

Quercetin and rutin as potential agents antifungal against *Cryptococcus* spp

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Abstract

Amphotericin B is a fungicidal substance that is treatment of choice for most systemic fungal infections affecting as cryptococcosis the immunocompromised patients. However, severe side effects have limited the utility of this drug. The aim of this study was to evaluate the antifungal effect of the combination of amphotericin B with quercetin or rutin and as a protective of cytotoxic effect. The antifungal activity to amphotericin B, quercetin and rutin alone and in combination was determined in *Candida* sp and *Cryptococcus neoformans* strains. Cytotoxicity test on erythrocytes was performed by spectrophotometric absorbance of hemoglobin. The amphotericin B MIC was reduced when used in combination with quercetin or rutin to *C. neoformans* ATCC strain and reduced when combined with rutin to a clinical isolate of *C. neoformans*. In addition, the combination of quercetin with amphotericin B may reduce the toxicity of amphotericin B to red blood cells. Our results suggest that quercetin and rutin are potential agents to combine with amphotericin B in order to reduce the amphotericin dose to lessen side effects and improve antifungal efficacy.

Keywords: amphotericin B, quercetin, rutin, *Cryptococcus neoformans*.

Quercetina e rutina: potenciais agentes para terapia antifúngica

Resumo

A anfotericina B é uma substância fungicida e é o tratamento de escolha para a maioria das infecções fúngicas sistêmicas que afetam os pacientes imunocomprometidos, como a criptococose. No entanto, as severas reações adversas têm limitado a utilização desta droga. O objetivo deste estudo foi avaliar o efeito antifúngico e o potencial efeito protetor de citotoxicidade da combinação de anfotericina B com quercetina ou rutina. A atividade antifúngica de anfotericina B, quercetina e rutina, isoladamente e em combinação foi determinada em cepas de *Candida* sp e *Cryptococcus neoformans*. O teste de citotoxicidade em eritrócitos foi realizado por espectrofotometria, através da determinação da absorbância da hemoglobina. A concentração inibitória mínima da anfotericina B foi reduzida quando utilizada em combinação com a quercetina e rutina em *C. neoformans* ATCC e reduzida quando combinados com rutina em um isolado clínico de *C. neoformans*. Além disso, a combinação de quercetina com anfotericina B pode reduzir a toxicidade da droga em eritrócitos. Os resultados sugerem que quercetina e rutina são potenciais agentes para combinação com anfotericina B, a fim de reduzir a dose de anfotericina, diminuindo os efeitos colaterais e melhorando sua eficácia antifúngica.

Palavras-chave: anfotericina B, quercetina, rutina, *Cryptococcus neoformans*.

1. Introduction

Cryptococcus neoformans is a fungal pathogen that causes meningoencephalitis as the most frequent clinical manifestation in immunocompromised patients, mainly in persons infected by HIV. The mortality of disseminated cryptococcosis is 17% in treated patients with systemic

antifungal agents. The treatment regimens for cryptococcal meningitis are focused on amphotericin B (amB) alone or associated with azoles, especially fluconazole (Negrini, 2012). However, the therapy with amB has been limited by severe toxic side effects especially nephrotoxicity

(Laniado-Laborín and Cabrales-Vargas, 2009). Attempts have been made to reduce amB toxicity by combination with other antifungal agents or natural products because combination therapy can promote synergistic antifungal effect with the advantage of reduced toxicity. This research approach ranges from evaluation of antifungal activity of plant extracts to isolated products plant. Höfling et al. (2010) shows strong activity of *Punica granatum*, *Syzygium cumini*, *Rosmarinus officinalis*, *Arrabidaea chica* *Mentha piperita* and *Tabebuia avellanedae* against *Candida* species. Giordani et al. (2006) demonstrated that essential oil from *Cinnamomum cassia* synergistically increased the *in vitro* antifungal activity of AmB against *Candida albicans*. Another study demonstrated a synergistic effect between allicin and amB against *C. albicans* (An et al., 2009). Quercetin and Rutin, both polyphenolic flavonoids, stand out among the natural products, through numerous studies. Studies about the antimicrobial activity have shown that quercetin has activity on *Staphylococcus aureus* and *Clostridium botulinum*, and *in vitro* antibacterial activity against the periodontal pathogens *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* (Naghski et al., 1947; Andersen and Berry, 1947; Geoghegan et al., 2010). Other authors also described the action of quercetin against *Helicobacter pylori* and *Escherichia coli* as well as synergistic activity with lecithin against *Bacillus subtilis* and the hepatitis A virus (Wu et al., 2008; Ramadan and Selim Asker, 2009). Rutin was shown to be effective against *Bacillus cereus*, *Salmonella enteritidis* and also in the treatment of arthritis caused by *Candida albicans* (Arima et al., 2002; Pereira et al., 2007; Han, 2009). The aim of this study was to evaluate the antifungal effect of the combination of amB with quercetin or rutin and the potential protective effect of quercetin and rutin on amB cytotoxicity.

