# Emodin Reduces the Activity of (1,3)-*β*-D-glucan Synthase from *Candida albicans* and Does Not Interact with Caspofungin

MONIKA JANECZKO*

Department of Molecular Biology, The John Paul II Catholic University of Lublin, Lublin, Poland

Abstract

Candidiasis is the most common opportunistic yeast infection, with *Candida albicans* as a paramount causative species. (1,3)-*β*-D-glucan is one of the three main targets of clinically available antifungal agents used to treat *Candida* infections. It is one of the most abundant fungal cell wall components. Echinocandins represent the newest class of antifungals affecting cell wall biosynthesis through non-competitive inhibition of (1,3)-*β*-D-glucan synthase. Therefore, treatment with echinocandins causes defects in fungal cell integrity. In the present study, similar activity of emodin (6-methyl-1,3,8-trihydroxyanthraquinone) has been revealed. Many reports have already shown the antifungal potential of this pleiotropic molecule, including its activity against *C. albicans*. The aim of this report was to evaluate the activity of emodin towards a new molecular target, i.e. (1,3)-*β*-D-glucan synthase isolated from *Candida* cells. Moreover, given the identical mechanism of the activity of both molecules, interaction of emodin with caspofungin was determined. The study revealed that emodin reduced (1,3)-*β*-D-glucan synthase activity and increased cell wall damage, which was evidenced by both a sorbitol protection assay and an aniline blue staining assay. Furthermore, the synergy testing method showed mainly independence of the action of both tested antifungal agents, i.e. emodin and caspofungin used in combination.

K e y  w o r d s:  *Candida albicans*, caspofungin, echinocandins, emodin, (1,3)-*β*-D-glucan synthase

## Introduction

Candidiasis is one of the most prevalent superficial and deep-seated fungal infections in humans and, as such, a major global health problem, which is additionally associated with a high mortality rate. The most pervasive and problematic cause of infections of all *Candida* species is *Candida albicans* – a part of the commensal microbiota of more than half of the healthy population. It is a cause of both opportunistic and invasive fungal infections (Pfaller and Diekema 2007; Sardi et al. 2013). Yeast infections frequently develop in immunocompromised patients with AIDS, cancer, and neutropenia as well as those receiving immunesuppressive and antibiotic therapy (Canela et al. 2018). Recent reports indicate that *Candida* infections are often associated with bipolar disorder and schizophrenia (Severance et al. 2016). The pathogenicity of *Candida* species is supported by a wide range of virulence factors and fitness attributes, such as biofilm formation, polymorphism, thigmotropism, phenotypic switching, secretion of hydrolytic enzymes, quick adaptation to fluctuations in environmental pH, metabolic flexibility, and strong stress response mechanisms (Mayer et al. 2013; Martins et al. 2014).

Due to the similarity of human and fungal cells, discovery of selective antifungal drugs is extremely difficult. Nevertheless, there are some elements differentiating both types of cells. One of them is the cell wall that does not exist in mammalian cells. (1,3)-*β*-D-glucan is the main polysaccharide in the fungal cell wall. It is synthesized in the fungal cell by glucan synthase located in the cell membrane. This enzyme is regarded as a molecular target in the search for compounds with potential antifungal activity. Echinocandins, the current antifungal drugs are the inhibitors of (1,3)-*β*-D-glucan synthase (Denning 2003).

Echinocandins represented by anidulafungin, caspofungin, and micafungin target the synthesis of (1,3)-*β*-D-glucan polymers through non-competitive

---

* Corresponding author: M. Janeczko, Department of Molecular Biology, The John Paul II Catholic University of Lublin, Lublin, Poland;
e-mail: mjanec@kul.pl

inhibition of the glucan synthase enzyme. Inhibition of a fungal-specific target by these antibiotics leads to defects in fungal cell wall integrity (Pianalto and Alspaugh 2016).

Despite the effectiveness of echinocandins in the control of many fungal infections, antifungal resistance and defensive mechanisms resulting from the use of these drugs have been observed in fungal cells. One of the beneficial alternatives for potentiating the antifungal drugs is combination therapy comprising an antibiotic with a natural product (Zacchino et al. 2017). A combination of several medicinal substances can significantly improve the therapeutic properties and reduce the effective concentration of antibiotics while eliminating their side effects (Martins et al. 2014; Singh and Yeh 2017).

In the light of these facts, the medicinal potential of phytochemicals, their synergistic action with antifungal agents, and their interrelated mechanisms of action have been extensively studied (Kanafani and Perfect 2008; Sher 2009; Agarwal et al. 2010). Emodin (6-methyl-1,3,8-trihydroxyanthraquinone) is one of the most promising natural compounds. Many reports regard emodin as a plant component with antioxidant, antibacterial, antiviral, antimutagenic, antitumor, and immunosuppressive properties (Shrimali et al. 2013; Dong et al. 2016). The latest research on the biological activity of this compound points to its anti-*C. albicans* activity (Kong et al. 2009; Janeczko et al. 2017).

