



Black and white teas as potential agents to combine with amphotericin B and protect red blood cells from amphotericin B-mediated toxicity

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Abstract

Amphotericin B is a fungicidal substance that is treatment of choice for most systemic fungal infections affecting 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 black tea or white tea and protective of cytotoxic effect. The present study shows that white and black teas have additive effects with amphotericin B against some species *Candida*. In addition, the combination of white and black tea with amphotericin B may reduce the toxicity of amphotericin B to red blood cells. Our results suggest that white and black tea is a potential agent to combine with amphotericin for antifungal efficacy and to reduce the amphotericin dose to lessen side effects.

Keywords: amphotericin B, antifungal, black tea, *Camellia sinensis*, white tea, toxicity.

Chá preto e branco de *Camellia sinensis* como potenciais agentes para associar com anfotericina B

Resumo

A anfotericina B é o tratamento de escolha para a maioria das infecções fúngicas sistêmicas que afetam os doentes imunocomprometidos. No entanto, efeitos secundários graves têm limitado a utilidade desta droga. O objetivo deste estudo foi avaliar o efeito antifúngico da combinação de anfotericina B com chá preto ou chá branco, bem como o efeito citotóxico desta combinação sobre hemáceas. O presente estudo demonstra que o chá branco e preto de *Camellia sinensis* têm efeitos aditivos com anfotericina B contra algumas espécies de *Candida* sp. Além disso, a combinação de chá branco e preto com anfotericina B pode reduzir a toxicidade da anfotericina B em hemáceas. Nossos resultados sugerem que o chá branco e preto são agentes potenciais para associação com anfotericina B contribuindo para eficácia antifúngica, bem como redução de toxicidade.

Palavras-chave: anfotericina B, antifúngico, chá preto, *Camellia sinensis*, chá branco, toxicidade.

1. Introduction

Candida species are the most common cause of opportunistic fungal disease. *Candida* spp. is responsible for approximately 80% of fungal infections in hospitals and are the fourth most common nosocomial pathogen (Colombo and Guimaraes, 2003). Furthermore, candidemia has mortality rates between 40 and 60%, the diagnosis and treatment are difficult and expensive (Colombo et al., 2006; Tumbarello et al., 2007; Wenzel, 1995).

The treatment of these infections is based on the use of azole antifungal drugs such as ketoconazole, miconazole, itraconazole, fluconazole, and voriconazole (Pfaller, 2012).

Following its discovery in 1953, amphotericin B (amB) has remained the treatment of choice for most systemic fungal infections affecting immunocompromised patients (Pagano et al., 2010). However, severe side effects, infusion-related toxicities, the frequent association with renal damage and the intravenous formulation of amB, have limited the utility of this drug (Sawaya et al., 1995). Therefore numerous attempts have been made in recent years to reduce amB toxicity. Among these new developments, formulations such as a liposomal encapsulation base, lipid complexes, colloidal dispersions and nanoparticles have

been shown to cause less toxicity and increase therapeutic efficacy, but the high cost of these formulations have limited their use (Filippin and Souza, 2006; Fukui et al., 2003; Jung et al., 2009). Thus, reducing the amB dose by combining it with new antifungal products appears to be an important approach, as shown by Rosato et al. (2008), who demonstrated that essential oils increased the *in vitro* antifungal activity of amB against *Candida* spp (Rosato et al., 2008).

Previous work has demonstrated the antifungal activity of white and black teas, which is due to the differential preparation of the terminal leaves and apical buds of the plant *Camellia sinensis* (L.) (Almajano et al., 2008; Friedman, 2007; Sitheque et al., 2009). One study demonstrated that the antifungal activity of these teas and their individual component, theaflavin digallate inhibited the growth of *Trichophyton mentagrophytes* and *Trichophyton rubrum* (Okubo et al., 1991). Another study showed that that a combined effect between epigallocatechin gallate and amB blocked the formation of *C. albicans* hyphae (Han, 2007a). Black tea also increased the antibacterial activity of the several antibiotics, such as chloramphenicol, gentamicin, methicillin and nalidixic acid against some enterobacteria (Tiwari et al., 2005). The aim of this study was to evaluate the antifungal effect of the combination of amphotericin B with black tea or white tea and the potential protective effect of black and white teas on amB cytotoxicity in red blood cells.