2. Material and Methods

2.1. Materials

Liquid RPMI 1640 medium with L-glutamine and without sodium bicarbonate, morpholinepropanesulfonic acid (MOPS), dimethyl sulfoxide (DMSO), quercetin and rutin was purchased from Sigma® (St. Louis, MO, USA). AmB (Iffect Chemphar Co. Ltd., China).

2.2. Antifungal activity

2.2.1. Strains

a) *Cryptococcus neoformans* ATCC 90112 and one clinical isolate of *C. neoformans*. Antifungal activity was determined by broth microdilution using serial dilutions in RPMI 1640 medium, as described by the European Committee for Antibiotic Susceptibility Testing (EUCAST) with some modifications (Rodríguez-Tudela et al., 2008). This protocol was chosen because it provides a better performance of growth for this yeast.

b) *Candida albicans* ATCC 14053, *C. albicans* ATCC 64546, *C. krusei* ATCC 6258 and one clinical isolate of *Candida tropicalis* that was obtained from a blood sample. Antifungal activity was determined by broth microdilution, as described by the Clinical and Laboratory Standards Institute (CLSI, 2008), method M27-A3 with some modifications. The Preparation of stock solution of amB also followed the recommendations of the CLSI.

2.2.2. Procedure

RPMI 1640 medium, buffered with 0.165M MOPS, was used for *in vitro* antifungal analyses. The medium was adjusted to pH 7.0 and filtered. An amB stock solution was prepared in DMSO, and stock solutions of quercetin and rutin were prepared in ethanol.

a) Cell suspensions of two strains of *C. neoformans* were prepared in RPMI medium with 2% glucose and adjusted to give final inoculum concentrations of 0.5×10^5 a 2.5×10^5 CFU.mL⁻¹ (EUCAST). The cells were incubated with different concentrations (125-31.25µg.mL⁻¹) of quercetin and rutin alone and in combination with amB (16-0.03µg.mL⁻¹) at 37 °C for 24 hours. The concentrations of natural compounds were chosen since they were the best concentrations in soluble RPMI. Medium alone inoculated with the fungal strain was used as a growth control, and uninoculated medium was used as the blank control. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that does not result in any visible growth of the yeast compared to the control. Each concentration was assessed in triplicate and in two independent experiments. The reading was always performed by more than one observer. To confirm the absence or reduction of growth, the content of each source was diluted 1:100 in saline and 20µL of each suspension was streaked in triplicate onto Sabouraud dextrose agar plates (Barchiesi et al., 2007). The plates were incubated at 35 ± 2 °C for 48h and the number of colonies on each plate was counted. The results were statistically analyzed by ANOVA with 95% confidence ($\alpha = 0.05$) and post hoc Tukey.

b) Cell suspensions of *Candida* spp. strains were prepared in RPMI medium and adjusted to give final inoculum concentrations of 0.5×10^3 to 5×10^3 CFU.mL⁻¹. The cells were incubated with different concentrations of quercetin and rutin and/or amB at 37 °C for 48 hours. Medium alone inoculated with the fungal strain was used as a growth control, and uninoculated medium was used as the blank control. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that does not result in any visible growth of the yeast compared to the control. Each concentration was assessed in triplicate and two independent experiments, the reading being performed always by more than one observer.

The quality control was performed with *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 in accordance with the recommendations of the CLSI M27-A3. The controls shown to be within the parameters established which makes

the validated experiment. The Ethics Committee of the Universidade Estadual do Centro-Oeste, Guarapuava, Brazil, approved all experiments. (Protocol COMEP 193/2011).