The aim of the present study was to evaluate the inhibitory properties of emodin against (1,3)-$\beta$-D-glucan synthase from *C. albicans* cells. Moreover, the influence of the compound on *C. albicans* cell wall destruction was evidenced with the use of a sorbitol protection assay and aniline blue staining. A checkerboard microliter plate assay was also used to determine the FICI (Fractional Inhibitory Concentration Index) in order to evaluate the combined activities of emodin and caspofungin against *C. albicans* strains by determining the FICI (Fractional Inhibitory Concentration Index).

## Experimental

### Materials and Methods

***C. albicans* strains.** The experiments were performed on *C. albicans* reference strain ATCC 10231. Additionally, 20 clinical strains isolated from urinary tracts and 20 clinical strains isolated from reproductive systems of gynecological patients of Jan Boży Independent Public Provincial Hospital in Lublin, Poland, were included in this study. The strains were identified using VITEK 2 YST IC CARDS (Biomerieux).

**Determination of Minimal Inhibitory Concentrations (MICs).** The MICs of emodin (Sigma-Aldrich,

USA), caspofungin (Sigma-Aldrich, USA), and amphotericin B (Sigma-Aldrich, USA) were determined with the broth dilution method as recommended by CLSI, with some modifications (CLSI 2017). Two-fold serial dilutions of emodin (0.8–400 µg/ml) or antibiotics (0.015–10 µg/ml) were prepared in 96-well microtiter plates using RPMI-1640 medium (with L-glutamine and phenol red, without bicarbonate) (Sigma-Aldrich, USA) buffered with 0.165 M 3-(N-morpholino)propane sulfonic acid (MOPS) (Sigma-Aldrich, USA). Adjacent wells of the microtiter plates contained 100 µl of each dilution. The inoculum was prepared by dilution of *C. albicans* cells with RPMI. The turbidity of this suspension was adjusted to $1–5 \times 10^3$ at a 530 nm wavelength. After addition of 20 µl of the inoculum to the wells, the plates were incubated at 37°C for 48 hours. 100 µl of uninoculated medium was included as a sterility control (blank). The MIC was taken as the lowest concentration of disinfectant that inhibits fungal growth. The experiments were performed in triplicate.

**Sorbitol protection assay.** The sorbitol protection assay was carried out to determine the effect of emodin on the destabilization of the fungal cell membrane. To this end, duplicate plates containing either emodin or amphotericin B and caspofungin were prepared as controls. One plate from each pair contained only the substance tested and the other plate contained an adequate antifungal and, additionally, 0.8 M sorbitol as an osmotic protectant (Frost et al. 1995). MICs for each trial were determined by the modified CLSI protocol as described above. Each assay was performed in triplicate.

**Preparation and quantification of (1,3)-$\beta$-D-glucan synthase.** (1,3)-$\beta$-D-glucan synthase from *C. albicans* cells (ATCC 10231) was prepared using the method proposed by Shedletzky et al. (1997) with some modifications described by Lee and Kim (2016). The enzyme was isolated from *C. albicans* cells cultivated in 1 l of Sabouraud Dextrose Agar Broth (Biocorp, Poland) at 37°C for 16 h. The cells were homogenized in a Bead Beater (Minilys Homogenizer, Bertin Instruments) in 12 cycles of 1 min with 0.5-mm acid-washed glass beads. The protein concentration in the microsome and membrane fraction was measured using the Bradford method in accordance with the manufacturer's instructions (Sigma-Aldrich, USA). The (1,3)-$\beta$-D-glucan synthase assay was performed according to the method developed by Frost et al. (1995) and modified by Lee and Kim (2016). The glucans stained specifically with aniline blue solution (0.1%) were a measure of the enzyme activity. Fluorescence was measured using a spectrofluorometer (Pharmacia Biotech) at 400-nm excitation and 460-nm emission wavelengths. The effect of emodin on the enzyme activity was determined at concentrations corresponding to the MIC/4, MIC/2, MIC, and 2×MIC, and DMSO was used as a control.

The assays were performed in triplicate in three independent experiments.

**Aniline blue staining of (1,3)-*β*-glucan in the *C. albicans* cell wall.** The aniline blue staining method and fluorescence microscopy were used to visualize the effect of emodin and caspofungin on (1,3)-*β*-D-glucans in the *C. albicans* (ATCC 10231) cell walls. The yeast cells at the exponential phase were harvested by centrifugation at $4500 \times g$ at 4°C for 5 min. Next, the cells were washed twice and resuspended in 0.85% NaCl. Emodin at concentrations corresponding to MIC/2 and MIC/4, caspofungin at MIC/2, and 1% DMSO as control were added to the cell suspensions and incubated at 37°C for 10 h. The cells were harvested and washed in 0.85% NaCl; next, the cell density of each experimental group was adjusted to $1 \times 10^8$ cells/ml and the cells were resuspended in an aniline blue solution (0.1%). The samples were stained at 50°C for 30 min. A drop of each suspension was squashed between the microscope slide and the cover glass. The preparation was sealed and examined in a fluorescence microscope under UV illumination (Nicon). Images were taken with a cooled monochrome camera.