2. Materials and Methods

2.1. Reagents and chemicals

Liquid RPMI1640 medium with L-glutamine and without sodium bicarbonate, morpholinepropanesulfonic acid (MOPS) and dimethyl sulfoxide (DMSO) was purchased from Sigma® (St. Louis, MO, USA), and trypan blue was purchased from Vetec® (São Paulo, Brazil). Amphotericin B (IfectChemphar Co. Ltd., China) was kindly donated by local vendor. All other chemicals were of analytical grade and of the highest commercially available purity. White and black teas (Prenda®, Brazil) were purchased from a retail market.

2.2. Tea infusion

The tea infusion stocks were prepared from 0.5g of dry leaves and 25ml of boiling sterile water or PBS (0.85% NaCl, 50mM sodium phosphate, pH 7.4). Each sample was incubated for 30 minutes at room temperature and then filtered. The tea infusions were then frozen at -20 °C (Bancirova, 2010). The concentrations are expressed according to the dry weight, according to guidelines established by the Brazilian Pharmacopoeia (ANVISA, 2010).

2.3. Antifungal activity

Strains: *Candida albicans* (ATCC 14053 and 64546) and *Candida krusei* (ATCC 6258) strains were used as a reference and eight different clinical isolates of *Candida* spp were evaluated in this study. Antifungal activities were determined by broth microdilution using serial dilutions in RPMI 1640 medium, as described by the Clinical and

Laboratory Standards Institute (2008), method M27-A3 with some modifications.

Medium: RPMI 1640 medium, buffered with 0.165M MOPS, was used for *in vitro* antifungal analyses. The medium was adjusted to pH 7.0 with 1.0M sodium hydroxide (NaOH) and filtered. An amB stock solution was prepared in DMSO, and stock solutions of white tea and black tea was prepared in sterilized water. Medium alone inoculated with the fungal strain was used as a growth control, and uninoculated medium was used as the blank control. Sterile 96-well microtiterplates were prepared with serial dilutions of the compounds. Cell suspensions of *Candida* spp strains were prepared in RPMI medium and adjusted to give final inoculum concentrations of 0.5×10^3 - 2.5×10^3 CFU/ml. The cells were incubated with different concentrations of white tea and black tea and/or amB at 37 °C for 24 hours. 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. Synergistic activity was determined by incubating cells with the teas and amB, both at subinhibitory concentrations. The fractional inhibitory concentration index (FICI) was defined as the sum of the MIC of each drug when used in combination divided by the MIC of the drug used alone. FICI values were interpreted as follows: $FICI \leq 0.5$, synergistic; $0.5 < FICI \leq 1$, synergistic to additive; $1 < FICI \leq 4$, indifferent; and $FICI > 4$, antagonistic (Arikan et al., 2002; Odds, 2003). The Ethics Committee of the Universidade Estadual do Centro-Oeste, Guarapuava, Brazil, approved all experiments.

2.4. Cytotoxicity to human erythrocytes *in vitro*

2.4.1. Preparation of erythrocyte suspension

Freshly collected human blood, obtained from one healthy donor, was treated with heparin 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).

2.4.2. Hemolysis assay

The hemolysis studies were performed with erythrocytes suspended in PBS (0.9mL) at a hematocrit of 5%. A 0.02mL volume of amB (16µg/mL) in DMSO was added to the cell suspension. The final DMSO concentration in the suspension had no effect on the erythrocytes. The teas were added to the suspensions at different concentrations (12.5 - $100 \mu\text{g} \cdot \text{mL}^{-1}$). Erythrocytes suspended in PBS were used as a blank control. The suspensions were incubated for 7 hours at 37 °C with continuous shaking in the dark. 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 % = $[(Ab-Aa)/Ab] \times 100$, where Ab is the absorption of the control and Aa is the absorption of the sample (Dyrbukt et al., 2005).

