**Original Article**

**In Vitro Effects of Farnesol Alone and in Combination with Antifungal Drugs Against *Aspergillus* Clinical Isolates**

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**ABSTRACT**

Farnesol is an extracellular quorum-sensing molecule produced by *Candida albicans*. Farnesol is also a sesquiterpene alcohol existing in many herbal products and has various activity against fungal cells. We aimed to investigate the efficacy of farnesol alone and the contribution of farnesol on the activity of voriconazole and amphotericin B against *Aspergillus* clinical isolates in vitro. A total of 45 *Aspergillus* clinical isolates were used in this study. The MIC values of voriconazole, amphotericin B, and farnesol were determined using reference broth microdilution method. The interactions of farnesol with voriconazole and amphotericin B were investigated by the checkerboard method and evaluated based on the fractional inhibitor concentration index (FICI). The MIC ranges of farnesol, voriconazole, and amphotericin B were 1,500-6,000 µM, 0.125-1 µg/mL, and 0.125-0.5 µg/mL against *Aspergillus fumigatus* isolates, 3,000-12,000 µM, 0.125-0.5 µg/mL, and 0.25-2 µg/mL against *Aspergillus flavus* isolates, respectively. The most common interaction in combination tests was “no interaction,” and synergistic interaction was not detected. The combinations of farnesol with voriconazole and amphotericin B had antagonistic activity against 38% and 27% of all isolates, respectively.

We concluded that the responses of different fungal species against farnesol are variable, and different interactions may be observed when it is combined with different antifungals. Therefore, it should be noted that farnesol may have an adverse effect on some fungi or interact negatively with antifungals used in combination.

**Key words**: amphotericin B, antifungal activity, *Aspergillus*, farnesol, voriconazole

**Introduction**

The frequency of invasive fungal infections (IFIs) has increased significantly due to invasive medical approaches, hematological malignancies, immunosuppressive therapy, and transplantation of hematopoietic stem cells or solid organs. After *Candida albicans*, *Aspergillus fumigatus* is the second most common invasive fungal pathogen with high mortality rates\(^1\). Currently, several antifungal drug classes including triazoles, polyenes, and echinocandins are available for use in the treatment of IFIs, and triazoles are the most important options for the treatment of invasive aspergillosis (IA). Although new antifungal drugs have been discovered, resistance against standard antifungal therapy is increasing, and no new classes of antifungal agents have been approved since 2006\(^2\). Treatment with available drugs is often complicated due to their high toxicity, low tolerability, drug interactions, and limited spectrums of activities. Moreover, some fungi are intrinsically resistant to these antifungal agents. Therefore, the need for new drug or treatment alternatives, especially those with a wider spectrum, have lower toxicity, and are cheaper, is increasing day by day. Particularly, the emerging resistance to triazoles (e.g., voriconazole) is an important problem emphasizing the need for new therapeutic options\(^2,3\).

Farnesol is an extracellular quorum-sensing molecule described nearly 20 years ago in the dimorphic yeast *C. albicans*, and it inhibits the yeast-to-hypha transition, consequently blocking biofilm formation in *C. albicans*\(^4\). Farnesol is also a sesquiterpene alcohol existing widely in fruits, vegetables, herbs, and essential oils\(^5\). An extensive variation has been reported in the activity of farnesol among fungal species: inhibition of conidiation in *Aspergillus niger*\(^6\), inhibition of germination in *Fusarium graminearum*\(^7\), apoptotic-like programmed cell death in *Aspergillus flavus*\(^8\).
Aspergillus nidulans\textsuperscript{9}, inhibition of gradual filamentation in Trichosporon spp.\textsuperscript{10}, decrease in the minimal inhibitory concentrations of echinocandins against Candida parapsilosis biofilm\textsuperscript{11}, and inhibition of biofilms in C. albicans resistant strains\textsuperscript{12}.

Because of these above-mentioned effects of farnesol, we think that it has the potential to become a new antifungal agent. Hence, we aimed to investigate the efficacy of farnesol alone and the contribution of farnesol on the activity of voriconazole and amphotericin B against clinical Aspergillus isolates in vitro.

