, Boston, MA, USA). A concentration-response curve was obtained to determine the concentration

of punicalagin or amphotericin B that inhibited cell growth by 50% compared to the untreated group (IC_{50}).

MTT assay of A549 human lung cells

Cytotoxicity analysis in A549 cells was performed using the MTT reduction test, adapted from Mosmann[20]. Briefly, A549 cells (1 x 10^5 cells/mL) were seeded in 96-well plates overnight and then treated with nine different concentrations of punicalagin (0.78-200 µg/mL) in complete medium for 45 h. Amphotericin B (0.78-200 µg/mL) was used as reference drug. Subsequently 10 µL of MTT (5 mg/mL) were added to each well, incubated for 3 h, the supernatant was removed and 100 µL of DMSO was added to each well. Absorbance was measured at 560 nm. Cell viability was expressed as a percentage of the control IC_{50} and selectivity index (SI) values were determined.

Hemolytic assay

Hemolytic assays were performed according to He *et al*.[21]. Briefly, 100 µL aliquots of sheep erythrocytes/ PBS 10% were added to 100 µL of a two-fold dilution series of punicalagin in concentrations ranging from 256 to 1 µg/mL or of amphotericin B ranging from 8 to 0.031 µg/mL in the same buffer and placed in Eppendorf tubes. The tubes were incubated for 30 min at 37 °C, and centrifuged for 5 min at 4500 g. From the supernatant fluid, 150 µLwere transferred to a flat bottom microtiter plate and absorbance was measured using a spectrophotometer. Total hemolysis was achieved with a 1% Triton X-100 solution.

Scanning electron microscopy

Scanning electron microscopy (SEM) analysis was performed according to Faganello *et al*.[22] with some modifications. The isolates were cultured in SDA containing a corresponding concentration to MIC (4 µg/mL) of punicalagin and incubated at 30 °C. Small blocks of each fungal sample were withdrawn and fixed in 2% glutaraldehyde and 2% paraformaldehyde, in 0.1 M sodium cacodylate buffer with 3% sucrose at pH 7.2 and kept overnight at 4 °C.

Samples were washed in the same buffer four times, dehydrated in ethyl alcohol and dried in a critical CO_2 point (Autosamdri®, 815, Series A) covered with gold (Denton Vacuum, Desk V) and analyzed in Jeol, JSM-6610 Scanning electron microscopy.

Statistical analysis

Data were expressed as mean or mean ± standard deviation (SD) in cytotoxicity assays. The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by Bonferroni's test using the GraphPad Prism 5.01 software (GraphPad Inc., San Diego, CA, USA). Statistical significance was established as $p<0.05$. All experiments were performed in three independent assays.

RESULTS

Broth microdilution assay

Punicalagin showed antifungal activity on species of the *C. neoformans* complex with a MIC range of 0.5 to 4.0 µg/mL and MFC> 256 µg/mL for all isolates. At the concentration of 0.5 µg/mL the compound inhibited 37% of the C. *neoformans* species complex isolates. Fluconazole showed a MIC range of 0.5 to 8.0 and a MFC range of 1 to 64 µg/mL.

Time kill curve

The time-kill curve (Figures 1A and 1B) showed reduction in the number of CFU/mL of the *C. neoformans* complex cells treated with punicalagin at concentrations corresponding to the MIC of 4 µg/mL and 2 × MIC (8 µg/mL). The major reduction was observed with *C. neoformans* ATCC 28957 at a concentration corresponding to 2 × MIC at 12 h. The difference was not statistically significant between treated and non-treated cells. Amphotericin B had maximum fungicidal activity after 12 h of incubation at concentrations equivalent to 1 µg/mL for *C. gattii* ATCC 24065 and *C. neoformans* ATCC 28957.

Citotoxicity using BALB/C 3T3 cells and A 549 cells

NRU analysis showed IC50 of 58.7 µg/mL for punicalagin and < 0.78 µg/mL for amphotericin B. Increased inhibition of 3T3 cell growth was observed with enhanced concentration of punicalagin as shown in Figure 2A.

MTT analysis on alveolar epithelial cells showed that the concentration of punicalagin that caused the death of 50% of these cells was > 200 µg/mL, while for amphotericin B, used as a cytotoxicity control, the IC was 59.6 µg/mL. Effects of different punicalagin concentrations after 48 hours exposure are shown in Figure 2B.

