

# Asarones from *Acorus calamus* in Combination with Azoles and Amphotericin B: A Novel Synergistic Combination to Compete Against Human Pathogenic *Candida* Species In Vitro

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**Abstract** The increase in drug resistance to current antifungal drugs brings enormous challenges to the management of *Candida* infection. Therefore, there is a continuous need for the discovery of new antimicrobial agents that are effective against *Candida* infections especially from natural source especially from medical plants. The present investigation describes the synergistic anticandidal activity of two asarones ( $\alpha$  and  $\beta$ ) purified from *Acorus calamus* in combination with three clinically used antifungal drugs (fluconazole, clotrimazole, and amphotericin B). The synergistic anticandidal activities of asarones and drugs were assessed using the checkerboard microdilution and time-kill assays. The results of the present study showed that the combined effects of asarones and drugs principally recorded substantial synergistic activity (fractional inhibitory concentration index (FICI) <0.5). Time-kill study by combination of the minimal inhibitory concentration (MIC) of asarones and drugs (1:1) recorded that the growth of the *Candida* species was significantly arrested between 0 and 2 h and almost completely attenuated between 2 and 6 h of treatment. These findings have potential implications in adjourning the development of resistance as the anticandidal activity is achieved with lower concentrations of asarones and drugs. The combination of asarones and drugs also significantly inhibit the biofilm formation by *Candida* species, and this would also help to fight against drug resistance because biofilms formed by *Candida* species are ubiquitous in nature and are characterized by their recalcitrance toward antimicrobial treatment. The in vitro synergistic activity of asarones and drugs against pathogenic *Candida* species is reported here for the first time.

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## Introduction

Drug-resistant pathogens and serious side effects are major problems associated with antifungal chemotherapy [1]. *Candida* species are the fourth leading cause of nosocomial blood stream infections in the USA, with treatment costs estimated to be more than two to four billion US dollars annually [2] and with attributable mortality rates estimated to be 38–49 % [3]. The epidemiology of invasive fungal infections is changing, although *Candida albicans* remains the most important fungal agent. However, a notable increase in infections caused by non-*albicans* species (*Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei*) has been reported, and infections by these species account for 36–63 % of all cases [4].

Notwithstanding the increasing need for effective therapy, the range of antifungal agents available is limited, and some of the most effective agents are also toxic. In addition, while the azoles have been used successfully for the treatment of *Candida* infections, numerous reports of treatment failures are now appearing in the literature [5]. Amphotericin B, a polyene fungicidal agent, has been the standard treatment for *Candida* infections for decades, but the toxicity of its conventional form and the costs of its lipid forms limit its use [6]. More recently, azole antifungal compounds, which have excellent efficacy–toxicity profiles, have emerged as the principal drugs used in the treatment of *Candida* infections in non-neutropenic patients [7]. As already emphasized, the administration of azoles for treating fungal infections has encountered certain limitations, such as their low water solubility, low bioavailability, and frequent side effects consequent on the requirement of high doses and/or long-term administration [8]. However, with the increasing clinical use of azole, resistance is emerging in clinical isolates from immunocompromised patients. In addition, azole is only fungistatic; this characteristic probably contributes to the development of resistance. Therefore, novel fungal therapies for effective management of *Candida* infections are urgently required to meet this dangerous situation.

*Acorus calamus* Linn., very popularly known as “sweet flag,” is native to Central Asia, North America, and Eastern Europe [9]. Rhizome of this plant is widely used in the Indian systems of medicine, such as Ayurveda, Siddha, and Unani [10]. Moreover, the rhizomes of this plant are widely used in the number of diseases like epilepsy, mental ailments, chronic diarrhea, and dysentery. *A. calamus* is also used in the conditions of stomatopathy, hoarseness, flatulence, dyspepsia, helminthiasis, amenorrhea, dysmenorrhoeal, nephropathy, calculi, and stragury [11]. Studies on chemical composition of *Acorus* spp. have revealed that  $\alpha$ - and  $\beta$ -asarones are the major bioactive compounds [12, 13]. A number of bioactivities are reported to  $\alpha$ - and  $\beta$ -asarone, like antibacterial, antihelminthic, antifungal properties [14], and anticancer activity [15]. However, little is known about its synergistic interaction with clinically used antibiotics against pathogenic fungi.

The present study was undertaken to evaluate in vitro synergistic effect of  $\alpha$ - and  $\beta$ -asarones isolated from *A. calamus* in combination with two azole and amphotericin B drugs against pathogenic *Candida* species. The present work highlights a promising role of asarones in combination with the three most widely used drugs for anticandidal drug therapy.

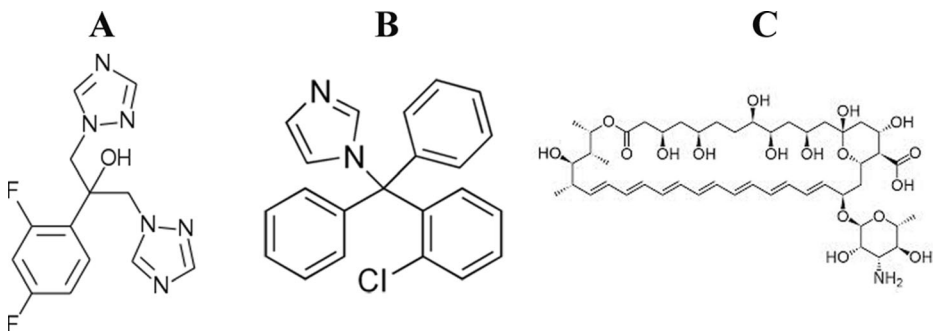
## Materials and Methods

### Chemical and Antifungal Agents

The microbiological media were purchased from HiMedia, Mumbai, India. The fluconazole and clotrimazole and polyene (amphotericin B) (Fig. 1) were purchased from Sigma-Aldrich, USA. Antifungal stock solutions were prepared in distilled water and sterilized by syringe filtration (Millipore). Three stock solutions were maintained at  $-20\text{ }^{\circ}\text{C}$ .

