

Article

Hydroethanolic Extract of *Punica granatum* Inhibits *Cryptococcus* by Depolarising Mitochondrial Membranes

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Abstract: Cryptococcal infections are distributed worldwide and mainly caused by *Cryptococcus neoformans* and *Cryptococcus gattii*. The reduced number of antifungals and increasing number of cases of resistance require the search for new therapeutic options, such as natural products. Among these, *Punica granatum* L. has demonstrated antifungal activity. The present study aimed to evaluate the in vitro activity of the hydroethanolic extract of the leaf of *P. granatum* (HEPg) alone or in antifungal combination against *C. neoformans* and *C. gattii* and the interference of *P. granatum* in the mitochondrial membrane of *Cryptococcus* using flow cytometry. The minimum inhibitory concentration was determined, which showed inhibitory activity against *Cryptococcus* isolates. The fractional inhibitory concentration resulted in an indifferent interaction between the combination of amphotericin B + HEPg, whereas the combination of fluconazole + HEPg was synergistic against *C. gattii*. The depolarisation of mitochondrial membranes was more pronounced when *C. gattii* was previously treated with *P. granatum*, either individually or in combination with antifungal agents. In contrast, prior treatment of *C. gattii* with fluconazole promoted the hyperpolarisation of mitochondrial membranes. Considering the growing search for alternative forms of treatment for cryptococcosis, this study highlights the antifungal potential of *P. granatum*.

Keywords: *Punica granatum*; cryptococcosis; antifungal activity; mitochondrial membrane



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1. Introduction

Cryptococcosis is one of the main endemic mycoses in several countries, including Brazil, and is caused by yeasts of the *Cryptococcus* genus, mainly *Cryptococcus neoformans* and *Cryptococcus gattii* [1]. Cryptococcal meningitis is particularly prevalent in individuals living with HIV, and responsible for high morbidity and mortality rates [2].

Cryptococcosis is a disabling and even lethal disease if the individual does not benefit from effective antimicrobial therapy. Mycosis is treated with antifungal agents, such as fluconazole (and other azoles), amphotericin B, and 5-fluorocytosine [1–3]. Fluconazole inhibits an enzyme essential for ergosterol biosynthesis [4]. In contrast, polyenes such as amphoteric B bind to ergosterol, forming pores in the membrane with intracellular ion

leakage [5]. Flucytosine acts as an antimetabolite and interrupts RNA and DNA [6]. In addition to the limited number of therapeutics available, cases of antifungal resistance have raised global alerts regarding major public health problems [7].

In this context, research on the antifungal activity of synthetic [8] and natural products, such as *Punica granatum* [9–11], popularly known as pomegranate, has gained prominence since their antifungal and anti-biofilm activities have already been demonstrated. *P. granatum* exhibits antimicrobial activity through different mechanisms, including enzyme inhibition, membrane disruption, anti-biofilm activity, and quorum sensing [12]. It exhibits activity in combination with calcium hydroxide ($\text{Ca}(\text{OH})_2$) against *Enterococcus faecalis* and *Candida albicans* polymicrobial biofilms [9], and *Cryptococcus* biofilms [10]; however, there is a lack of research on the mechanisms of action of the *P. granatum* extract against cryptococcal cells.

This study aimed to evaluate the in vitro activity of the hydroethanolic extract of the leaf of *P. granatum* alone or in combination with antifungal agents against *C. neoformans* and *C. gattii*, and *Cryptococcus* mitochondrial membrane interference by *P. granatum* using flow cytometry.

2. Materials and Methods

2.1. Preparation of Hydroalcoholic Extract from the Leaves of *P. granatum* L.

The hydroethanolic extract of the leaf of the *P. granatum* (HEPg) plant was prepared from collections at the Ático Seabra Herbarium of the Universidade Federal do Maranhão in São Luís, Maranhão, Brazil, and a voucher specimen was deposited (voucher number 01002) [9]. The leaves underwent the entire drying process at 40 °C, grinding to obtain a powder, mixing in 70% alcohol, with occasional agitation for 7 days at room temperature. The lyophilised extract obtained by rotary evaporation and drying was kindly provided by Lídio G. Lima Neto (UNICEUMA). The extract of *P. granatum* L. was previously characterised by Marques et al. [13] and Pinheiro et al. [14]. For the antifungal test, the HEPg powder was diluted in distilled water to a concentration of 100 mg/mL.