3. Results

Antifungal susceptibility tests showed that both the white and black teas inhibited the growth of the yeast cells and that black tea has a higher antifungal activity than white tea in all the strains tested. This antifungal activity was dose-dependent and was less effective than the antifungal activity of amB. The amB and black tea or white tea combination markedly reduced MICs for two clinical isolates and two ATCC strains. Based on the FICIs, an additive effect was observed for amB and black tea in two strains and for amB and white tea in four strains. No effect was observed in the remaining isolates. Regardless of the MIC endpoints, no antagonism was observed between amB and either of the teas (Table 1).

To explore the toxicity of black and white teas, as well as their possible protective effect on amB-induced cytotoxicity in red blood cells, hemolysis induced by the drug was measured in the absence and presence of the teas. The inhibition of hemolysis by both the black and white teas was dose-dependent, and black tea showed a greater inhibition of hemolysis than white tea (Figure 1). Black and white teas showed an inhibition of hemolysis at a

concentration $100\mu\text{g.mL}^{-1}$ of 63.31% and 52.18% respectively, while in the concentration of $50\mu\text{g.mL}^{-1}$ the inhibitions were respectively 59.25% and 31.95%. No inhibitory effect was observed at the other tested concentrations.

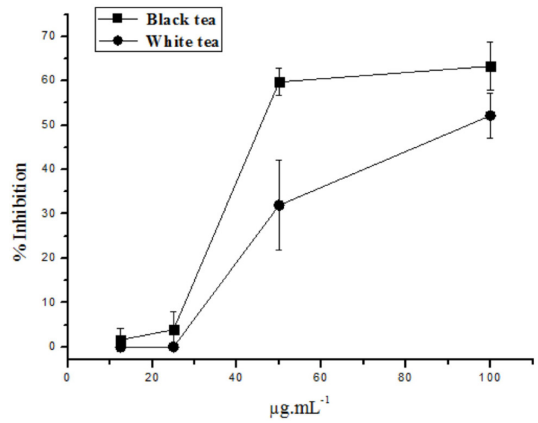


Figure 1. Inhibitory effect of white and black teas in hemolysis induced by amphotericin B.

Table 1. Antifungal activity of amB in the absence or presence of black and white teas.

Strains	MIC Black tea ($\mu\text{g.mL}^{-1}$)	MIC White tea ($\mu\text{g.mL}^{-1}$)	MIC amB ($\mu\text{g.mL}^{-1}$)	MIC amB + Black tea ($\mu\text{g.mL}^{-1}$)	FICI index Black tea + amB	MIC amB + White tea ($\mu\text{g.mL}^{-1}$)	FICI index White tea + amB
<i>Candida albicans</i> (ATCC 14053)	29.6	158.4	0.025	0.025	2 (I)	0.025	2 (I)
<i>Candida albicans</i> (ATCC 64546)	29.6	79.2	0.025	1.56×10^{-4}	0.506 (A)	1.56×10^{-4}	0.506 (A)
<i>Candida krusei</i> (ATCC 6258)	14.8	79.2	0.25	0.25	2 (I)	3.12×10^{-4}	0.501 (A)
<i>Candida albicans</i> (LAC15)	29.6	158.4	0.02	0.02	2 (I)	4×10^{-3}	0.7 (A)
<i>Candida parapsilosis</i> (LAC1)	29.6	79.2	0.02	5×10^{-4}	0.525 (A)	1×10^{-3}	0.55 (A)
<i>Candida utilis</i> (LAC2)	29.6	158.4	0.1	0.1	2 (I)	0.1	2 (I)
<i>Candida kefyr</i> (LAC4)	14.8	79.2	0.1	0.1	2 (I)	0.1	2 (I)
<i>Candida albicans</i> (LAC3)	14.8	158.4	0.02	0.02	2 (I)	0.02	2 (I)
<i>Candida tropicalis</i> (LAC6)	118.4	> 316.8	0.5	0.5	2 (I)	0.5	2 (I)
<i>Candida albicans</i> (LAC5)	14.8	79.2	0.02	0.02	2 (I)	0.02	2 (I)
<i>Candida albicans</i> (LAC13)	14.8	158.4	0.02	0.02	2 (I)	0.02	2 (I)

(I) indifferent effect; (A) additive effect.