Hemolysis of sheep erythrocytes

The hemolytic effect of punicalagin was tested on sheep erythrocytes and the concentration of 256 µg/mL produced hemolysis in 2.46% of cells, while for amphotericin B,

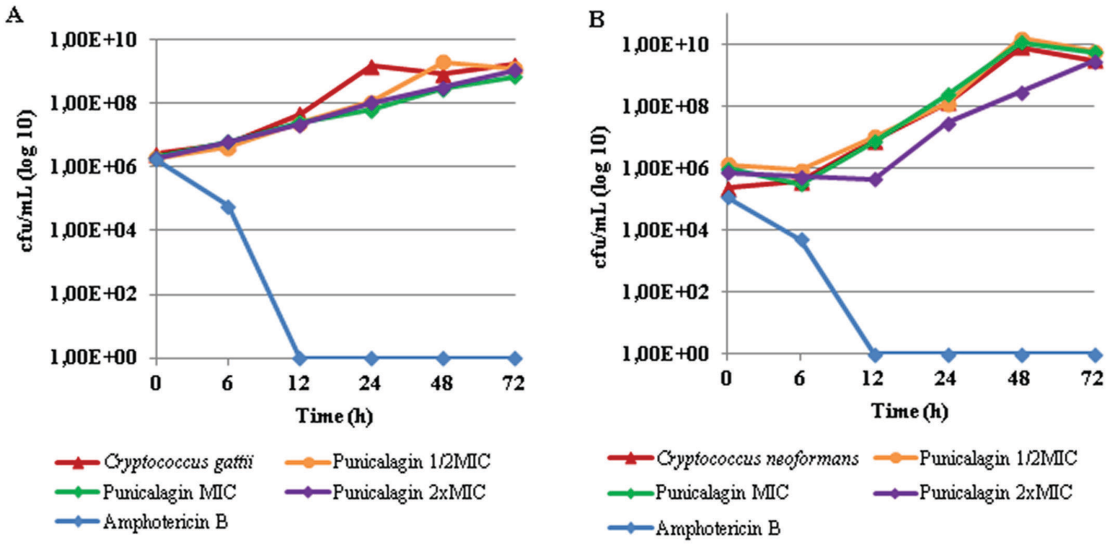

Figure 1 - Growth curve of *C. gattii* ATCC 24065 (A) and *C. neoformans* ATCC 28957 (B) without treatment and treated with punicalagin at the values corresponding to ½ of the minimum inhibitory concentration - MIC (0.25), MIC (0.5 µg/mL), and 2 × MIC (1 µg/mL), and amphotericin B in MIC (0.5 µg/mL) after 6, 12, 24, 48, and 72 h of incubation (A and B). For *C. gattii* ATCC 24065, (ANOVA p=0.481) and for *C. neoformans* ATCC 28957, (ANOVA p=0.461)

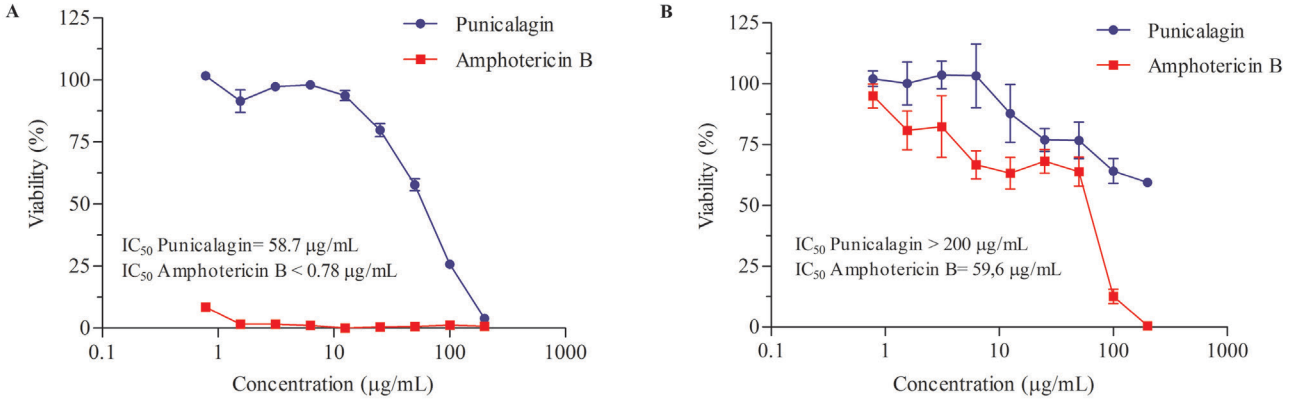

Figure 2 - A) Viability of BALB/c3T3 cells exposed with different concentrations of punicalagin and amphotericin B (both at 0.78-200 µg/mL) for 48 h using 3T3 neutral red uptake (NRU) assay; B) Cell viability curve of human alveolar epithelium A549 at different concentrations of punicalagin and amphotericin B (both at 0.78-200 µg/mL) for 48 h using the MTT assay

hemolysis of 0.87% of erythrocyte cells was found at concentration of 8 µg/mL.