**Caspofungin – emodin combination assay (a checkerboard method).** Interactions between caspofungin and emodin were measured by calculation of the fractional inhibitory concentration index (FICI). A total of l00 µl of RPMI-1640 medium was distributed into each well of the microdilution plates. The first antibiotic of the combination – caspofungin was serially diluted along the ordinate at a concentration range of 0–1.2 µg/ml, while the other drug – emodin was diluted along the abscissa at a concentration range of 0–100 µg/ml. The inoculum was prepared from *C. albicans* in RPMI-1640 medium as described in the MIC assay. Each microtiter well was inoculated with 20 µl of the yeast inoculum and the plates were incubated at 37°C for 48 h. The MIC values were detected with the naked eye. The FICI values were calculated for each well with the equation FICI = FICA + FICB = (MICA+B/MICA) + (MICB + + A/MICB), where MICA and MICB are the MICs of drugs A and B alone, respectively, and MICA + B and MICB + A are the concentrations of the drugs applied in combination, respectively, in all the wells corresponding to the MIC. A combination of two drugs is considered synergistic when the FICI is ≤ 0.5, indifferent when the FICI is > 0.5 to ≤ 4, and antagonistic when the FICI is > 4 (Odss 2003; Petersen et al. 2006).

## Results and Discussion

Emodin is a natural anthraquinone derivative found mainly in the roots and rhizomes of numerous plants. Pharmacological studies have demonstrated that emo-din with its various biological functions has been used in the treatment of cancers and inflammatory diseases (Wei et al. 2013; Dong et al. 2016; Monisha et al. 2016). The unique therapeutic potential of emodin results from its ability to interact with many molecular targets, e.g. protein kinases, NADH-oxidase, topoisomerase II, survivin, XIAP, STAT3, p53, and p21 (Shrimali et al. 2013). Furthermore, emodin was found to have antimicrobial activity (Alves et al. 2004; Kong et al. 2009; Liu et al. 2013; Cao et al. 2015; Liu et al. 2015; Janeczko et al. 2017).

In this study, the antifungal activity of emodin against the reference and clinical strains of *C. albicans* has been confirmed by the CLSI method in RPMI medium. The minimal inhibitory concentration against the standard strain was 12.5 µg/ml. The control antibiotics, caspofungin and amphotericin B, inhibited yeast growth at concentrations of 0.15 µg/ml and 1 µg/ml, respectively (Table I). Moreover, emodin suppressed the growth of all clinical strains isolated from the urinary tracts or the vaginas of the gynecological patients. The activity against these species has been shown at values of MICs between 6.25 and 50 µg/ml. Also, all isolates were susceptible to caspofungin. The MICs ranged from 0.03 to 0.6 µg/ml. The MIC values of the antifungal agents tested individually are summarized in Table II and Table III. These results were comparable to MICs obtained in our previous work (Janeczko et al. 2017). As demonstrated in the previous study, emodin suppressed the growth of *C. albicans* and other reference strains, such as *C. krusei*, *C. parapsilosis*, and *C. tropicalis*, as well as clinical *Candida* strains. In addition, fungicidal activity against these species has been shown at values of MICs and MFCs (Minimal Fungicidal Concentrations) between 12.5 and 200 µg/ml. Moreover, we have proved that this compound has anti-virulent potential by reducing hyphal formation, suppressing adhesion, which is the first and critical phase of fungal infection, and destabilizing fully established biofilm. In terms of the high pleiotropic nature of emodin, it has been confirmed that this compound is an effective inhibitor of protein kinase 2 (CK2) isolated from *C. albicans* cells (Janeczko et al. 2017).

In order to verify the influence of emodin on *C. albicans* cells, the previous research on the molecular

Table I
Effect of sorbitol on the MICs of emodin and antibiotics against
*C. albicans* ATCC 10231.

|  | MIC (µg/ml) | |
|---|---|---|
|  | Without sorbitol | With sorbitol |
| Emodin | 12.5 | 25 |
| Caspofungin | 0.15 | 0.6 |
| Amphotericin B | 1 | 1 |

Table II
*In vitro* activity of emodin and caspofungin alone and in combination assessed by the broth
dilution assay against clinical strains of *C. albicans* isolated from urinary tracts.