2.2. Strains

The standard American Type Culture Collection (ATCC) strain of *C. neoformans* ATCC 24067 and three clinical isolates (*C. neoformans* 62066, *C. gattii* 196 L, and *C. gattii* 23109) provided by the Culture Collection of the Mycology Laboratory of the Federal University of Minas Gerais and maintained in the Culture Collection of the Environmental Microbiology Laboratory (LAMAM) of CEUMA University were used. The samples were cultured on Sabouraud Dextrose Agar (SDA) medium for growth at 37 °C for 48 h.

2.3. Inoculum Preparation

The inocula were prepared from isolates grown on SDA, where an aliquot of each sample was transferred to 4 mL of sterile saline solution until reaching the turbidity level corresponding to the 0.5 tube on the McFarland scale, thus obtaining a concentration of 10^6 CFU/mL corresponding to yeasts. Then, 10 µL of the inoculum was diluted in 9990 µL of RPMI to reach a concentration of 10^3 CFU/mL.

2.4. Determination of Minimum Inhibitory Concentration (MIC)

To determine the MIC, 96-well microplates were used for a microdilution test in RPMI medium (Sigma-Aldrich, Burlington, MA, USA), according to CLSI [15]. The drug concentrations ranged from 0.000125 to 0.064 mg/mL for fluconazole (FLC; Sigma-Aldrich), 0.03 to 16 µg/mL for amphotericin B (Sigma-Aldrich), and 0.00003 to 0.016 mg/mL for HEPg.

Fungal inoculum was added to all wells at a concentration of 10^3 CFU/mL, except for the negative control (NC).

2.5. Determination of Fractional Inhibitory Concentration (FIC)

The antifungal agents and HEPg were evaluated individually and in combination. The 'checkerboard' microdilution method [16], which provides a matrix with all possible combinations of the drug and extract in the required concentration range, was used to test the susceptibility of *Cryptococcus*. In the NC wells, only the RPMI medium was added, and in the growth control wells, 100 μ L of RPMI plus 100 μ L of the inoculum was added. In the combination wells, 100 μ L of the inoculum, 50 μ L of the extract, and 50 μ L of the antifungal agent were added. The reading was performed to determine 100% inhibition of growth. The interactions between these substances were quantitatively evaluated by determining the FIC. The formula for calculating FIC was as follows: $FIC = [\text{antifungal MIC in combination} / \text{antifungal MIC}] + [\text{extract MIC in combination} / \text{extract MIC}]$. The FIC was calculated for all possible combinations of the same isolate, and the final result was expressed as the mean of the FICs. In addition, an interaction curve was constructed. The interaction was classified as synergism when the $FIC \leq 0.5$; indifferent if $0.5 > FIC \leq 4.0$; and antagonism for $FIC > 4.0$ [17].

2.6. Evaluation of Mitochondrial Membrane Potential ($\Delta\Psi_m$) Using Flow Cytometry

For the flow cytometry assay, the Rhodamine 123 probe was used to determine the mitochondrial membrane potential ($\Delta\Psi_m$).

Two isolates were chosen: *C. gattii* (196L) and *C. neoformans* (62066). The isolates were subcultured in the SDA medium and incubated for 48 h at 37 °C. The inocula were prepared in test tubes containing 4 mL of sterile saline solution, to which an aliquot of each sample was transferred until it reached the turbidity level corresponding to tube 0.5 on the McFarland scale, thus obtaining a concentration of 10^6 CFU/mL.

The following groups were tested: control (RPMI + inoculum), fluconazole control (RPMI + inoculum + fluconazole), amphotericin B control (RPMI + inoculum + amphotericin B), HEPg control (RPMI + inoculum HEPg), combination of HEPg and fluconazole (RPMI + inoculum + HEPg + fluconazole), and combination of HEPg and amphotericin B (RPMI + inoculum + HEPg + amphotericin B). The samples were then incubated for 12 h at 37 °C. Fluconazole, amphotericin B, and HEPg were added at the MIC, individually or in combination.