### Isolation of $\alpha$ - and $\beta$ -Asarones

The *A. calamus* samples were purchased from the local market of Trivandrum, India. A voucher specimen has been deposited at the Agroprocessing and Natural Product Division, NIIST, Trivandrum. The rhizomes (250 mg) of *A. calamus* were shade-dried, and the powdered *A. calamus* sample was extracted with 90 % aqueous methanol (MeOH) on a shaker for 4 days. The MeOH extract was filtered through Whatman no. 1 filter paper and concentrated in vacuum using a rotavapor (BUCHI). The remaining residue was dissolved in distilled water (pH 5.0) and extracted twice with an equal volume of EtOAc at room temperature. The ethyl acetate (EtOAc) layers were pooled and extracted with distilled water to remove the hydrophilic compounds. The resulting organic solvent layers were evaporated under reduced pressure. The concentrated EtOAc extract was subjected to flash column chromatography over silica gel (60<sub>F254</sub>, 100–200  $\mu\text{m}$ , Merck) with successive elutions with  $\text{CHCl}_3/\text{MeOH}$  in increasing order of polarity (90:10, 80:20, 70:30, 50:50, 30:70, 10:90, v/v). Each of the  $\text{CHCl}_3/\text{MeOH}$  elutes was concentrated and evaluated for antifungal activity. The active  $\text{CHCl}_3/\text{MeOH}$  (80:20) and  $\text{CHCl}_3/\text{MeOH}$  (30:70) elutes that exhibited antifungal activity against *Candida* spp. were further purified on a silica gel column (15  $\times$  200 mm) with a stepwise gradient of *n*-hexane and  $\text{Me}_2\text{CO}$  (100:0, 90:10, 80:20, 70:30, 50:50, 30:70, 10:90, 100:0 v/v). The concentrates of 90 % hexane/10 % acetone and 100 % acetone elutes were separated by the gel filtration of the C26/100 column packed with Sephadex LH-20 resin using MeOH as a mobile phase at a 0.5 ml/min rate. The 2 ml fractions were collected using a fraction collector (Bio-Rad). Each of all the fractions was bioassayed for antifungal activity against *Candida* spp. The active fractions 47–75 and 103–155 were subjected to preparative TLC on glass plates (20  $\times$  20  $\times$  0.2 mm) coated with silica gel (60<sub>GF254</sub>, Merck). The TLC plates developed in the *n*-hexane/EtOAc (8:2) solvent system were visualized under UV light. The activity of bands on TLC plates was examined by bioautographic technique. Scraped active bands were collected



**Fig. 1** Structure of azoles and amphotericin B drugs purchased from Sigma-Aldrich are used in the present study. **a** Fluconazole, **b** clotrimazole, and **c** amphotericin B

and extracted with 100 % MeOH at 28 °C in a shaking incubator for 24 h. The pooled extract was bioassayed against *Candida* spp. The concentrated extracts from antifungal-active TLC bands were further purified on Sephadex LH-20 column (10×950 mm) chromatography. The Sephadex LH-20 column was eluted with MeOH at a flow of 0.2 ml/min. The isolation of the antifungal compounds  $\alpha$  and  $\beta$ -asarone was performed by HPLC system on a Prep C18 column (7  $\mu$ m, 7.8×300 mm, Waters) using a linear gradient solvent system from 50 % CH<sub>3</sub>CN in H<sub>2</sub>O to 70 % CH<sub>3</sub>CN in H<sub>2</sub>O at a flow rate of 2 ml/min. The separation was monitored at an absorbance of 270 nm by a UV detector (Schimadzu). Isolated pure  $\alpha$ - and  $\beta$ -asarone were confirmed by comparing the retention time of isolated asarones with authentic  $\alpha$ - and  $\beta$ -asarone from Sigma-Aldrich.

#### Test *Candida* spp.

The *Candida* strains used in the study were *C. albicans* MTCC 277 and *C. tropicalis* MTCC 184, which were procured from the Microbial Type Culture Collection and Gene Bank (MTCC) Division, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India. The two *Candida* species was subcultured in potato dextrose agar (PDA) and broth (PDB) (HiMedia Laboratories Limited, Mumbai, India) at 37 °C for 48 h to ensure viability and purity prior to activity testing.

#### Inoculum Preparation

Stock inoculum of the *Candida* species were prepared by picking freshly growing colonies from 24-h-old cultures grown on PDA plates and suspended in 5 ml of sterile saline (0.85 %). Cell density was adjusted using a spectrophotometric method at 600 nm wavelength to achieve the turbidity equivalent 0.5 McFarland standard. The dilution of the *Candida* species stock suspension was adjusted from 1–5×10<sup>6</sup> cells/ml.

#### Antifungal Susceptibility Testing by Determining the MIC

The in vitro antifungal activities of the azoles, amphotericin B, and  $\alpha$ - and  $\beta$ -asarones were determined using a broth microdilution method, which was performed in accordance with Clinical and Laboratory Standards Institute guidelines [16]. Minimal inhibitory concentration (MIC) values were determined in 96-well flat-bottomed microtiter plates by measuring the optical density (OD) of the *Candida* cultures. In all experiments, the test medium was Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich) containing L-glutamine but lacking sodium bicarbonate, buffered to pH 7.0 with 0.165 M MOPS (Sigma-Aldrich). *Candida* cultures were prepared from 1-day-old cultures grown on PDA slants. *Candida* suspensions were diluted in RPMI 1640 to give a final inoculum of 5×10<sup>3</sup> CFU/ml. Series of twofold dilutions were prepared in RPMI 1640 and were mixed with equal amounts of cell. The final concentrations for azoles and amphotericin B in the wells were 0.5–128  $\mu$ g/ml, and for asarones, it was 2–1000  $\mu$ g/ml.