| Strain No. | MIC of the drug used alone (µg/ml) | | MIC of the drug used in combination (µg/ml) | FICI | Interaction |
|---|---|---|---|---|---|
| | Emodin | Caspofungin | Emodin + Caspofungin | | |
| 1 | 12.5 | 0.15 | 12.5 + 0.3 | 3 | Indifferent |
| 2 | 12.5 | 0.07 | 12.5 + 0.3 | 5.28 | Antagonism |
| 3 | 25 | 0.3 | 25 + 0.6 | 3 | Indifferent |
| 4 | 25 | 0.3 | 25 + 0.3 | 2 | Indifferent |
| 5 | 50 | 0.6 | 50 + 1.2 | 3 | Indifferent |
| 6 | 50 | 0.15 | 50 + 0.3 | 3 | Indifferent |
| 7 | 12.5 | 0.15 | 12.5 + 0.3 | 3 | Indifferent |
| 8 | 12.5 | 0.07 | 12.5 + 0.3 | 5.28 | Antagonism |
| 9 | 25 | 0.03 | 25 + 0.15 | 6 | Antagonism |
| 10 | 25 | 0.3 | 25 + 0.15 | 1.5 | Indifferent |
| 11 | 25 | 0.15 | 25 + 0.3 | 3 | Indifferent |
| 12 | 50 | 0.6 | 50 + 0.3 | 1.5 | Indifferent |
| 13 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 14 | 50 | 0.6 | 50 + 0.6 | 2 | Indifferent |
| 15 | 25 | 0.3 | 25 + 0.15 | 1.5 | Indifferent |
| 16 | 6.25 | 0.03 | 12.5 + 0.06 | 4 | Indifferent |
| 17 | 50 | 0.6 | 50 + 0.6 | 2 | Indifferent |
| 18 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 19 | 50 | 0.15 | 25 + 0.15 | 1.5 | Indifferent |
| 20 | 25 | 0.6 | 50 + 0.6 | 3 | Indifferent |

impact of this substance has been extended to examination of its effect on cell wall damage. The yeast cell wall serves many functions, inter alia providing cell rigidity and shape, metabolism, ion exchange, and interactions with host defense mechanisms. Since the cell wall is not present in mammalian cells, it is an excellent target for specific antifungal antibiotics with higher selectivity towards pathogen cells and lower toxicity against host cells (Denning and Hope 2010). The damaging effects on the *C. albicans* cell wall were evaluated on the basis of the MICs of emodin, caspofungin (positive control), and amphotericin B (negative control) in the absence and presence of sorbitol. Reduction of crucial cell wall components by antifungal agents will lyse cells in the absence of an osmoprotectant. These effects can be recovered in the presence of such osmoprotectants as sorbitol: in this case, cells will continue to grow. As shown in Table I, the MIC of emodin increased twice in relation to the sample without any osmoprotectant after 2 days of incubation. The MIC of caspofungin increased three times without any sorbitol after the same incubation time. In contrast, the MIC of amphotericin B did not change. The increase in the MIC in the presence of sorbitol demonstrated that emodin was involved in cell wall synthesis.

The fungal cell wall is a unique structure built of $\alpha$- and $\beta$-linked glucans, chitin, polysaccharides, and mucopolysaccharides. Many of these biopolymers are essential for proper functioning of fungal cells. Enzymes synthesizing these biopolymers could be desirable antifungal targets (Wiederhold 2018). Since emodin is a highly pleiotropic molecule capable of interacting with several major molecular targets and damaging *C. albicans* cell walls, the influence of the compound on the activity of (1,3)-$\beta$-D-glucan synthase (GS) has been analyzed. This enzyme is a glucosyltransferase involved in synthesis of 1,3-$\beta$-D-glucan in fungi – one of the main molecular targets used in clinically available antifungals and also a pharmacological target for echinocandins (Denning 2003). Inhibition of GS activity and the following depletion of $\beta$-glucans from the fungal cell wall result in cell lysis under osmotic stress (Frost et al. 1995).

An aniline blue assay was used to determine the effect of emodin on the activity of (1,3)-$\beta$-D-glucan synthase obtained from a microsomal membrane fraction from *C. albicans*. The decrease in the GS activity after the treatment with the anthraquinone tested was shown as a percentage of the DMSO control. As shown in Fig. 1, emodin reduced the GS activity approxi-

Table III
*In vitro* activity of emodin and caspofungin alone and in combination assessed by the broth
dilution method against clinical strains of *C. albicans* isolated from vaginas.

| Strain No. | MIC of the drug used alone (µg/ml) | | MIC of the drug used in combination (µg/ml) | FICI | Interaction |
|---|---|---|---|---|---|
| | Emodin | Caspofungin | Emodin + Caspofungin | | |
| 1 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 2 | 50 | 0.6 | 50 + 0.6 | 2 | Indifferent |
| 3 | 25 | 0.15 | 25 + 0.3 | 3 | Indifferent |
| 4 | 50 | 0.3 | 50 + 0.3 | 2 | Indifferent |
| 5 | 25 | 0.15 | 25 + 0.3 | 3 | Indifferent |
| 6 | 50 | 0.07 | 25 + 0.15 | 2.64 | Indifferent |
| 7 | 50 | 0.3 | 50 + 0.6 | 3 | Indifferent |
| 8 | 50 | 0.6 | 25 + 0.3 | 1 | Indifferent |
| 9 | 12.5 | 0.3 | 25 + 0.3 | 3 | Indifferent |
| 10 | 50 | 0.15 | 50 + 0.6 | 5 | Antagonism |
| 11 | 50 | 0.07 | 50 + 0.15 | 3 | Indifferent |
| 12 | 50 | 0.6 | 50 + 0.3 | 1.5 | Indifferent |
| 13 | 25 | 0.3 | 50 + 0.3 | 3 | Indifferent |
| 14 | 25 | 0.6 | 50 + 0.15 | 2.25 | Indifferent |
| 15 | 25 | 0.3 | 50 + 0.15 | 2.5 | Indifferent |
| 16 | 12.5 | 0.03 | 25 + 0.15 | 5 | Antagonism |
| 17 | 12.5 | 0.6 | 12.5 + 0.6 | 2 | Indifferent |
| 18 | 50 | 0.3 | 50 + 0.6 | 3 | Indifferent |
| 19 | 50 | 0.15 | 25 + 0.07 | 1 | Indifferent |
| 20 | 50 | 0.6 | 50 + 0.3 | 1.5 | Indifferent |