Materials and methods

Isolates and media

The most common agents of IA were chosen for this study; a total of 45 Aspergillus clinical isolates (A. fumigatus, n = 31 and A. flavus, n = 14) were used. These isolates were non-repetitive and isolated from respiratory specimens (spum or bronchoalveolar lavage fluid) of different patients in our tertiary hospital. All isolates were identified using conventional methods, such as based on their microscopic morphological characteristics, macroscopic colony characteristics, and growth temperatures\textsuperscript{13}. In addition, most of these isolates have been previously identified using molecular methods\textsuperscript{14}. All of the clinically important fungal isolates are stored in our culture collection. Candida krusei ATCC 6258 and C. parapsilosis ATCC 22019 were used as quality-control isolates for antifungal susceptibility testing. Sabouraud dextrose agar (Merck, Darmstadt, Germany) was used for subculturing the strains for viability and purity. Potato dextrose agar (PDA, Merck, Darmstadt, Germany) was used for fresh cultures of all isolates prior to testing. RPMI-1640 medium (Merck, Darmstadt, Germany) buffered to pH 7.0 with MOPS (3-N-morpholinopropanesulfonic acid) was used for susceptibility testing and combination method.

Antifungals and farnesol

Stock solutions of voriconazole (VOR) and amphotericin B (AmB) (Sigma Chemical Co., St Louis, MO, USA) were prepared in dimethyl sulfoxide at a concentration of 3,200 µg/mL. Antifungal stock solutions were dispensed into 1-mL tubes and stored at -70°C until they were used. Farnesol was commercially obtained (Sigma Chemical Co.) as a 4 M stock solution, dissolved in methanol and diluted with RPMI-1640 medium to working concentration in which the methanol concentration was 1%. Each drug-free control well contained 1% methanol.

Antifungal susceptibility testing

Antifungal susceptibilities of all isolates against VOR, AmB, and farnesol were tested using the broth microdilution method in accordance with the M38-A2 document of the Clinical and Laboratory Standards Institute (CLSI)\textsuperscript{15}. Working dilutions were prepared by using RPMI 1640 medium to yield the final two-fold drug concentrations of 0.03-16 µg/mL for VOR and AmB, and 47-12,000 µM for farnesol. The solutions were dispensed into 96-well microtiter plates.

Fresh cultures of each isolate were grown on PDA for seven days at 35°C. The final fungal suspensions were prepared with sterile 0.85% saline and adjusted spectrophotometrically to an optical density (OD) at 530 nm ranging from 0.09 to 0.13. These suspensions were diluted 1:50 in RPMI 1640 medium, and the final inoculum concentrations ranged from approximately 0.4-5 x 10^4 CFU/mL, which are more concentrated than the study density. Each well of the microtiter plates was inoculated with 0.1 ml fungal suspension and incubated at 35°C. Growth (drug-free) and sterility (microorganism-free) control wells were included for each isolate, and all of the strains were evaluated twice for susceptibility testing. The minimal inhibitory concentrations (MICs) of VOR and AmB were evaluated visually after 48-h incubation and read at the lowest drug concentration that prevented any discernible growth (100% inhibition). For farnesol, 50% or more growth inhibitions were assessed both visually and under a stereomicroscope.

Combination testing

The combinations of farnesol with VOR or AmB were performed against all isolates using the checkerboard method in accordance with the M38-A2 document of CLSI. One microtiter plate was prepared for each combination against each isolate. The concentration ranges of farnesol and drugs were determined in accordance with predetermined MIC values and prepared in RPMI-1640 medium to become 4-fold of the final concentrations in the microplates. Antifungal agents and farnesol were dispensed in the rows and columns, respectively, at 50 µL per well. One hundred microliters of fungal suspension (0.4-5 x 10^4 CFU/mL) for each isolate were inoculated in each well of a plate. For all combinations, MIC was determined after 48 h of incubation at 35°C with the endpoint criterion mentioned above for VOR and AmB.

Drug interactions were classified as synergistic, no interaction (indifference) or antagonistic based on the fractional inhibitory concentration index (FICI). FIC values were obtained by dividing the MIC value of the drug combination by the MIC value that was detected when the drug was tested alone. FICI was obtained by summing the FIC values of each drug (FICI = FIC A + FIC B). Synergy was defined as an FICI of ≤ 0.5; no interaction (indifference) was defined as an FICI > 0.5 but < 4; and antagonism was defined as an FICI ≥ 4. Off-scale MIC values were converted to the next highest two-fold concentration.
Results