The microplates were incubated for 48 h at 37 °C, and the OD was measured at 600 nm with a microtiter plate reader (Bio-Rad). Uninoculated medium was used as the background for the spectrophotometric calibration; the growth control wells contained inoculum suspension in the drug-free medium. The solvent control wells contained inoculum suspension in the drug-free solvent containing (1 %) medium to prove that solvent had no inhibitory effect on the investigated fungi at the applied concentration. For calculation of the extent of inhibition, the OD<sub>600 nm</sub> of the drug-free control cultures was set at 100 % growth. The MICs for asarones

were the lowest concentration of drugs that produced an optically clear well, while the MICs for azoles and amphotericin B were the lowest concentration of drugs that produced a prominent decrease in turbidity. The quality control strains were included every time an isolate was tested. All experiments were repeated at least three times.

### Synergistic Activity Between Asarones and Azoles and Amphotericin B

To evaluate the combined effects of azoles and amphotericin B with asarones, checkerboard experiments were carried out [17, 18]. Briefly, cells were grown overnight in PDA medium at 37 °C with constant agitation. After incubation, the cells were washed in PBS and suspended as  $4 \times 10^3$  cells/ml solution. A 100  $\mu$ l aliquot of microtiter plate containing RPMI medium in the absence and presence of different concentrations of asarones, azoles, and amphotericin B, alone or in combination. In brief, serial double dilutions of the test compounds were prepared (in  $\mu$ g/ml): 0.015–2 (azoles and amphotericin B) and 0.12–32 (asarones). Serial dilutions of the combination of asarones with azoles and amphotericin B were mixed in PDB. Plates were incubated at 37 °C for 24 h, and the MIC was determined by measuring the optical density at 600 nm. The MIC was defined as the lowest concentration of drug, alone or in combination with other agents, inhibiting the growth of yeast (100 % inhibition of growth compared to the control).

Drug interaction was based on the fractional inhibitory concentration index (FICI) (Odds 2003), a non-parametric model defined by the equation:

$$\text{FICI} = \text{FIC of asarones} + \text{FIC of azoles/amphotericin B}$$

where FIC of asarones is defined as MIC of asarones in combination with FLC divided by the MIC of asarones alone and FIC of azoles/amphotericin B as MIC of azoles/amphotericin B in combination with asarones divided by the MIC of azoles/amphotericin B alone. The values calculated by the FICI equation will determine the interaction relations as follows: values  $\leq 0.5$  represent synergistic interactions, 4.0 antagonistic effect, and values in between these two represent no interaction [19].

### Time-Kill Curve Analysis

A time-kill curve (CFU as a function of time) is evaluated to study the rate and extent of reduction in *Candida* spp. burden when treated with asarones and azoles/amphotericin B individually and in combinations. The experiments were conducted in PDB for 48 h. The concentrations used are the MIC values of corresponding asarones plus azole drugs in alone (see Table 1). An initial inoculum of approximately  $1 \times 10^6$  CFU/ml was taken for all the experiments. Samples (0.1 ml) were collected at 0, 2, 4, 6, 8, 12, 24, and 48 h and serially diluted in normal saline and aliquoted in PDA. These plates were then incubated at 37 °C for 48 h. The colonies were counted after 48 h incubation. The broth without any agent was used as the control. The data were plotted as log CFU/ml versus time (h) for each time point. Tests were performed three times. Synergism was defined as a decrease of  $\geq 1 \log_{10}$  CFU/ml in antifungal activity produced by the combination compared with the agent alone after 48 h. Lowest limit of quantification of time-kill assay was  $0.5 \log_{10}$  CFU/ml (99.99 % reduction).

### Inhibition of Biofilm Formation Assay

*Candida* biofilms were developed on polystyrene surfaces of 96-well plates as per standard methodologies [20]. One hundred microliters of a cell suspension ( $1 \times 10^6$  cells/ml) in PBS was

**Table 1** Synergistic activity of the  $\infty$ - and  $\beta$ -asarone with azoles and amphotericin B against *Candida* species

Test bacteria	Agent	MIC ( $\mu\text{g/ml}$ )		Outcome
		Alone	Combination <sup>a</sup>	
<i>C. albicans</i>	$\infty$ -Asarone FLU	250 1	8 0.06	Synergistic
	$\infty$ -Asarone CLO	250 2	16 0.12	Indifferent
	$\infty$ -Asarone AMP	250 1	8 0.03	Synergistic
<i>C. tropicalis</i>	$\infty$ -Asarone FLU	500 1	16 0.12	Synergistic
	$\infty$ -Asarone CLO	500 1	8 0.25	Synergistic
	$\infty$ -Asarone AMP	500 2	4 0.25	Synergistic
<i>C. albicans</i>	$\beta$ -Asarone FLU	125 1	2 0.03	Synergistic
	$\beta$ -Asarone CLO	125 2	4 0.06	Synergistic
	$\beta$ -Asarone AMP	125 2	8 0.06	Synergistic
<i>C. tropicalis</i>	$\beta$ -Asarone FLU	64 2	2 0.03	Synergistic
	$\beta$ -Asarone CLO	64 4	2 0.06	Synergistic
	$\beta$ -Asarone AMP	64 2	1 0.03	Synergistic