After the incubation period, the cells were washed three times with PBS (pH 7.2) and centrifuged at 6000 rpm for 10 min. After washing the cells, PBS (500 μ L) was added, and they were labelled with Rhodamine 123 (10 μ g/mL) in the dark for 10 min (Sigma-Aldrich). After incubation, the cells were washed three times with PBS. Then, PBS was added, and the analysis was performed on a flow cytometer (BD AccuriTM, Piscataway, NJ, USA; FL1 for Rhodamine 123). Cryptococcal cell population was delimited based on its size (FSC) and granularity (SSC), and cell debris stayed out of the gathered region. Controls of unstained and Rhodamine 123-stained non-fluorescent cryptococcal cells (FL1 channel of the BD Accuri C6 cytometer), untreated with HEPg, were the reference of a normal polarised mitochondrial potential, while the cells that presented Rhodamine 123 fluorescence were the reference of a disturbed mitochondrial potential. A total of 10,000 events were analysed for each sample. Changes in the fluorescence intensity of Rhodamine 123 were quantified using the variation index (VI) obtained using the equation $(MT-MC)/MC$, where MC is the mean fluorescence intensity of the control and MT is the mean of the treated cells. Negative VI values correspond to membrane depolarisation. Data represent the mean of two independent experiments. * $p < 0.05$ was significant.

2.7. Statistical Analysis

The results are presented as mean $\pm$ standard deviation. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA, USA). The results were statistically analysed for significant differences between groups using analysis of variance and *t*-tests. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Phytochemical Analysis of *P. Granatum* Leaf (PgL)

According to Marques et al. [13], flavonoids (flavanone, flavone, flavonol, and xanthones), phenolic acids, and coumarins were detected by phytochemical analyses of the hydroalcoholic leaf extract of *P. granatum* (HEPg), based on colour intensity and/or precipitate formation. In addition to the compounds, *P. granatum* leaves also contain other active compounds, such as tannins and phenolics, including gallic and ellagic acid, while flavonoids are rich in quercetin, kaempferol, and catechin [12].

3.2. HEPg Alone and in Combination with Antifungals Showed Activity Against *Cryptococcus*

The antifungal activity of the hydroethanolic extract of *P. granatum* (HEPg) was observed against *Cryptococcus* isolates, with MIC values of 0.03 mg/mL (*C. gattii* 23109), 0.06 mg/mL (*C. neoformans* ATCC 24067), and 1 mg/mL (*C. gattii* 196 L), except in *C. neoformans* 62066, which did not show growth inhibition at the concentrations tested (Table 1).

Table 1. Minimum inhibitory concentration (MIC) of the hydroethanolic extract of *P. granatum* (HEPg), fluconazole, and amphotericin B against *Cryptococcus* spp.

Minimum Inhibitory Concentration (MIC)			
Isolates	<i>P. granatum</i> (mg/mL)	Fluconazole (mg/mL)	Amphotericin B (mg/mL)
<i>Cryptococcus neoformans</i> 24067 ATCC	0.06	0.016	0.0005
<i>Cryptococcus neoformans</i> 62066	>16	0.004	0.00025
<i>Cryptococcus gattii</i> 196L	1	0.032	0.0125
<i>Cryptococcus gattii</i> 23109	0.03	0.016	0.00025

3.3. HEPg in Combination with Fluconazole Showed Synergism Against *C. gattii*

Regarding the interaction between HEPg and antifungal activity against *Cryptococcus*, synergism was observed only in the combination of the extract with fluconazole (HEPg + FLC) with an FIC of 0.18 against *C. gattii* 196 L, whereas there was no difference in the interaction with amphotericin B (HEPg + AMB). The combinations (HEPg + FLC and HEPg + AMB) against the other isolates also showed no difference ($0.5 > \text{FIC} \leq 4.0$) based on the values listed in Table 2. However, when the FIC values were individually considered between HEPg + FLC against *C. gattii* 23109, there was a tendency towards synergism (medium FIC: 0.24) at lower azole concentrations (0.002 and 0.004 mg/mL).

Table 2. Fractional inhibitory concentration (FIC) of HEPg hydroethanolic extract of *P. granatum* (HEPg) in combination with antifungals.

Isolated	FIC Fluconazole	Interaction	FIC Amphotericin B	Interaction
C. n 24067 ATCC	2.65	Indifferent	0.99	Indifferent
C. g 196L	0.18	Synergism	1.16	Indifferent
C. g 23109 1	1.10	Indifferent	1.16	Indifferent
C. n 62066	2.83	Indifferent	1.49	Indifferent

C. n: *Cryptococcus neoformans*; C. g: *Cryptococcus gattii*. Synergism: ≤ 0.5 ; Indifferent: $0.5 < x < 4.0$.

For *C. neoformans* 62066, the mean FIC values did not differ between the two combinations. Although the FIC values were considered individually, the effect showed no difference between concentrations.

To analyse the effects of HEPg alone or in combination with antifungal drugs on the mitochondrial membrane potential, yeast cells were stained with Rhodamine 123 (Figure 1).

