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Original Article

Evaluation of synergistic anticandidal and apoptotic effects of ferulic acid and caspofungin against *Candida albicans*

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ABSTRACT

This study aimed to investigate the synergy between anticandidal and apoptotic effects of ferulic acid and caspofungin against *Candida albicans* and *Candida glabrata*, with the help of a quantitative checkerboard microdilution assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as a viability dye. Apoptotic effects of caspofungin and ferulic acid concentrations (alone and combined) were analyzed for *C. albicans* and *C. glabrata* based on annexin V–propidium iodide binding capacities using flow cytometric analysis. *C. albicans* showed a synergistic effect, represented by a fractional inhibitory concentration index of < 0.5, but *C. glabrata* showed no synergistic effect (fractional inhibitory concentration index > 0.5). Early and late apoptotic effects of caspofungin and ferulic acid concentrations (1 μg/mL and 1000 μg/mL) were calculated as 55.7% and 18.3%, respectively, while their necrotic effects were determined as 5.8% and 51.6%, respectively, using flow cytometric analyses. The apoptotic effects of the combination of caspofungin and ferulic acid at concentrations of 1 μg/mL and 1000 μg/mL on *C. albicans* and *C. glabrata* were 73.0% and 48.7%, respectively. Ferulic acid also demonstrated a synergistic effect in combination with caspofungin against *C. albicans*. Another possibility is to combine the existing anticandidal drug with phytochemicals to enhance the efficacy of anticandidal drug.

1. Introduction

A considerable increase in the incidence of deep fungal infections has been observed in the last two decades in hospital environments due to the increase in organ transplantations, rise in the incidence of AIDS, and use of invasive devices (such as catheters, artificial joints, and valves), and also in immunocompromised patients [1,2].

Candida albicans and *Candida glabrata* have been highly associated with several opportunistic fungal infections [3]. *Candida* species are the basis for the development of new antifungal drugs. However, increasing levels of *Candida* species resistant to the current antifungal drugs have been

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observed, making these agents ineffective [4]. Therefore, other therapies, which are more effective and safer than the current ones, are being explored; namely the use of plant extracts enriched in phenolic compounds [5,6].

Although the advances in medical and chemical fields have increased life expectancy, an increasing resistance of pathogenic microorganisms to conventional drugs has been observed due to the random use of chemicals and drugs, leading to the development of other complications [7].

Ferulic acid is a phenolic acid widely distributed in the plant kingdom [8]. It is one of the most abundant phenolic acids. It may be found in high concentrations in foods such as navy bean, corn bran, wheat bran, eggplant, artichokes, and beets [9]. It presents a wide variety of potential therapeutic effects helpful in the treatment of cancer, diabetes, and lung and cardiovascular diseases; hepatoprotective, neuroprotective, and photoprotective effects; and antimicrobial and anti-inflammatory properties [10,11].

Caspofungin and other agents in the echinocandin class of antifungals have assumed an increasingly important role in the therapy of invasive candidiasis [12]. These agents are nontoxic and show potent fungicidal activity against *C. albicans* and other *Candida* species [13]. Although the mechanism of action of echinocandins is known, the physiological mechanisms by which they cause cell death are not defined [14].

The minimum inhibitory concentration (MIC) values of caspofungin and ferulic acid using the microbroth dilution assay, fractional inhibitory concentration (FIC) index using the checkerboard microdilution assay, and mechanisms of *C. albicans* and *C. glabrata* cell death caused by caspofungin and ferulic acid were determined in this study. The flow cytometric analysis showed that caspofungin caused both apoptosis and necrosis of *C. albicans* and *C. glabrata* cells.

2. Materials and Methods

2.1. Fungal strains and chemicals

C. albicans (ATCC 90028) and *C. glabrata* (ATCC 90030) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Caspofungin diacetate (SML0425) and ferulic acid (1270311) were purchased from Sigma (St. Louis, MO, USA).

2.2. Antimicrobial assay

MIC is defined as the lowest concentration of the antimicrobial agent that inhibits the bacterial growth, as detected by the lack of visual turbidity. The microbiological assay was performed according to the Clinical and Laboratory Standards Institute M7-A7 broth microdilution method [15].

2.3. Checkerboard microdilution assay for ferulic acid and caspofungin

Synergy was tested by the checkerboard method; a two-dimensional array of serial concentrations of test compounds, which has been used most frequently to assess antimicrobial combinations *in vitro*. The tested dilutions were based on the MIC of the two compounds. The checkerboard test was used as the basis to calculate the FIC index [16]. The effects of the combination of caspofungin with ferulic acid were investigated by the checkerboard broth microdilution method. Drug interaction was classified as synergistic, additive, or less-than-additive based on the FIC index, which is the sum of FIC indexes for each drug. The FIC index of each drug was calculated as the MIC of that drug in combined treatment divided by the MIC of the drug used alone. Drug–drug interactions were considered synergistic if the FIC index was < 0.5; additive if the FIC index was $= 1.0$; and less-than-additive if the FIC index was > 0.5.

2.4. Analysis of apoptosis caused by Candida species using flow cytometry

C. albicans and *C. glabrata* cells (2×10^6/mL) were incubated in Sabouraud Dextrose Broth with 1 μg/mL caspofungin and 1000 μg/mL ferulic acid for 24 hours at 30°C. *C. albicans* and *C. glabrata* cells were harvested by centrifugation and washed in 0.1M potassium phosphate buffer. Annexin V/propidium iodide (PI) assays were performed according to the staining kit protocol, using 5 μg annexin V and 5 μg PI at 37°C for 20 minutes. The cells were analyzed using a BD Accuri C6 flow cytometer (Becton–Dickinson, Mansfield, MA, USA) [17].

3. Results

The primary aim of this study was to determine the MIC and FIC index of caspofungin and ferulic acid, which induced both apoptosis and necrosis, using flow cytometry. The checkerboard microdilution assay showed that ferulic acid and caspofungin exhibited antifungal activity against *C. albicans* with an MIC value of 40 μg/mL and 2 μg/mL, respectively, and against *C. glabrata* with an MIC value of 20 μg/mL and 4 μg/mL, respectively. The FIC index of ferulic acid and caspofungin was 0.0375 (Table 1). The apoptotic effects of caspofungin and ferulic acid concentrations (alone and combined) after the 24-hour incubation period, which were analyzed for *C. albicans* and *C. glabrata* based on annexin V–PI binding capacities using flow cytometry, are depicted in Figure 1. Flow cytometric analyses revealed early and late apoptotic effects of 1 μg/mL caspofungin on *C. albicans* as 2.1% and 53.6%, respectively, while their necrotic effects were determined as 5.8%. Especially late apoptotic effects on *C. albicans* were found to be increased (Figure 1). The late apoptotic effects of the combination of caspofungin and ferulic acid (1 μg/mL and 1000 μg/mL) on *C. albicans* and *C. glabrata* were 63.4% and 44.9%, respectively (Table 2 and Figure 1). These findings indicated that the anticandidal effect of the combination of caspofungin and ferulic acid on *C. albicans* than on *C. glabrata* increased depending on the concentration and prolonged incubation period. The results of flow cytometric analysis were used to determine the apoptotic effects of caspofungin and ferulic acid alone and combined against *C. albicans* and *C. glabrata*. Caspofungin exerted apoptotic activity against *C. albicans* by directly killing the cells (resulting in necrosis) and causing others to undergo programmed cell death (apoptosis).

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