MIC value of  $\infty$  and  $\beta$ -asarone, azoles, and amphotericin B alone was conducted by macrobroth dilution method, and MIC value of combination was conducted by checkerboard assay. FICI index were obtained from the checkerboard assay: To evaluate the interaction of asarones with azoles against *Candida* species, checkerboard experiments were performed. FIC index = (MIC of azoles and amphotericin B in combination with  $\infty$  and  $\beta$ -asarone / MIC of azoles and amphotericin B alone) + (MIC of  $\infty$  and  $\beta$ -asarone in combination with azoles / MIC of  $\infty$  and  $\beta$ -asarone alone). A FICI less than or equal to 0.5 is indicated as synergy

<sup>a</sup>The MIC of asarones in combination with azoles and amphotericin B

inoculated, and plates were incubated at 37 °C for 90 min to allow attachment of cells onto the surface. Non-adhered cells were removed by washing the wells with sterile PBS, two to three times. RPMI 1640 medium (200  $\mu\text{l}$ ) was added to each well, and the plates were incubated at 37 °C for 24 h to allow biofilm formation. RPMI 1640 medium with 1:1 concentrations of test compound was added immediately after the adhesion phase to observe any effects on the development of biofilms. After incubation, wells were washed to remove any released cells, and biofilm growth was analyzed by MTT-metabolic assay.

#### Statistical Analysis

All statistical analyses were performed with SPSS (Version 17.0; SPSS, Inc., Chicago, IL, USA). In MIC and FICI determinations, when the results were different in both experiments, we made another test for final result. One-way ANOVA-Duncan's multiple range test was used

in time-kill assay. Data for time-kill analysis was presented as means±standard deviations. Statistical significance was defined as  $p < 0.05$ .

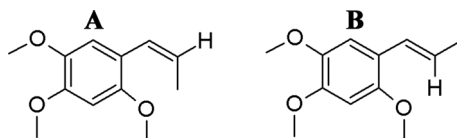
## Results

### Purification of $\alpha$ - and $\beta$ -Asarone from *A. calamus*

*A. calamus* was extracted with 90 % MeOH. The concentrated crude extracts were then partitioned with EtOAc. Antifungal activity against *Candida* spp. was detected in the EtOAc layer. The active organic layers were pooled, concentrated, and further chromatographed on a silica gel (100–200, Merck) column. The silica gel column was eluted with stepwise gradients of  $\text{CHCl}_3$  and MeOH. The fractions eluted with  $\text{CHCl}_3$  and MeOH (80:20 and 30:70 v/v) showed a high level of antifungal activity against *Candida* spp. and was rechromatographed on a silica gel column using a stepwise gradient elution system of *n*-hexane and  $\text{Me}_2\text{CO}$ . The 90 % hexane/10 % acetone and 100 % acetone fractions that were highly active against *Candida* spp. were further purified by Sephadex LH-20 column (26×950 mm) chromatography. Antifungal activity against *Candida* spp. was detected in fractions 47–75 and 103–155. The antifungal compounds of Sephadex LH-20 fractions active against *Candida* spp. were further purified by TLC. Silica gel plates loaded with the antifungal-active fractions were developed by a solvent system of *n*-hexane/EtOAc (8:2, v/v). MeOH elutes of the active band scraped from preparative TLC plates were bioassayed against *Candida* spp. using a paper disc method and then purified by gel filtration chromatography of Sephadex LH-20 (10×950 mm column). The antifungal compound active against 47–75 and 103–155 was detected by bioautography on a silica TLC plate. The clear inhibition zone occurred at the position of  $R_f$  0.40 and 52 on the silica gel TLC plates developed by a solvent system of *n*-hexane/EtOAc (8:2, v/v). The HPLC profile of the antifungal compound  $\alpha$ - and  $\beta$ -asarone showed an antifungal active peak at the retention time of 12.34 and 15.86 min, respectively at 270 nm, which matched with the authentic  $\alpha$ - and  $\beta$ -asarone from Sigma-Aldrich. The yield of  $\alpha$ - and  $\beta$ -asarone was 21.1 and 43.56 mg, respectively. The chemical structure of  $\alpha$ - and  $\beta$ -asarone is shown in Fig. 2.

### Antifungal Activity

The MIC values of  $\alpha$ - and  $\beta$ -asarone, azoles, and amphoteric B alone are shown in Table 1. It is evident from the table that the highest activity was recorded for  $\beta$ -asarone in the range of 64–125  $\mu\text{g/ml}$ . While  $\alpha$ -asarone exhibited activity at higher concentration (250–500  $\mu\text{g/ml}$ ), for azole drugs, the activity ranges from 1 to 4  $\mu\text{g/ml}$  and for amphotericin B, the activity ranges from 1 to 2  $\mu\text{g/ml}$ .



**Fig. 2** Structure of asarones. **a**  $\alpha$ -asarone and **b**  $\beta$ -asarone



## Checkerboard Assay

The combined activities of  $\alpha$ - and  $\beta$ -asarone with azoles and amphotericin B from the in vitro checkerboard interactions against the *Candida* species are summarized in Table 1. FIC, FIC index, and interpretations for the activities of  $\alpha$ - and  $\beta$ -asarones with azoles and amphotericin B against the test *Candida* species recorded significant synergistic interaction. Significant synergistic interaction was recorded by  $\beta$ -asarone with azoles and amphotericin B. Antagonism and indifference was not recorded for any combinations. In the combination of  $\alpha$  and  $\beta$ -asarone with azoles and amphotericin B, the MIC values have reduced to more than eight times.

## Time-Kill Assay

Time-kill assays for the synergistic combinations ( $\alpha$ - and  $\beta$ -asarone with azoles and amphotericin B) on *Candida* species are shown in Fig. 3. The maximum reduction of *Candida* species growth by  $\alpha$ -asarone with azoles and amphotericin B was recorded between 2 and 4 h ( $p < 0.05$ ) (Fig. 3a). At 48 h, this combination reduced 99.99 % of *Candida* species. Significant reduction was recorded by the combination  $\alpha$ -asarone plus clotrimazole and amphotericin B, and at 48 h, this combination resulted in 100 % inhibition. Regrowth of the *Candida* species was recorded by  $\alpha$ -asarone alone after 12 h.