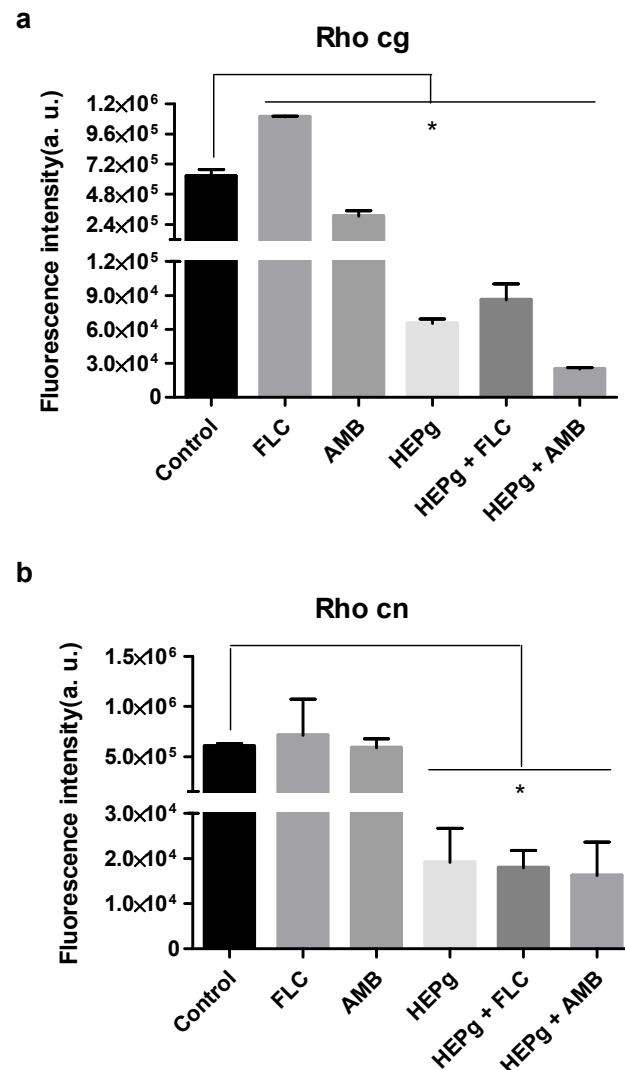


Figure 1. Determination of mitochondrial membrane potential ($\Delta\Psi_m$) in isolates *C. gattii* 196L (a) and *C. neoformans* 62066 (b), respectively, using the Rhodamine 123 probe. Arbitrary units of fluorescence (AU). An asterisk represents statistical differences between the treatments and the control * ($p < 0.05$). The tests were performed in duplicate. Control, fungus + RPMI; FLC, fluconazole + fungus + RPMI; AMB, amphotericin B + Fungus + RPMI; HEPg, extract + fungus + RPMI; HEPg + FLC, extract + fluconazole + fungus + RPMI; HEPg+AMB, extract + amphotericin B + fungus + RPMI. *: ($p < 0.05$).

The decrease in Rhodamine 123 fluorescence, indicative of mitochondrial membrane depolarisation, was more pronounced when *C. gattii* was previously treated with HEPg, either alone ($p = 0.004$) or in combination with FLC ($p = 0.004$) and AMB ($p = 0.003$), and was more subtle after prior treatment with AMB alone ($p = 0.0213$) when compared with the fluorescence of the FLC-alone ($p = 0.006$) and untreated control groups ($p < 0.05$). In contrast, prior treatment of *C. gattii* with FLC promoted the hyperpolarisation of mitochondrial membranes ($p > 0.05$), as illustrated in Figure 1a. The pretreatment of *C. neoformans* with HEPg alone ($p = 0.001$) or in combination with FLC ($p = 0.001$) and AMB ($p = 0.001$) promoted the depolarisation of mitochondrial membranes compared with that of the control ($p < 0.05$).

In contrast, treatment with fluconazole ($p = 0.718$) tended to cause the hyperpolarisation of the mitochondrial membranes, whereas AMB alone ($p = 0.862$) did not cause significant disturbances in membrane polarisation (Figure 1b).

4. Discussion

The increasing antifungal resistance of pathogens that cause disabling mycoses in humans is one of the biggest challenges for healthcare centres worldwide, given the difficulties in developing and licencing more effective antifungal drugs. In this scenario, medicinal plants may offer a less laborious route to obtain compounds with antifungal activity that are well tolerated by the host. In Brazil, despite the recent introduction of 5-flucytosine in Unified Health [18], the low availability of this drug restricts its use in the treatment of patients with cryptococcal meningitis, with fluconazole remaining the first choice for this purpose. Additionally, the emergence of strains resistant to this azole [19,20] led to the search for natural compounds with antifungal activity.