Scanning electron microscopy

This analysis demonstrated that the untreated cells of *C. neoformans* ATCC 28957 (Figure 3A) and *C. gattii* 24065 (Figure 3C) showed normal cell morphology with intact smooth wall and spherical bodies present in large amounts. After exposure to punicalagin, the yeasts had rough walls and were shrivel as shown in Figures 3B and 3D.

DISCUSSION

Several antifungal agents have been used for the prophylaxis and treatment of *Cryptococcus neoformans* species complex infections[23]. Current pharmacological treatments are effective, but intrinsically resistant species are emerging rapidly. In addition, the high cost of treatment and host-associated cytotoxicity makes it necessary to search for new microbial compounds. The activity of punicalagin has been studied against some *Candida* species and against *C. neoformans*[4,6,7,24] but there are no reports described for *C. gattii*. Interestingly, the isolate *C. gattii* L20 showed high MIC value to fluconazole, with low MIC value for punicalagin. Fluconazole is the drug of choice for the maintenance therapy of cryptococcal disease[25].

Moreover, our findings have shown that punicalagin promotes fungistatic effects. CFM was >256 for all isolates and although the kill curve showed reduction in the numbers of CFU of yeast cells, this difference was not statistically significant in treated and untreated cells for all yeasts

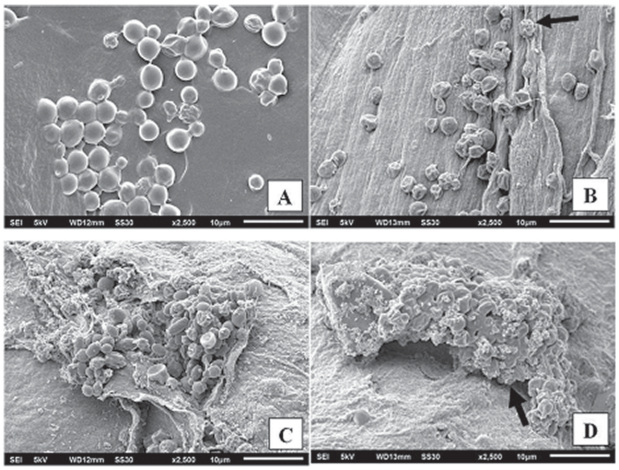

Figure 3 - Cell morphology analysis of *C. neoformans* ATCC 28957 and *C. gattii* 24065. Untreated cells appear ovoid and intact (A and C); and cells treated with punicalagin at minimum inhibitory concentration – MIC (4 µg/mL) show rough and shrivel yeasts (B and D)

studied (ANOVA). Our results are similar to those found by Endo *et al*.[6], who found a small reduction of CFU in the presence of punicalagin. Fungistatic effects of naturally occurring tannins against yeasts were reported by Baba-Moussa *et al*.[26] and Morey *et al*.[27]. The extract of *Terminalia avicennioides* stem bark was fungicidal on *Epidermophyton floccosum*, *Microsporum gypseum* and *Trichophyton mentagrophytes*, but only fungistatic on *Trichophyton rubrum* and *Candida albicans* (Baba-Moussa *et al*.[26]). Fraction of *Stryphnodendron adstringens* exhibited a fungistatic effect with the minimum inhibitory concentration ranging from 0.5 to 8.0 µg/mL in *Candida tropicalis* (Morey *et al*.[27]).

In this study, we found that punicalagin has low toxic activity on human lung carcinoma A549 cells, 3T3 fibroblast cells, and no hemolytic potential to animal cells. The concentration of punicalagin with 50% cytotoxicity (IC50) on BALB/c 3T3 cells was 58.7 µg/mL, showing that this compound was 14.6 times greater than their MIC (4 µg/mL) against yeasts. Similar results were found with carcinoma cells, where IC50 was 200 µg/mL, therefore, 50-fold larger than the MIC. Similar data to this study confirm the results of low *in vitro* toxicity observed for punicalagin. The low cytotoxic activity of punicalagin was verified by Endo *et al*.[6] on Vero and macrophages J774G8 cell monolayers and Foss *et al*.[5] on Vero cells.

We have also demonstrated that punicalagin exhibited low toxicity on sheep erythrocytes cells. Lysis of sheep erythrocytes cells is easily obtained by measuring the release of hemoglobin, constituting a good tool for toxicity studies. Determination of hemolysis activity is quick, reproducible, and inexpensive[28].

The reduced number of cells with rough and shrivel yeasts in the presence of punicalagin indicated that the compound caused damage to the cell structure. These alterations could represent the first step towards the discovery of new drugs. Compounds that inhibit microbial growth and cause morphological changes can be proposed as potential antifungal agents.

Based on the data obtained, we believed that punicalagin has antifungal potential on important pathogenic yeasts and presents a low cytotoxicity profile associated with no hemolytic potential to animal cells. The excellence of punicalagin suggests that it may be a promising drug against yeasts and invites further research.

CONFLICT OF INTERESTS

The authors declare no conflicts of interest.

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