For  $\beta$ -asarone with azoles and amphotericin B, maximum reduction in the *Candida albicans* was recorded by  $\beta$ -asarone and fluconazole and the maximum reduction was recorded between 0 and 2 h. This combination reduced 99.99 % of *C. albicans* at 12 h ( $p < 0.05$ ) (Fig. 3b). Whereas against *C. tropicalis*, maximum reduction was recorded by  $\beta$ -asarone plus fluconazole and amphotericin B and the maximum reduction was recorded between 0 and 2 h and reduced 99.99 % of *C. tropicalis* at 12 h ( $p < 0.05$ ) (Fig. 3b). The combination of asarone with azoles and amphotericin B never recorded regrowth even after 48 h.

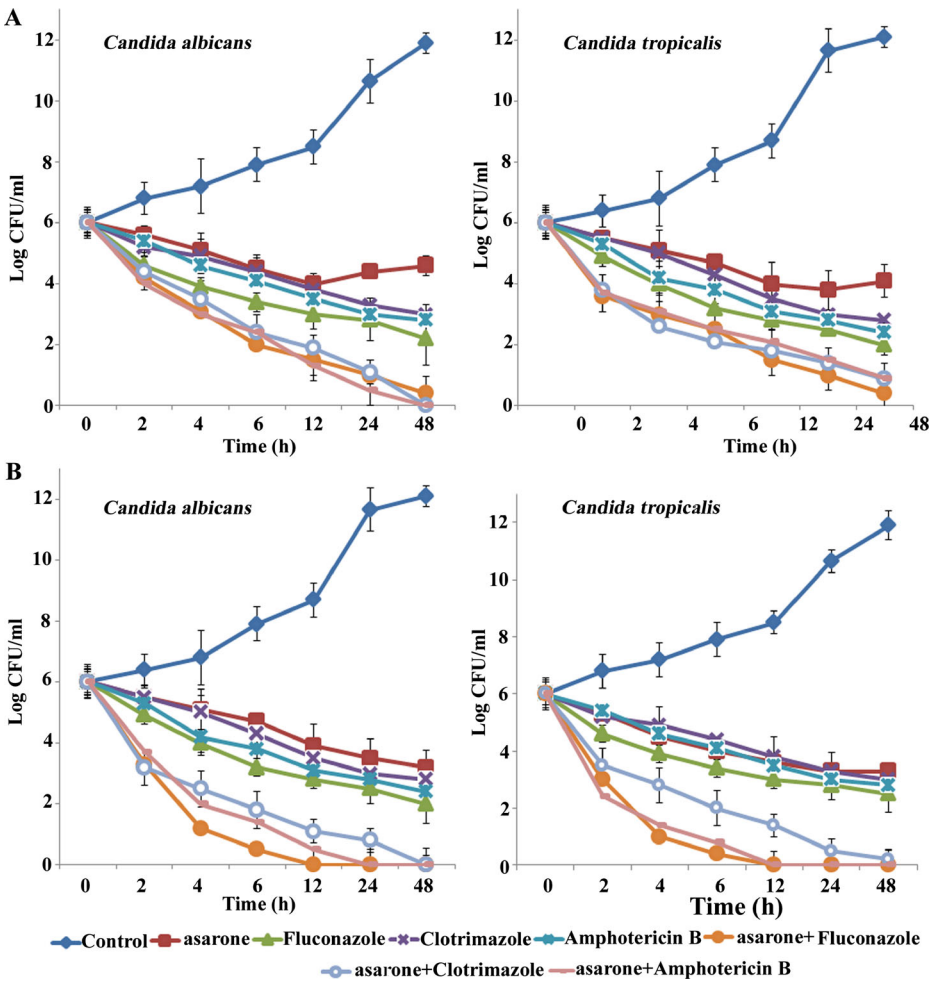
## Biofilm Inhibition

$\alpha$ - and  $\beta$ -asarone alone recorded the inhibition of biofilms of *Candida* sp. at MIC concentration of 69–80 % (Fig. 4). Whereas, the combination of  $\alpha$ - and  $\beta$ -asarone with azoles and amphotericin B recorded significant inhibition of biofilms than the individual compounds. Significant inhibition was recorded by  $\beta$ -asarone plus fluconazole against *C. tropicalis* (12 %) (Fig. 4). This clearly indicated that combination of asarones and antibiotics has better inhibition of biofilm formation.