Although the antimicrobial activity of *P. granatum* has been extensively studied, data on its mechanisms of action are scarce. Sousa et al. [9] observed that the extracts of *P. granatum* displayed low cytotoxicity at a concentration of 1 mg/mL. This concentration was effective against all three *Cryptococcus* species tested in this study. Our group observed that an ethanolic extract of *P. granatum* leaves, in combination with calcium hydroxide, showed activity against mono- and polymicrobial biofilms formed by *E. faecalis* and *C. albicans* [9]. Kommalapati et al. [21] observed that the albedo extract of *P. granatum* was effective against *Candida* spp. isolated from the oral cavity of patients. Farhat et al. [22] observed that the dried powder of *P. granatum* (POMANOX® P30) exhibited antifungal activity against *C. albicans* and *C. glabrata*.

Lin et al. [23] observed that tryptanthrin, an alkaloid obtained as yellow needle-like crystals from the sublimation of indigo, exhibits antifungal activity against *C. neoformans*, *C. deuterogattii*, and *C. gattii* by inducing cell cycle arrest in the G1/S phase. Previously, we noticed that the acetate fraction of *P. granatum* extract inhibited growth and disrupted *Cryptococcus laurentii* biofilm formation [10]. Based on a survey of medicinal plants commonly used against fungal infections, Cruz et al. [24] certified that the water infusion extracts of *Ziziphus joazeiro* and *Caesalpinia pyramidalis* showed antifungal activity against *Trichophyton rubrum*, *Candida guilliermondii*, *C. albicans*, *C. neoformans*, and *Fonsecaea pedrosoi*, when compared to amphotericin B. In the present study, we showed that HEPg inhibited *Cryptococcus* growth by depolarising the mitochondrial membranes. The combination of HEPg and antifungal drugs tended to enhance the antimicrobial activity against *Cryptococcus*, at least when fluconazole was used, although the interaction of HEPg with AMB did not improve the antifungal activity. Santos et al. [25] evaluated the dynamic interaction between fluconazole and amphotericin B against *C. gattii* and showed that both drugs showed a tendency towards antagonism, but the interaction was concentration-dependent.

Further studies on the isolation and biological activity of the active compounds from *P. granatum* leaves are required. Ellagic acid is a phenolic found and has antifungal potential. A liposomal formulation of ellagic acid was effective in the treatment of *C. neoformans* in leukopenic mice and may represent a promising therapeutic formulation for cryptococcosis in immunocompromised individuals [26].

The results of the present study show that *P. granatum* interferes with the polarisation of the mitochondrial membrane of *Cryptococcus* spp., and this mechanism is species-dependent. Electron transport in complex III is partially inhibited by mitochondrial hyperpolarisation, leading to the accumulation of electrons that generate reactive oxygen species (ROS) [27]. In addition, studies have shown that antifungals, such as fluconazole, induce the accumulation of intracellular ROS in *C. neoformans* [28], which corroborates the data on mitochondrial

hyperpolarisation caused by azole in the present study. According to Silva et al. [29], in a study that evaluated the antifungal activity of berberine against a strain of *C. albicans* resistant to fluconazole, after 24 h of exposure to this clinically used antifungal, no change was observed in the mitochondrial membrane potential. However, when treated with various concentrations of berberine, mitochondrial dysfunction was observed. Folly et al. [30] did not observe any changes in mitochondrial membrane depolarisation when *Xyloasma prockia* (Turcz). was tested against the four strains of *C. neoformans* and *C. gattii* after 24 h of treatment.

To our knowledge, this study is the first to highlight mitochondrial damage in *C. caused by P. granatum*. Understanding the mechanisms of action of these natural products may contribute to the development of therapeutic strategies for cryptococcosis.

5. Conclusions

Considering the growing search for alternative ways to treat cryptococcosis, the present study highlights the antifungal potential of *P. granatum* alone and in combination with antifungal drugs against *Cryptococcus* spp. isolates, owing to its antimicrobial action and the depolarisation or hyperpolarisation of mitochondrial membranes. Knowledge of the mechanisms of action of *P. granatum* against cryptococcal cells will contribute to research on new therapeutic alternatives for cryptococcosis. Further studies are needed to elucidate the adjacent mechanisms of action and isolate active compounds to evaluate their anticryptococcal activity, as well as in vivo testing, such as alternative and murine models of fungal infections.

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