The results of this study are presented in Table 1. The MIC ranges of farnesol, VOR, and AmB were 1,500-6,000 µM (geometric mean = 3,587 µM), 0.125-1 µg/mL (geometric mean = 0.36 µg/mL) and 0.125-0.5 µg/mL (geometric mean = 0.26 µg/mL) against A. fumigatus isolates, 3,000-12,000 µM (geometric mean = 5,434 µM), 0.125-0.5 µg/mL (geometric mean = 0.30 µg/mL) and 0.25-2 µg/mL (geometric mean = 0.61 µg/mL) against A. flavus isolates, respectively. The most common interaction in combination tests was “no interaction,” and synergistic interaction was not detected in any combination. Geometric means of FICI values in the combination of farnesol with voriconazole and amphotericin B were 2.6 (range: 1.02-8.5) and 2.2 (range: 0.56-8.03) for A. fumigatus, 2.4 (range: 1.08-5.0) and 2.8 (range: 1.02-10) for A. flavus, respectively. The combination of farnesol with VOR had antagonistic activity against 13 (42%) A. fumigatus isolates with FICI values of 4.01 to 8.5, and 4 (29%) A. flavus isolates with FICI values of 4.008 to 5. Similarly, the combination of farnesol with AmB had antagonistic activity against 7 (23%) A. fumigatus isolates with FICI values of 4.01 to 8.03, and 5 (36%) A. flavus isolates with FICI values of 4.5 to 10.

Discussion

The increase in the frequency of fungal infections has been brought about by problems related to treatment and resistance. Because of the eukaryotic nature of the fungal cell, although several antifungal drugs in different chemical classes have been approved by the Food and Drug Administration, treatment is often difficult due to their high toxicity, low tolerability, or narrow spectrum of action. These issues have encouraged studies on the use of combination therapies or non-drug substances for the treatment of invasive fungal infections. An alternative approach involves increasing the effectiveness or widening the spectrum of action of antifungals. Natural products are unique chemicals with different biological activities, and the potential antimicrobial effects of certain natural compounds have attracted serious attention within the scientific community. Significant progress is foreseen in the discovery of new antifungal drugs with the contribution of inexpensive, nontoxic, and easily accessible natural compounds.

Farnesol is a 15-carbon isoprenoid alcohol endogenously synthesized by C. albicans via enzymatic dephosphorylation of farnesy1 pyrophosphate, which is part of steroid biosynthesis, and it also exists in many natural sources. It has been shown that farnesol exogenously inhibits the conidiation in A. niger and the germination of macroconidia in F. graminearum, and induces apoptotic-like programmed cell death in A. flavus, A. nidulans, and F. graminearum. Furthermore, it has also been shown that farnesol has antifungal activity against many yeasts. In a study evaluating the combinations of farnesol with fluconazole, anidulafungin, and AmB against C. albicans biofilm, synergic interactions were observed for farnesol with fluconazole and micafungin combinations, and no interaction for farnesol with AmB combination based on FICI indexes. Farnesol inhibits gradual filamentation at a concentration range between 600 and 1,200 µM and causes the reduction of filament structures at every stage of biofilm development in Trichosporon spp., decreases the MICs of caspofungin and micafungin against C. parapsilosis biofilm, and inhibits the development of biofilms formed by C. albicans resistant strains. Cordeiro et al. evaluated the antifungal activity of farnesol alone and in combination with fluconazole, itraconazole, AmB, and caspofungin against drug-resistant strains of Candida species (n = 45); the MICs of farnesol ranged between 4.68 and 150 µM, and farnesol significantly reduced the MICs of all antifungals against all isolates. Furthermore, they observed significant rates of synergistic interactions without any antagonistic interaction in all combinations. The MIC-decreasing effects of farnesol on echinocandin drugs have also been reported for both planktonic and sessile cells of C. parapsilosis; the median MICs of caspofungin and micafungin in combination with farnesol showed up to 64-fold decreases. In addition, it has been reported that farnesol blocks the paradoxical growth caused by caspofungin at high concentrations in both C. parapsilosis and A. fumigatus.

It has been suggested that farnesol may also be have antifungal activity against Aspergillus spp. because of the effects mentioned above on the growth or biofilm formation of various fungi as reported in many studies. In the present study, we investigated the activity of farnesol against Aspergillus clinical isolates and its contribution to the efficacy of antifungal drugs in vitro. However, we did not observe any promising results. The MIC values of farnesol alone were high against our Aspergillus isolates. Moreover, no synergistic interaction was detected in combinations of farnesol with VOR or AmB against Aspergillus isolates in this study. Conversely, considerably antagonistic interaction was observed: 38% for farnesol with VOR combinations, 27% for farnesol with AmB combinations. There may be two different explanations for this antagonistic interaction in our results. First, regarding the mechanism of action of farnesol on the fungal cells, farnesol and its derivatives have been thought to be precursors in the biosynthetic pathway of sterols, and it has been suggested that exogenous farnesol causes alterations in the cell membrane by inhibiting the synthesis of ergosterol. AmB is an antifungal drug causing pore formation at the cell membrane and induction of ergosterol sequestration after binding to ergosterol. While farnesol decreases the biosynth-
Table 1. Results of antifungal susceptibility testing and combination studies