## Discussion

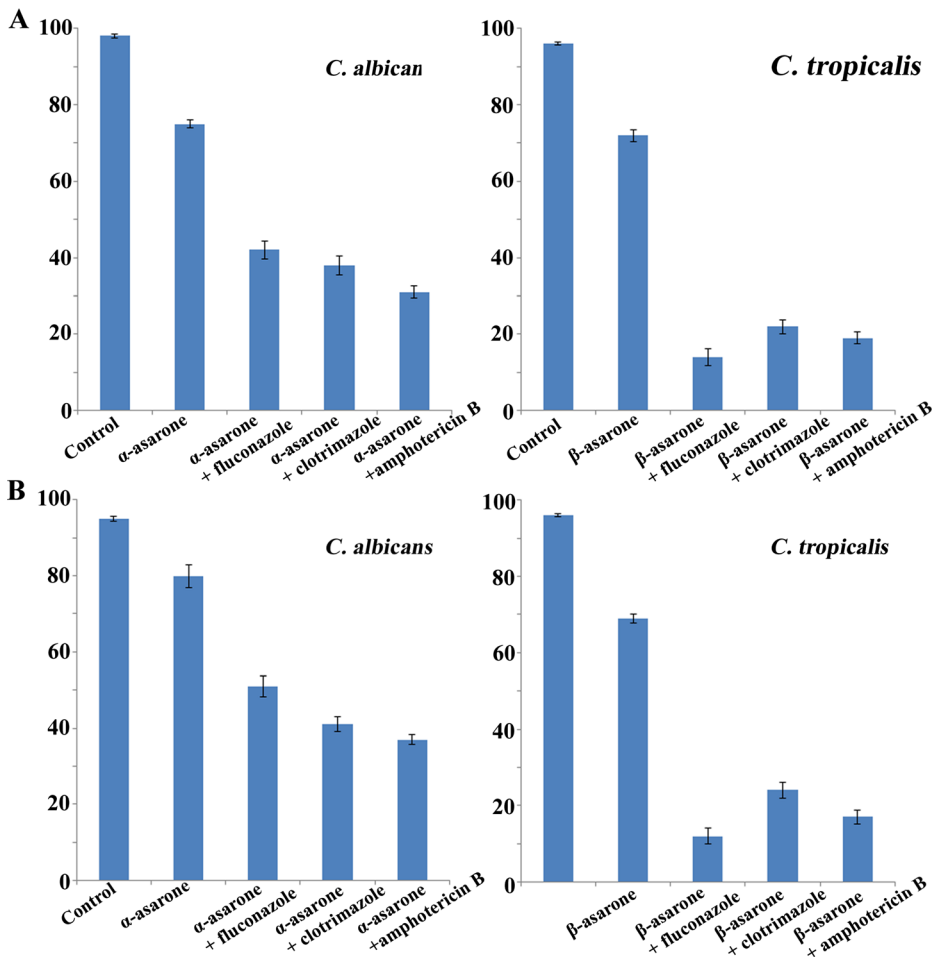
In recent years, candidiasis has reemerged with higher prevalence and mortality rates that are nearly 45 % among immunocompromised population groups [21]. Combination therapy is one approach that can be used to improve the efficacy of antimicrobial therapy for difficult-to-treat infections [22]. Attempts have been made to cope with treatment failures either by combining different antifungals or by combining antifungals with non-antifungals [23]. However, assessing the nature and intensity of drug interactions is still a debated issue. In the present study, we investigated the combined effects of asarones plus azoles and amphotericin B against *Candida* species by the checkerboard microdilution method and the time-killing test.





**Fig. 3** Time-kill curve of asarones, azoles, and amphotericin B alone and in combination against *Candida* species. The strains at a starting inoculum density of  $10^6$  CFU/ml were used. At 0, 2, 4, 6, 8, 12, 24, and 48 h, aliquots were removed from each tube to examine the cell viability. The experiments were performed three times. Data are expressed as mean±standard deviation. **a**  $\alpha$ -asarone and **b**  $\beta$ -asarone. The *x*-axis represents time, and the *y*-axis represents logarithmic *Candidal* survival. CFU colony-forming unit

Phongpaichit et al. [24] reports the significant antifungal activity of *A. calamus* extracts against various pathogenic fungi like *Trichophyton rubrum*, *Microsporium gypseum*, and *Penicillium marneffeii* with  $IC_{50}$  values of 0.2, 0.2, and 0.4 mg/ml, respectively. However, this extract recorded moderate activity against yeasts: *C. albicans*, *Cryptococcus neoformans*, and *Saccharomyces cerevisiae* (MIC 0.1–1 mg/ml). In addition, authentic  $\alpha$ - and  $\beta$ -asarones were also tested for their antimicrobial potential. Both  $\alpha$ - and  $\beta$ -asarones exhibited good antimicrobial activities against the fungi and yeasts than those of rhizome and leaf extracts. The study clearly suggested that *A. calamus* rhizomes and leaves must possess active principle  $\alpha$ - and  $\beta$ -asarones which is believed to be responsible for their antimicrobial activities; further, it was established that  $\beta$ -asarone has high antimicrobial activity as compared



**Fig. 4** Effect of the combination of asarones and drugs on the inhibition of biofilm formation on *Candida* species. **a**  $\alpha$ -asarone and **b**  $\beta$ -asarone

to the  $\alpha$ -asarone [14]. Similar in our study, also  $\alpha$ - and  $\beta$ -asarones are the major compounds present in *A. calamus* and  $\beta$ -asarone exhibited high anticandidal activity than the  $\alpha$ -asarone.

Rajput and Karuppaiyl [25] reported the anticandidal properties of *A. calamus* rhizome (ethyl acetate) extract and its active principle,  $\beta$ -asarone.  $\beta$ -asarone exhibited promising growth inhibitory activity at 0.5 mg/ml. Similarly, in the present study, also  $\beta$ -asarone recorded more activity. Minimum fungicidal concentration (MFC) of  $\beta$ -asarone was highly toxic to *C. albicans*, killing 99.9 % inoculum within 120 min of exposure.  $\beta$ -asarone inhibited *C. albicans* morphogenesis and biofilm development at subinhibitory concentrations [25].