mately to 67.6% ± 3.4%, 76% ± 4%, 87.4% ± 5,2%, and 91.2% ± 3,8% at concentrations equal to 2 × MIC, MIC, MIC/2, and MIC/4, respectively, when compared to the DMSO control cells. The reduced activity of GS proved that emodin prevented the synthesis of the *C. albicans* cell wall, and therefore, the effect of its activity corresponds to that of echinocandins.

Additionally, aniline blue was used to verify the *in vivo* influence of emodin on changes in the content of (1,3)-$\beta$-D-glucans in *C. albicans* by inhibition of their
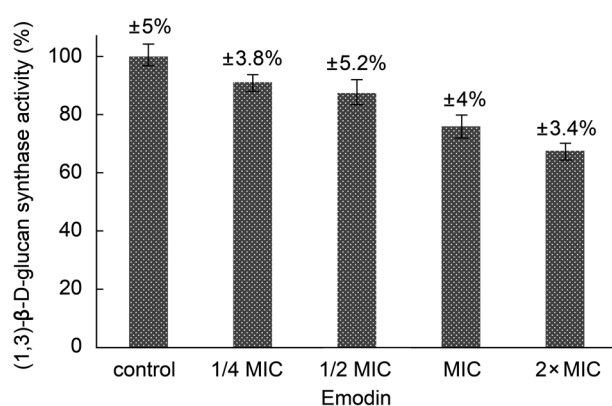


Fig. 1. Effect of emodin on (1,3)- $\beta$-D-glucan synthase activity.

biosynthesis. This fluorescent dye binds to (1,3)-$\beta$-D-glucans in the cell wall. The biosynthesis of *C. albicans* cell wall components was shut down by inhibition of GS. The fungi were grown in the presence of emodin at the concentrations of 6.25 µg/ml and 3.12 µg/ml corresponding to MIC/2 and MIC/4, respectively or in the presence of caspofungin at 0.07 µg/ml (MIC/2). The control contained DMSO at the same concentration as in the samples with emodin. As shown in Fig. 2, the intensity of fluorescence of fungal cells stained with blue aniline was lower in cells treated with emodin and caspofungin than in the controls. The apparent significant loss of fluorescence of the fungal cell walls and, above all, the disintegration of cells under the pressure of caspofungin at MIC/2 correlated with the sensitivity of this species to echinocandin, i.e. a 1,3-$\beta$-D-glucan synthesis inhibitor (Fig. 2B). The treatment of *C. albicans* cells with emodin at concentrations MIC/2 and MIC/4 resulted in reduction of the number of cells, but the reduction of the glucan content in the cell walls was almost imperceptible (Fig. 2C and Fig. 2D).