2.3. Cytotoxicity in vitro on human erythrocytes

2.3.1. Preparation of erythrocyte suspension

Freshly collected human blood, obtained from one healthy donor, was treated with 10µL of heparin (5.000UI/mL, Cristália Ltda) and centrifuged at 2500rpm for 5 min to separate the plasma from the erythrocytes. The pelleted erythrocytes were washed three times with isotonic PBS (pH7.4), and the supernatant was carefully removed after each wash. After the final wash, the packed cells were re-suspended in PBS, and the hematocrit was determined (Koga et al., 1998). The Ethics Committee of the Universidade Estadual do Centro-Oeste, Guarapuava, Brazil, approved all experiments. (Protocol COMEP 193/2011).

2.3.2. Hemolysis measurements

The hemolysis studies were performed with erythrocytes suspended in PBS (3mL) at a hematocrit of 2%. A 0.037mL volume of free AmB (10µg.mL⁻¹) in DMSO was added to the cell suspension. The final DMSO concentration (1.0%) in the suspension had no effect on the erythrocytes. The quercetin and rutin in ethanol were added to the suspensions at different concentrations (6.25-200µg.mL⁻¹). Erythrocytes suspended in PBS were used as a blank control. Suspensions containing only free AmB were used as positive controls of hemolysis. The suspensions were incubated at 37 °C with continuous shaking in the dark and were monitored until the controls showed significant hemolysis. After incubation, the erythrocyte suspensions were centrifuged at 2500rpm for 5 min and the hemolysis degree was estimated by visible spectroscopy at 540nm from the hemoglobin released into the supernatant (Koga et al., 1998). The results are expressed as the percentage of inhibition of AmB-induced hemolysis, calculated according to the following formula: hemolysis inhibition % = [(Ab-Aa)/Ab]×100, where Ab is the absorption of the control and Aa is the absorption of the sample (Dyppbukt et al., 2005).

3. Results and Discussion

The results show that quercetin and rutin alone showed no antifungal effects against *C. neoformans*. However, when these compounds were tested associated with amB they showed improvement in antifungal activity of amB. According to Table 1, in *C. neoformans* ATCC 90112, the amB MIC was 0.25µg.mL⁻¹; however, when used in combination with quercetin

(at concentrations of 31.25, 62.5 and 125µg.mL⁻¹) or rutin (at a concentration of 62.5 and 125µg.mL⁻¹), this value was reduced to 0.125 µg.mL⁻¹ and 0.0625µg.mL⁻¹, respectively. When rutin was tested at a concentration of 31.25µg.mL⁻¹, the amB MIC was reduced to 0.125µg.mL⁻¹. In relation to the clinical isolate of *C. neoformans*, the amB MIC was also 0.25µg.mL⁻¹, and in combination with rutin (125µg.mL⁻¹) this concentration was reduced to 0.0625µg.

Table 1. Results of susceptibility test for determining the MIC of amB, quercetin and rutin alone and in combination against two strains of *Cryptococcus neoformans* by broth microdilution assay.

Compounds	CIM (µg.mL ⁻¹)	
	<i>Cryptococcus neoformans</i> ATCC 90012	<i>Cryptococcus neoformans</i> Clinical isolate
Amphotericin (AmB)	0.25	0.25
Quercetin (QR)	> 125	> 125
Rutin (RUT)	>125	>125
AmB+ QR (125µg/mL)	0.125	0.25
AmB+ QR (62.5µg/mL)	0.125	0.25
AmB+ QR (31.25µg/mL)	0.125	0.25
AmB+ RUT (125µg/mL)	0.0625	0.0625
AmB+ RUT (62.5µg/mL)	0.0625	0.25
AmB+RUT (31.25µg/mL)	0.125	0.25

mL⁻¹. These results were confirmed by counting the CFU that are presented in Figure 1. It was observed that amB tested at a subinibitory concentration showed no effect on the CFU count; however, when used in combination with quercetin (Figure 1b) and rutin (Figures 1a and c) the CFU count was reduced significantly (p< 0,05). Thus the CFU counts confirm and complement the results obtained in tests microdilution broth which also corroborates their validation. Although quercetin and rutin alone and in combination with amB showed no effects on the *Candida* strains. The MIC value for amB on *Candida albicans* ATCC 14053 and ATCC 64546 was 0.025 µg.mL⁻¹ and on *Candida kruzei* ATCC 6258 and *Candida tropicalis* were 0.25 µg.mL⁻¹ and 0.5 µg.mL⁻¹ respectively. The MICs values for amB combined with quercetin and rutin which were the same as found for amphotericin tested alone on all strains tested.