In this study, we have investigated the in vitro anticandidal activity of  $\alpha$ - and  $\beta$ -asarone, azoles, and amphotericin B alone and in combination by checkerboard assay to determine whether synergism, indifference, or antagonism would be the principal response against two *Candida* species. Very interestingly, the results of our study clearly indicated that asarones in combination with azoles and amphotericin B predominantly recorded synergistic effect

(Table 1). Significant synergistic activity was recorded by  $\beta$ -asarone and amphotericin B against *C. tropicalis*. Synergy will give significantly greater activity provided by two agents combined than that provided by the sum of each agent alone [26]. Currently, no information regarding the anticandidal activity of asarones in combination with azoles and amphotericin B drugs is available in literature. The in vitro synergistic activity of asarones plus azoles and amphotericin B against *Candida* species is reported for the first time. Further we have checked the inhibition of asarones and combination of asarones and antibiotics against *Candida* species, and the results clearly indicated that the combination of asarones and antibiotics inhibited the biofilm formation significantly than the asarones alone. *C. albicans* biofilms on implanted medical devices are a major source of life-threatening blood stream infections. Fluconazole, a frontline drug for the treatment of *C. albicans* infections, was relatively ineffective against the yeast in such biofilms, while amphotericin B was active against them [27]. Similarly, in the present study, also asarones in combination with amphotericin B recorded significant inhibition in biofilm formation.

In our study, significant synergy was recorded by asarones in combination with antibiotics. Positive interactions by checkerboard microdilution were confirmed by time-killing test in our study. Time-kill curves can provide growth kinetic information overtime. This method is often used to detect the differences in the rate and extent of antifungal activity over time. In our experiment, there was great agreement between the FICI method and the time-kill curves. The previous literatures also demonstrated the accordance between the two methods [28, 29].

Improving the efficiency of antifungal compounds can be done by using combination therapy, i.e., by combining agents with different antifungal mechanisms [30] or combining antifungal and non-antifungal compounds [29, 31]. In the present study, clinically used antifungal agents were combined with the  $\alpha$ - and  $\beta$ -asarone, and the numerous synergistic interactions noted during the study demonstrated that asarones might be of use in natural compound and antifungal combination therapy. In the present study, the significant enhancement in the activity of asarones and azoles and amphotericin B may be due to the inhibition of ergosterol. Ergosterol is an essential component of the fungal plasma membranes, and the inhibition of its synthesis negatively influences the membrane fluidity [32]. The antifungal effect of asarones are due, in part, to their role on ergosterol levels, as well as to their indirect effect on cell signaling, proliferation, and differentiation through inhibition of the synthesis of important terpenoids. The precise mechanism of action of asarones and azoles and amphotericin B on yeast cells still need to be elucidated deeply.

## Conclusions

In conclusion, to the best of our knowledge, this study reports for the first time the ability of asarones to sensitize in vitro *Candida* species to azoles and amphotericin B. This study suggests that asarones is not only a natural compound capable of inhibiting *Candida* species growth but is also able to act synergistically with azoles and amphotericin B in very low doses. Further studies are warranted to explore the mechanism behind the antifungal activity of asarones plus antibiotics (azoles and amphotericin B). Our findings are very much encouraging in view of the growing treatment failures and antibiotic resistance in *Candida* species and suggest a way of treating resistant *Candida* infections through an asarones plus azoles and amphotericin B combination approach.

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**Conflict of Interest** The authors declare that they have no conflicts of interest.

## References

1. Afeltra, J., & Verwiji, P. E. (2003). Antifungal activity of non-antifungal drugs. *European Journal of Clinical and Microbiological Infectious Diseases*, *22*, 397–407.
2. Wilson, L. S., Reyes, C. M., Stolpman, M., Speckman, J., & Allen, K. (2002). The direct cost and incidence of systemic fungal infections. The direct cost and incidence of systemic fungal infections. *Value in Health*, *5*, 26–34.
3. Gudlaugsson, O., Gillespie, S., Lee, K., Vande Berg, J., & Allen, K. (2003). The direct cost and incidence of systemic fungal infections. *Value in Health*, *37*, 1172–1177.
4. Chi, H. W., Yang, Y. S., Shang, S. T., Chen, K. H., Yeh, K. M., Chang, F. Y., & Lin, J. C. (2011). *Candida albicans* versus non-*albicans* bloodstream infections: the comparison of risk factors and outcome. *Journal of Microbiology and Immunological Infections*, *44*, 369–375.
5. Iwazaki, R. S., Eliana, H. E., Ueda-Nakamura, T., Nakamura, C. V., Garcia, L. B., & Filho, B. P. D. (2010). In vitro antifungal activity of the berberine and its synergism with fluconazole. *Antonie van Leeuwenhoek*, *97*, 201–205.
6. Marchetti, O., Moreillon, P., Entenza, J. M., Vouillamoz, J., Glauser, M. P., & Bille, J. (2003). Fungicidal synergism of FLU and cyclosporine in *Candida albicans* is not dependent on multidrug efflux transporters encoded by the CDR1, CDR2, CaMDR1, and FLU1 genes. *Antimicrobial Agents and Chemotherapy*, *47*, 1565–1570.
7. Georgopapadakou, N. H., & Tkacz, J. S. (1995). The fungal cell wall as a drug target. *Trends in Microbiology*, *3*, 98–104.
8. Cabrala, M. E., Lucia, I. C., Figueroaa, B., Julia, I., & Farina, A. (2013). Synergistic antifungal activity of statin-azole associations as witnessed by *Saccharomyces cerevisiae*- and *Candida utilis*-bioassays and ergosterol quantification. *Revista Iberoamericana de Micología*, *30*(1), 31–38.
9. Gilani, A. U., Shah, A. J., Ahmad, M., & Shaheen, F. (2006). Antispasmodic effect of *Acorus calamus* is mediated through calcium channel blockade. *Phytotherapy Research*, *20*, 1080–1084.
10. Meena, A. K., Rao, M. M., Singh, A., & Kumar, S. (2010). Physiochemical and preliminary phytochemical studies on the rhizome of *Acorus calamus*. *International Journal Pharmacy and Pharmaceutical Science*, *2*, 130–131.
11. Palani, S., Kumar, R., Parameswaran, R. P., & Kumar, B. S. (2010). Therapeutic efficacy of *Acorus calamus* on acetaminophen induced nephrotoxicity and oxidative stress in male albino rats. *Acta Pharmaceutical Science*, *52*, 89–100.
12. Lee, S. H., Kim, K. Y., Yoon, Y., Hahm, D. H., & Kong, S. A. (2010). Asarone inhibits adipogenesis and stimulates lipolysis in 3T3-L-1 adipocytes. *Cellular and Molecular Biology*, *24*, 1215–1222.
13. Geng, Y., Li, C., Liu, J., Xing, L., Zhou, L., Dong, M., Li, X., & Niu, Y. (2010). Beta-asarone improves cognitive function by suppressing neuronal apoptosis in the beta-amyloid hippocampus injection in rats. *Biology and Pharmaceutical Bulletin*, *33*, 836–843.
14. Devi, S. A., & Ganjewa, D. (2009). Antimicrobial activity of *Acorus Calamus* (L.) rhizome and leaf extract. *Acta Biologica Szegediensis*, *53*, 45–49.
15. Aqil, F., Maryam, Z., & Ahmad, I. (2008). Antimutagenic activity of methanolic extracts of four medicinal plants. *Indian Journal of Experimental Biology*, *46*(9), 668–672.
16. Clinical and Laboratory Standards Institute (CLSI) (2012). Reference method for broth dilution antifungal susceptibility testing of yeasts, as the document is M27-S4. Wayne, PA, USA.
17. Orhan, G., Bayram, A., & Zer, Y. (2005). Synergy test by E test and checkerboard methods of antimicrobial combinations against *Brucella melitensis*. *Journal of Clinical Microbiology*, *43*, 140–143.
18. Mukherjee, P. K., Sheehan, D. J., Hitchcock, C. A., & Ghannoum, M. (2005). Combination treatment of invasive fungal infections. *Clinical Microbiology Review*, *18*, 163–194.