Recently, natural product screening has also been a source of a number of distinct GS inhibitors. Antifungal activity was shown by natural lipopeptides and triterpenes containing a polar (acidic) moiety (Vicente
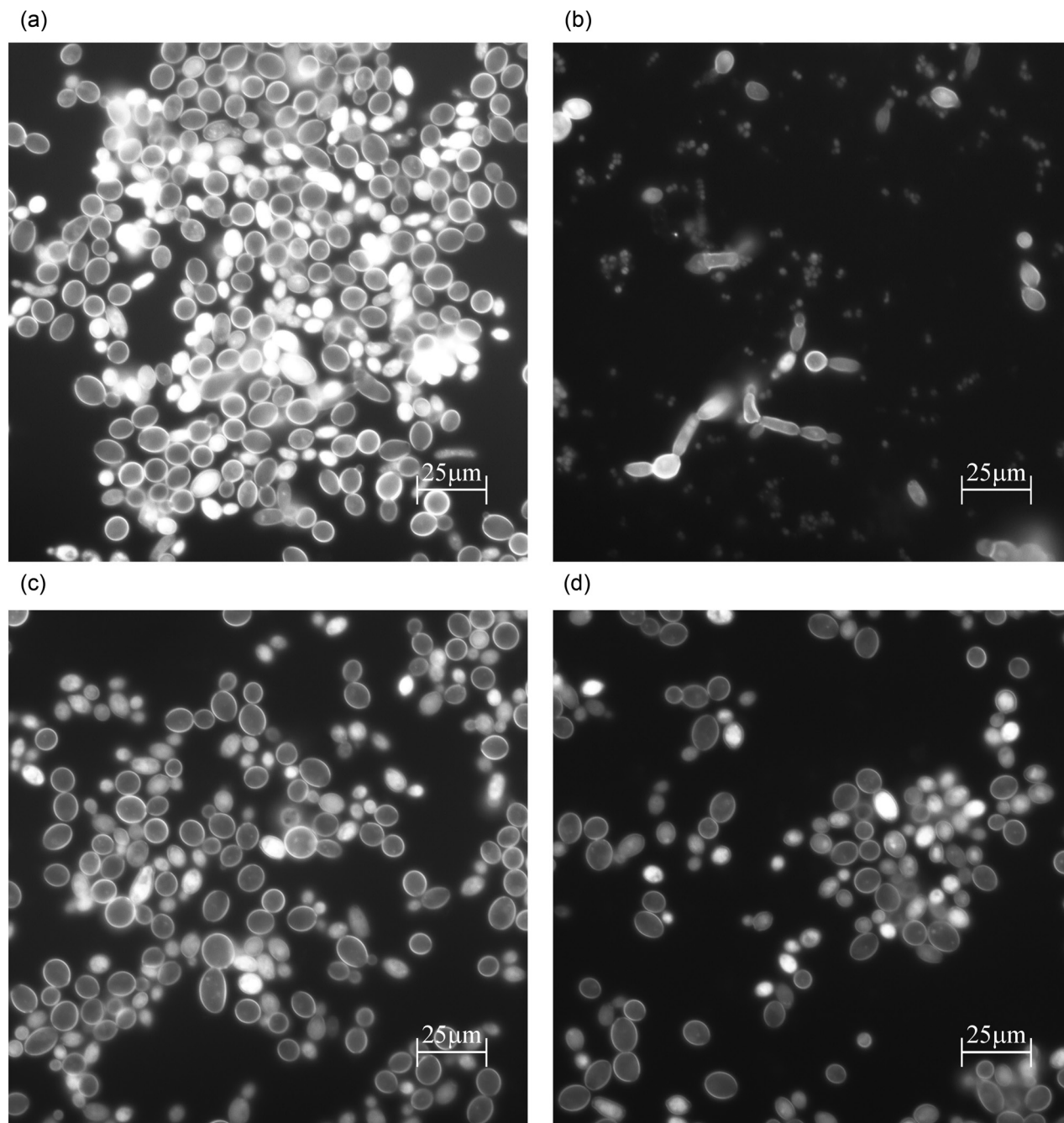
Fig. 2. Aniline blue staining of *C. albicans* cell walls. A) treatment with DMSO at 1% (control); B) caspofungin at 0.07 µg/ml; C) emodin at 6.25 µg/ml; D) emodin at 3.12 µg/ml.

et al. 2003). In addition, several new investigational agents are currently under development. Among these, there are semi-synthetic enfumafungins modified by replacement with amino ethers (Apgar et al. 2015), SCY-078, which derives from enfumafungin, as well and a cyclic hexapeptide rezafungin (CD101, biafungin, previously SP3025) (Wiederhold 2018).

The clinical success of the echinocandins is associated with their fewer toxic side effects in comparison to polyenes and their fewer drug-drug interactions compared to azoles. These drugs are primarily used for the treatment of invasive candidiasis and as an alternative therapy for aspergillosis treatment (Odds

et al. 2003; Denning and Hope 2010). Unfortunately, the effectiveness of these antibiotics is compromised due to a critical increase in the emergence of drug-resistant *Candida* strains. In the face of this problem, another strategy has been developed to overcome the treatment failures by combining different antifungals. Many reviews indicate that the combination of antibiotics, phytochemicals, or both natural plant products and well-known antibiotics offers significant potential for the development of novel antimicrobial therapies and treatment of several diseases caused by microorganisms. The advantages of the synergistic action of antibiotics and plant extracts include reduction of
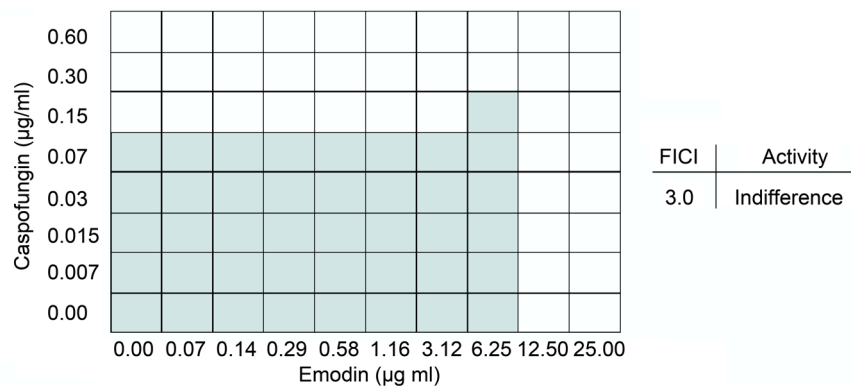
Fig. 3. The checkerboard method showing unresponsiveness of the emodin-caspofungin combination.
The resulting checkerboard included each combination of emodin and caspofungin, with wells containing
the highest concentration of each compounds in opposite corners (darkened fields mean cell growth;
light fields mean no growth).

undesirable effects and increased efficiency. It is also important to increase the stability and bioavailability of free agents and achieve an adequate therapeutic effect with relatively small doses compared to any synthetic medication (Hemaiswarya et al. 2008).