The good effect found for *Cryptococcus* strains suggests that the mechanism of action of these compounds can be related to the structure of these yeasts. These results corroborate the study by Faria et al. (2011) that show enhanced activity of antifungal amB using natural phenolics compounds against strains of *Cryptococcus* sp. The amB is toxic because of its high affinity for the ergosterol that is cholesterol analogue present in mammalian cell membranes. Thus, its use has been limited in many patients because it almost always results in some degree of renal impairment, which ranges in severity depending on the total dose (Park et al., 2011). Thus, a more rational treatment could be the combination of amB with compounds that enhance its action, so that smaller doses of drug can

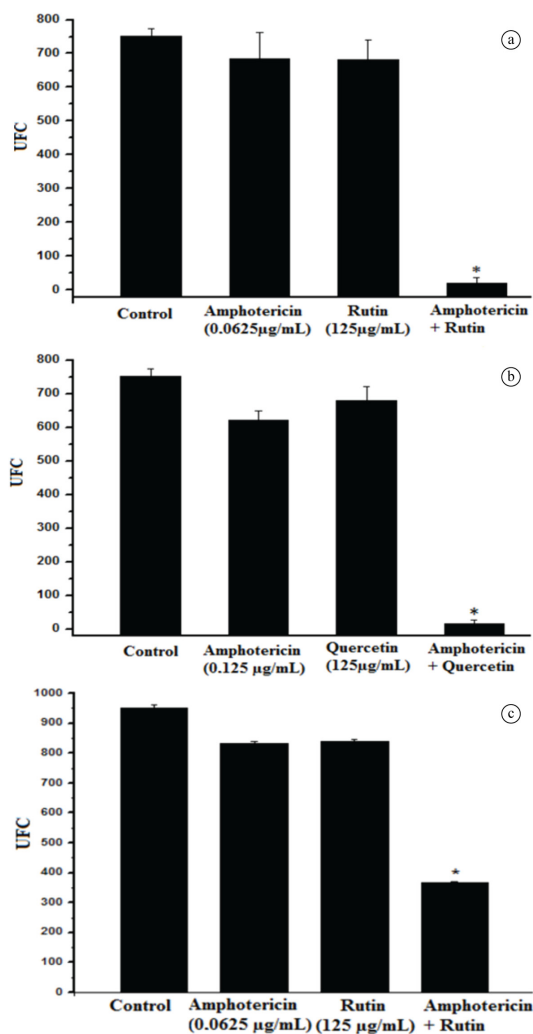


Figure 1. Effect of quercetin, rutin and amB alone and combination on mean growth (CFU) of *C. neoformans* ATCC 90012 (a and b) and clinical isolate *C. neoformans* (c) in sabouraud dextrose agar plate. Colony counts were determined after incubation at 37 °C for 48h, of triplicate cultures \pm standard deviation. *Combination of amphotericin (0.0625 $\mu\text{g}\cdot\text{mL}^{-1}$) + rutin (125 $\mu\text{g}\cdot\text{mL}^{-1}$) or amphotericin (0.125 $\mu\text{g}\cdot\text{mL}^{-1}$) + quercetin (125 $\mu\text{g}\cdot\text{mL}^{-1}$) was significantly different from all other groups. ($p < 0.05$).

be used, or with compounds that protect cells or tissues that are targets of amB-mediated toxicity. Some studies have already explored this possibility. For example, one group demonstrated the synergistic activity of grape seed extract (*Vitis vinifera*) and amB against *Candida* sp (Han, 2007b). This effect was also observed when amB was combined with the natural compounds berberine, alicin, epigallocatechin gallate and with essential oils of *Melaleuca alternifolia*, *Origanum vulgare*, *Pelargonium graveolens*, *Cinnamomum cassia*, *Coriandrum sativum*, *Thymus maroccanus* e *Thymus broussonetii* (Han and Lee, 2005; Han, 2007a; Rosato et al., 2008; An et al., 2009; Saad et al., 2010; Silva et al., 2011).