19. Odds, F. C. (2003). Synergy, antagonism, and what the checkerboard puts between them. *Journal of Antimicrobial Agents and Chemotherapy*, *52*, 1.
20. Hawser, S. P., & Dauglas, L. J. (1994). Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infection and Immunology*, *62*, 915–921.
21. Wenzel, R. P., & Gennings, C. (2005). Bloodstream infections due to *Candida* species in the intensive care unit: identifying especially high-risk patients to determine prevention strategies. *Clinical Infectious Diseases*, *41*, S389–S393.
22. Afeltra, J. R. G., Vitale, J. W. M., & Verweij, P. E. (2004). Potent synergistic in vitro interaction between non antimicrobial membrane-active compounds and itraconazole against clinical isolates of *Aspergillus fumigatus* resistant to itraconazole. *Antimicrobial Agents and Chemotherapy*, *48*, 1335–1343.
23. Sun, S., Yan, L., Qiongjie, G., Changwen, S., Jinlong, Y., & Lin, M. (2008). In vitro interactions between tacrolimus and azoles against *Candida albicans* determined by different methods. *Antimicrobial Agents and Chemotherapy*, *52*, 409–417.
24. Phongpaichit, S., Pujenjob, N., Rukachaisirikul, V., Ongsakul, M., & Songklanakarin. (2005). Antimicrobial activities of the crude methanol extract of *Acorus calamus* Linn. Songklanakarin. *Journal of Science and Technology*, *27*, 517–523.
25. Rajput, S. B., & Karuppaiyl, S. M. (2013).  $\beta$ -asarone, an active principle of *Acorus calamus* rhizome, inhibit morphogenesis, biofilm formation and ergosterol biosynthesis in *Candida albicans*. *Phytomedicine*, *20*, 139–142.
26. Sweeney, M. T., & Zurenko, G. E. (2003). *In vitro* activities of linezolid combined with other antimicrobial agents against *Staphylococci*, *Enterococci*, *Pneumococci*, and selected gram-negative organisms. *Antimicrobial Agents and Chemotherapy*, *47*(6), 1902–1906.
27. Tebbets, B., Zhiguo, Y., Douglas, S., Lixing, Z., Yi, J., Lihua, X., David, A., Ben, S., & Bruce, K. (2013). Identification of antifungal natural products via *Saccharomyces cerevisiae* bioassay: insights into macrotetrolide drug spectrum, potency and mode of action. *Medical Mycology*, *51*, 280–289.
28. Guo, Q., Sun, S., Yu, J., Li, Y., & Cao, L. (2008). Synergistic activity of azoles with amiodarone against clinically resistant *Candida albicans* tested by checkerboard and time-kill methods. *Journal of Medical Microbiology*, *57*, 457–462.
29. Quan, H., Cao, Y. Y., Xu, Z., Zhao, J. X., Gao, P. H., Qin, X. F., & Jiang, Y. Y. (2006). Potent in vitro synergism of fluconazole and berberine chloride against clinical isolates of *Candida albicans* resistant to fluconazole. *Antimicrobial Agents and Chemotherapy*, *50*, 1096–1099.
30. Girmenia, C., Venditti, M., & Martino, P. (2003). Fluconazole in combination with flucytosine in the treatment of fluconazole-resistant *Candida* infections. *Diagnostic Microbiology and Infectious Diseases*, *46*, 227–231.
31. Cruz, M. C., Goldstein, A. L., Blankenship, J. R., Del Poeta, M., Davis, D., Cardenas, M. E., Perfect, J. R., McCusker, J. H., & Heitman, J. (2002). Calcineurin is essential for survival during membrane stress in *Candida albicans*. *EMBO Journal*, *21*, 546–559.
32. Gyetvai, A., Emri, T., & Takács, K. (2006). Lovastatin possesses a fungistatic effect against *Candida albicans* but does not trigger apoptosis in this opportunistic human pathogen. *FEMS Yeast Research*, *6*, 1140–1148.