Since emodin affects the activity of $(1,3)$-$\beta$-D-glucan synthase in the same range as caspofungin, the interactions between this anthraquinone and the antibiotic were investigated. In this case, two possibilities of interaction could be expected – synergism or antagonism in the action against *C. albicans* cells. The third option was indifferent interaction between the two tested substances. Synergism is defined as a positive interaction occurring when two agents combined together exert an inhibitory effect that is greater than the sum of their individual effects. In turn, the term antagonism is used when the effect of both drugs together is worse than the effect of either alone. Then, indifference means that no effect is exhibited. The caspofungin-emodin combination effect was measured using the checkerboard microtiter plate method and calculation of the fractional inhibitory concentration index (FICI). The course and result of the experiment for the reference *C. albicans* strain is schematically shown in Fig. 3. The combination of both antifungals showed a tendency towards indifference between the tested compounds at most concentrations and ratios. Thus, emodin at 0.19–3.12 µg/ml did not affect the caspofungin activity against *C. albicans*. Similarly, caspofungin at 0.007–0.6 µg/ml did not change the MIC values for emodin. Only in one case, the MIC of caspofungin increased from 0.15 µg/ml to 0.3 µg/ml in the presence of 6.25 µg/ml emodin. Based on the MIC values in various concentration combinations of both antifungal compounds, the FICI was 3 and did not show any interactions between the compounds.

The checkerboard assays evaluated against 40 clinical isolates of *C. albicans* showed that the combination of emodin with caspofungin changed mainly the MIC values with respect to caspofungin. MICs increased for 18 strains, decreased for nine strains, and remained unchanged for 13 isolates. The composite emodin/caspofungin caused a change in the MICs with respect to emodin to a lesser extent. The MIC values decreased for four strains and increased for five strains. They were ca. 2–4-fold lower or higher than the values for the compounds applied alone. The FICI values of the combinations of the antifungal drugs ranged from 1 to 6. This combination showed predominantly indifferent interactions between emodin and caspofungin (87.5% isolates) with the FICI in the range from 1 to 4. An antagonistic effect was proved only against five strains tested (12.5%) with FICI > 4. Otherwise, no synergism was observed (FICI < 0.5). These results were comparable to the FICI of the *C. albicans* reference strain. The MIC and FICI values of the antifungal agents tested in combination are summarized in Table II and Table III.

In conclusion, as shown above, the antifungal activity of emodin against *C. albicans* may be related to the inhibition of $(1,3)$-$\beta$-D-glucan synthase activity, leading to disruption of $(1,3)$-$\beta$-D-glucans in the fungal cell wall. This completely new molecular target for emodin is highly desirable due to the high specificity of this type of antifungals in relation to host cells. The novel mechanism of emodin action could hypothetically amplify the activity of echinocandins; however, in combination with caspofungin, this anthraquinone shows indifferent or antagonistic interactions. The data from the studies of the interactions between emodin/caspofungin suggest that these combinations could not be an effective strategy against *C. albicans* infections.

**Conflict of interest**

# Literature

**Agarwal V, Lal P, Pruthi V.** 2010. Effect of plant oils on *Candida albicans*. J Microbiol Immunol Infect. 43(5):447–451.

**Alves DS, Perez-Fons L, Estepa A, Micol V.** 2004. Membrane-related effects underlying the biological activity of the anthraquinones emodin and barbaloin. Biochem Pharmacol. 68:549–561.

**Apgar JM, Wilkening RR, Greenlee ML, Balkovec JM, Flattery AM, Abruzzo GK, Galgoci AM, Giacobbe RA, Gill CJ, Hsu MJ, et al.** 2015. Novel orally active inhibitors of $\beta$-1,3-glucan synthesis derived from enfumafungin. Bioorg Med Chem Lett. 25(24):5813–5818.

**Canela HMS, Cardoso B, Vitali LH, Coelho HC, Martinez R, Ferreira MEDS.** 2018. Prevalence, virulence factors and antifungal susceptibility of *Candida* spp. isolated from bloodstream infections in a tertiary care hospital in Brazil. Mycoses. 61(1):11–21.

**Cao F, Peng W, Li X, Liu M, Li B, Qin R, Jiang W, Cen Y, Pan X, Yan Z, et al.** 2015. Emodin is identified as the active component of ether extracts from Rhizoma Polygoni Cuspidati, for anti-MRSA activity. Can J Physiol Pharmacol. 93(6):485–493.

**Denning DW, Hope WW.** 2010. Therapy for fungal diseases: opportunities and priorities. Trends Microbiol. 18(5):195–204.

**Denning DW.** 2003. Echinocandin antifungal drugs. Lancet. 362(9390):1142–1151.

**Dong X, Fu J, Yin X, Cao S, Li X, Lin L; Huyiligeqi, Ni J.** 2016. Emodin: A Review of its pharmacology, toxicity and pharmacokinetics. Phytother Res. 30(8):1207–1218.