| Isolates | Farnesol MIC (µM) | VOR MIC (µg/mL) | Combination of farnesol and VOR MICs | FICI Interpretation | AmB MIC (µg/mL) | Combination of farnesol and AmB MICs | FICI Interpretation |
|----------|------------------|----------------|-------------------------------------|---------------------|----------------|-------------------------------------|---------------------|
| **A. fumigatus** | | | | | | | |
| 91422 | 3000 | 0.125 | 94 / 0.25 | 2.031 | Indifference | 0.125 | 94 / 0.25 | 2.031 | Indifference |
| 29895 | 6000 | 0.5 | 94 / 1 | 2.031 | Indifference | 0.5 | 94 / 1 | 2.031 | Indifference |
| 1663 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.125 | 94 / 0.25 | 2.031 | Indifference |
| 1354 | 6000 | 0.5 | 94 / 1 | 2.015 | Indifference | 0.25 | 94 / 0.5 | 2.015 | Indifference |
| 1689 | 6000 | 1 | 94 / 1 | 1.015 | Indifference | 0.25 | 94 / 0.5 | 2.015 | Indifference |
| 96513 | 3000 | 0.25 | 6000 / 1 | 6 | Antagonism | 0.5 | 94 / 1 | 2.031 | Antagonism |
| 1700 | 3000 | 0.5 | 6000 / 0.5 | 3 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 83757 | 3000 | 0.5 | 94 / 0.5 | 1.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1370 | 6000 | 0.25 | 94 / 0.5 | 2.015 | Indifference | 0.5 | 94 / 1 | 2.015 | Indifference |
| 72127 | 6000 | 0.5 | 94 / 0.5 | 1.015 | Indifference | 0.5 | 375 / 0.5 | 0.56 | Indifference |
| 1375 | 1500 | 0.25 | 94 / 0.5 | 2.06 | Indifference | 0.5 | 94 / 1 | 2.06 | Indifference |
| 1340 | 6000 | 0.25 | 94 / 1 | 4.01 | Antagonism | 0.25 | 94 / 1 | 4.01 | Antagonism |
| 1344 | 3000 | 0.5 | 94 / 2 | 4.031 | Antagonism | 0.125 | 94 / 0.25 | 2.31 | Indifference |
| 1369 | 3000 | 0.25 | 94 / 1 | 4.031 | Antagonism | 0.125 | 94 / 0.5 | 4.031 | Antagonism |
| 1326 | 3000 | 0.25 | 94 / 1 | 4.031 | Antagonism | 0.125 | 94 / 0.5 | 4.031 | Antagonism |
| 1357 | 3000 | 0.25 | 750 / 2 | 8.25 | Antagonism | 0.25 | 94 / 1 | 4.031 | Antagonism |
| 1338 | 6000 | 0.25 | 94 / 1 | 4.015 | Antagonism | 0.125 | 175 / 1 | 8.029 | Antagonism |
| 1342 | 3000 | 0.5 | 94 / 2 | 4.031 | Antagonism | 0.5 | 94 / 1 | 2.031 | Indifference |
| 1349 | 3000 | 0.5 | 94 / 2 | 4.031 | Antagonism | 0.125 | 94 / 0.5 | 4.031 | Antagonism |
| 1347 | 3000 | 0.5 | 94 / 0.5 | 1.031 | Indifference | 0.5 | 94 / 0.5 | 1.031 | Indifference |
| 1345 | 3000 | 0.25 | 94 / 1 | 4.031 | Antagonism | 0.5 | 94 / 1 | 4.031 | Antagonism |
| 1367 | 3000 | 0.25 | 94 / 1 | 4.031 | Antagonism | 0.25 | 94 / 1 | 4.031 | Antagonism |
| 1377 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1817 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1666 | 3000 | 1 | 94 / 2 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1339 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1701 | 3000 | 0.5 | 94 / 1 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 5928 | 3000 | 0.5 | 94 / 1 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1331 | 6000 | 0.5 | 94 / 2 | 2.031 | Antagonism | 0.125 | 94 / 0.25 | 2.015 | Indifference |
| 95832 | 6000 | 0.25 | 3000 / 2 | 8.5 | Antagonism | 0.25 | 94 / 0.5 | 2.015 | Indifference |
| 1816 | 3000 | 0.5 | 94 / 0.5 | 1.031 | Indifference | 0.5 | 94 / 1 | 2.031 | Indifference |
| **A. flavus** | | | | | | | |
| 1378 | 12000 | 0.5 | 94 / 0.5 | 1.008 | Indifference | 1 | 94 / 2 | 2.078 | Indifference |
| 3730 | 6000 | 0.25 | 94 / 0.5 | 2.015 | Indifference | 0.5 | 3000 / 2 | 4.5 | Antagonism |
| 16904 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.5 | 1500 / 1 | 2.5 | Indifference |
| 1352 | 6000 | 0.5 | 94 / 1 | 2.015 | Indifference | 0.5 | 94 / 0.5 | 1.015 | Indifference |
| 1374 | 6000 | 0.5 | 6000 / 2 | 5 | Antagonism | 0.5 | 94 / 1 | 2.015 | Indifference |
| 1702 | 6000 | 0.25 | 375 / 0.5 | 2.06 | Indifference | 1 | 375 / 2 | 2.06 | Indifference |
| 1703 | 12000 | 0.125 | 94 / 0.5 | 4.008 | Antagonism | 1 | 375 / 1 | 1.31 | Indifference |
| 35874 | 3000 | 0.25 | 94 / 1 | 4.031 | Antagonism | 0.5 | 94 / 1 | 2.031 | Indifference |
| 1704 | 12000 | 0.25 | 94 / 0.5 | 2.008 | Indifference | 0.5 | 94 / 1 | 2.078 | Indifference |
| 47756 | 6000 | 0.5 | 175 / 1 | 2.02 | Indifference | 2 | 3000 / 16 | 8.5 | Antagonism |
| 1664 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.25 | 94 / 0.5 | 2.031 | Indifference |
| 1705 | 3000 | 0.25 | 94 / 0.5 | 2.031 | Indifference | 0.5 | 3000 / 4 | 9 | Antagonism |
| 1566 | 6000 | 0.25 | 94 / 1 | 4.031 | Antagonism | 0.5 | 94 / 1 | 2.031 | Indifference |
| 1706 | 3000 | 0.5 | 94 / 0.5 | 2.25 | Indifference | 0.5 | 6000 / 4 | 10 | Antagonism |