4. Discussion

The amB is toxic because of its high affinity for the ergosterol present in fungal and 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 (Sawaya et al., 1995). Thus, a more rational treatment could be the combination of amB with compounds that enhance its action, so that smaller doses of drug can 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 venifera*) and amB against *Candida* sp (Han, 2007b). This effect was also observed when amB was combined with the natural compound berberine (Han and Lee, 2005). The present study demonstrated the effect of black and white teas on the antifungal activity of amB and its toxicity to human erythrocytes.

The antifungal susceptibility tests showed that white and black teas have antifungal activities for different species. Others studies also suggest that a compound isolated from the white tea, has an antifungal effect on *C. albicans* due to the inhibition of ergosterol synthesis (Navarro-Martinez et al., 2006; Park et al., 2006). Tea and some phenolic compounds as theaflavin digallate, has also display antibacterial and antifungal activity (Okubo et al., 1991; Hassani et al., 2009). Although compounds isolated from teas exhibit antifungal activity, the tea itself may be more effective. Due to the complexity of the tea composition, many interactions can occur between the compounds in tea infusions, leading to synergistic or antagonistic effects (Williamson, 2001). The composition of the teas of this study was carried out in other published work, through the Folin-Ciocalteu assay. This method showed a high overall content of phenols in the tested teas (Camargo et al., 2016). This study also shows the effect of black and white teas on the MIC of amB for some *Candida* sp. strains. According to the FICI values, this effect is additive. Although black tea has a higher antifungal activity, an additive effect of white tea and amB was observed in a greater number of strains. Han (2007a) also showed that epigallocatechin gallate, a compound isolated from white tea, has a synergistic effect with amB both *in vitro* and *in vivo*. An additive effect was also observed in some strains of *C. albicans*, *C. krusei*, and *C. parapsilosis*. This suggests that the additive effect may be restricted to certain types of species, which implies varying susceptibility patterns for each species. The genetic diversity within and among the different species analyzed must also be considered (Wang et al., 2007).

In addition, our results suggested that white and black tea was a potential agent to combine with amB for increase antifungal efficacy and to reduce the amB dose to lessen side effects. The combination may reduce the toxicity in red blood cells.

The results from the hemolysis tests show that black and white teas have a protective effect on the toxicity

induced *in vitro* by amB. Brajtburg et al. (1985), suggests oxidative damage as a component in the anticellular effect of amB and particularly its lytic action. It is demonstrated that drug-induced hemolysis was delayed in the presence of the antioxidant catalase, while accelerated of lysis was observed in the presence of pro-oxidants such as ascorbate. This suggests that the protective effect observed for white and black teas may be related to antioxidant mechanisms because this activity is well established in *C. sinensis* teas.

However, a limitation on the use of tea infusions for pharmaceutical applications is the low oral bioavailability of most tea components. Due of their large molecular weight, thearubigins and some catechins are not absorbed in the intestine (Zhu et al., 2000). Mulder et al. (2001) reported that the maximum concentration of theaflavin in human plasma and urine was only 1ng.mL⁻¹ and 4.2ng.mL⁻¹ respectively, following consumption of 700mg of a pure theaflavin mixture, equivalent to approximately 30 cups of black tea. This suggests that large quantities of black or white teas would need to be consumed to achieve the benefits shown in this study. However, the bioavailability and biotransformation of tea compounds need to be studied further because quantitative data on the blood and tissue levels of tea compounds are still scarce.

5. Conclusion

The present study showed that white and black teas have additive antifungal effects with amB against some *Candida* sp. strains and protect red blood cells from amphotericin B-mediated toxicity. Our results suggested that white and black teas could potentially be combined with amphotericin B to enhance antifungal efficacy, so that a lower dose of amphotericin B could be used to lessen side effects. Additional *in vitro* tests are required to perform synergy tests on other fungal strains and to evaluate the antimicrobial potential of tea infusions for new therapeutic applications.

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