**Frost DJ, Brandt KD, Cugier D, Goldman R.** 1995. A whole-cell *Candida albicans* assay for the detection of inhibitors towards fungal cell wall synthesis and assembly. J Antibiot. 48(4):306–310.

**Hemaiswarya S, Kruthiventi AK, Doble M.** 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine. 15(8):639–652.

**Janeczko M, Masłyk M, Kubiński K, Golczyk H.** 2017. Emodin, a natural inhibitor of protein kinase CK2, suppresses growth, hyphal development, and biofilm formation of *Candida albicans*. Yeast. 34:253–265.

**Kanafani ZA, Perfect JR.** 2008. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis. 46(1):120–128.

**Kong WJ, Wang JB, Jin C, Zhao YL, Dai CM, Xiao XH, Li ZL.** 2009. Effect of emodin on *Candida albicans* growth investigated by microcalorimetry combined with chemometric analysis. Appl Microbiol Biotechnol. 83(6):1183–1190.

**Lee HS, Kim Y.** 2016. Antifungal activity of *Salvia miltiorrhiza* against *Candida albicans* is associated with the alteration of membrane permeability and (1,3)-$\beta$-D-Glucan synthase activity. J Microbiol Biotechnol. 26(3):610–617.

**Liu Z, Ma N, Zhong Y, Yang Zhan-qin Y.** 2015. Antiviral effect of emodin from *Rheum palmatus* against coxaskievirus B5 and human respiratory syncytial virus in vitro. J Huazhong University Sci Technol (Medical Sciences). 35:916–922.

**Liu Z, Wei F, Chen LJ, Xiong HR, Liu YY, Luo F, Hou W, Xiao H, Yang ZQ.** 2013. In vitro and in vivo studies of the inhibitory effects of emodin isolated from *Polygonum cuspidatum* on coxsakievirus b$_4$. Molecules. 18(10):11842–11858.

**Martins N, Ferreira IC, Barros L, Silva S, Henriques M.** 2014. Candidiasis: predisposing factors, prevention, diagnosis and alternative treatment. Mycopathologia. 177(5–6):223–240.

**Mayer FL, Wilson D, Hube B.** 2013. *Candida albicans* pathogenicity mechanisms. Virulence. 4(2):119–128.

**Monisha BA, Kumar N, Tiku AB.** 2016. Emodin and its role in chronic diseases. Adv Exp Med Biol. 928:47–73.

**Odds FC.** 2003. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother. 52(1):1.

**Petersen PJ, Labthavikul P, Jones CH, Bradford PA.** 2006. *In vitro* antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic analysis. J Antimicrob Chemother. 57(3):573–576.

**Pfaller MA, Diekema DJ.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 20(1):133–163.

**Pianalto KM, Alspaugh JAJ.** 2016. New horizons in antifungal therapy. J Fungi (Basel). 2:2(4).

**Sardi JC, Scorzoni L, Bernardi T, Fusco-Almeida AM, Mendes Giannini MJ.** 2013. Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol. 62(1):10–24.

**Severance EG, Gressitt KL, Stallings CR, Katsafanas E, Schweinfurth LA, Savage CL, Adamos MB, Sweeney KM, Origoni AE, Khushalani S, et al.** 2016. *Candida albicans* exposures, sex specificity and cognitive deficits in schizophrenia and bipolar disorder. Schizophr. 2:16018.

**Shedletzky E, Unger C, Delmer DP.** 1997. A microtiter-based fluorescence assay for (1,3)-beta-glucan synthases. Anal Biochem. 249(1):88–93.

**Sher A.** 2009. Antimicrobial activity of natural products from medicinal plants. Gomal J Med Sci. 7:72–78.

**Shrimali D, Shanmugam MK, Kumar AP, Zhang J, Tan BK, Ahn KS, Sethi G.** 2013. Targeted abrogation of diverse signal transduction cascades by emodin for the treatment of inflammatory disorders and cancer. Cancer Lett. 341(2):139–149.

**Singh N, Yeh PJ.** 2017. Suppressive drug combinations and their potential to combat antibiotic resistance. J Antibiot (Tokyo). 70(11):1033–1042.

**Vicente MF, Basilio A, Cabello A, Peláez F.** 2003. Microbial natural products as a source of antifungals. Clin Microbiol Infect. 9(1):15–32.

**CLSI.** 2017. Reference method for broth dilution antifungal susceptibility testing of yeasts. CLSI standard M27. 4th ed. Wayne (USA): Clinical and Laboratory Standards Institute.

**Wei WT, Lin SZ, Liu DL, Wang ZH.** 2013. The distinct mechanisms of the antitumor activity of emodin in different types of cancer (Review). Oncol Rep. 30(6):2555–2562.

**Wiederhold NP.** 2018. The antifungal arsenal: alternative drugs and future targets. Int J Antimicrob Agents. 51(3):333–339.

**Zacchino SA, Butassi E, Cordisco E, Svetaz LA.** 2017. Hybrid combinations containing natural products and antimicrobial drugs that interfere with bacterial and fungal biofilms. Phytomedicine. 37:14–26.