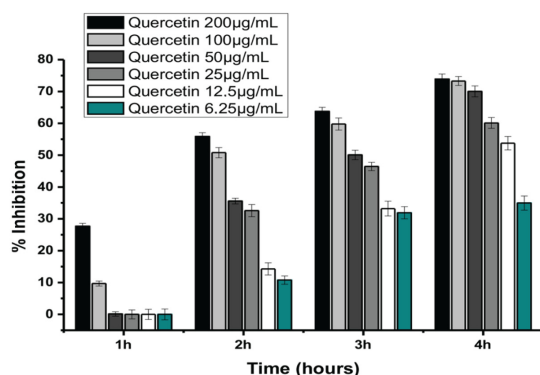


Figure 2. Inhibitory effect of different concentrations (6.25-200 $\mu\text{g}\cdot\text{mL}^{-1}$) of quercetin in hemolysis induced by amphotericin B (10 $\mu\text{g}\cdot\text{mL}^{-1}$).

The number of studies with natural products that show increased activity of amB on strains of *Cryptococcus* sp is much lower than found for strains of *Candida* sp. Davis et al. (1994) show an *in vitro* synergistic fungistatic activity effect of concentrated *Allium sativum* extract with amB against *C. neoformans*. Another study also showed an increase in antifungal activity of amB in the presence of acteoside, a phenylethanoid glycoside from *Colebrookea oppositifolia* (Ali et al., 2011).

The mechanisms involved in synergy with amphotericin and most of the natural compounds are not well understood. However, several studies suggest that the subinhibitory concentrations of amB facilitated the uptake of natural compounds, which resulted in increased killing of the fungal cells (Hirasawa and Takada, 2004; Ali et al., 2011).

Our results suggest that quercetin and rutin enhance the antifungal activity of amB. Additional *in vivo* tests are required to perform synergy tests and to evaluate the antimicrobial potential of these compounds for optimize the therapeutic conduct and reduce the toxic effects of the drug. The toxicity tests show that quercetin protects red blood cells from amB-mediated toxicity. The inhibition of hemolysis by quercetin was dose-dependent and increased with time (Figure 2). Initially, quercetin showed an inhibition of hemolysis of 27.69%, 8.82% and 1.65% at concentrations of 200 $\mu\text{g}\cdot\text{mL}^{-1}$, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ and 50 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. After four hour the inhibition of hemolysis was 55.86%, 50.79%, 35.57%, 32.58%, 14.63% and 10.77% at concentrations of 200 $\mu\text{g}\cdot\text{mL}^{-1}$, 100 $\mu\text{g}\cdot\text{mL}^{-1}$, 50 $\mu\text{g}\cdot\text{mL}^{-1}$, 25 $\mu\text{g}\cdot\text{mL}^{-1}$, 12.5 $\mu\text{g}\cdot\text{mL}^{-1}$ and 6.25 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively. Inhibitory effect was not observed with rutin. Studies have shown the involvement of oxidative stress in the antifungal activity of amB. This process may be explained by the generation of reactive oxygen species and hydroxyl radicals, promoted by the drug, leading to fungal cell damage through oxidation of proteins, peroxidation of membrane lipids or DNA or RNA cleavage (Brajtburg et al., 1985). Our results show that when amB is used with quercetin (potent antioxidant compound) cellular toxic effects are reduced. This reveals that quercetin is a potential adjunct to antifungal therapy with amB.

4. Conclusion

The current limitations in antifungal therapy stimulate the search for new alternatives to achieve therapeutic success. Among them are natural products that are attractive prototypes for this purpose, due to their broad spectrum of biological activity and their abundance in nature and low cost. Our results do suggest that quercetin and rutin are potential therapeutic agents for use in combination with amB since it has the advantage of reducing the toxicity of the drug and also improves their antifungal activity. But more studies are needed to determine the potential utility of this combination therapy.

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