VOR: voriconazole, AmB: amphotericin B, MIC: minimum inhibitory concentration, FICI: fractional inhibitory concentration index.
esis of ergosterol, AmB inhibits fungi by binding the fungal sterol, eventually, farnesol causes resistance to AmB by reduction of its binding site\(^2\). In accordance with the mechanism of action of farnesol mentioned above, Brilhante et al.\(^20\) showed that ergosterol concentration in the fungal cell decreased in strains of *Coccidioides posadasii* exposed to external farnesol. VOR is an antifungal drug inhibiting the biosynthesis of ergosterol by blocking the action of cytochrome P450-dependent enzyme 14-alpha-demethylase. In addition, inhibition of 14-alpha-demethylase leads to accumulation of methylated sterol intermediates (zymosterol, lanosterol, and squalene), resulting in the disruption of plasma membrane\(^21\). Because farnesol inhibits the ergosterol biosynthetic pathway and might decrease the levels of these intermediates, its combination with VOR may result in antagonistic interaction. Antagonistic interaction of farnesol with antifungal drugs has been reported previously. Xia et al.\(^22\) detected synergistic interactions between farnesol and fluconazole or 5-fluorocytosine, but antagonistic interactions between farnesol and terbinafine, itraconazole, or caspofungin on the biofilms formed by the resistant strains of *C. albicans*. They reported that the interaction of farnesol with each antifungal drug was different not only on the biofilms formed by the resistant and standard strains, but also on the biofilms at the different growth phases\(^20\).

Another explanation may be that farnesol may have different effects on the different fungal species. Rossignol et al.\(^23\) showed that there are substantial differences between the responses of *C. albicans* and *C. parapsilosis* to the addition of exogenous farnesol; while farnesol inhibits growth in *C. parapsilosis* and has no effect on its morphology, growth is exogenous farnesol; while farnesol inhibits growth in *C. albicans* and the morphological transition to hyphae is reduced. Although, externally, farnesol induced an apoptosis-like cell death in *A. flavus* and *A. nidulans*, it did not induce apoptosis in *A. niger*\(^7,9\). Dichtl et al.\(^24\) observed for *A. fumigatus* that farnesol has a devastating effect on some cell wall mutants, whereas its impact on wild-type strains is limited. They suggested that farnesol is not suitable for treatment of *A. fumigatus* infections\(^27\).

Therefore, these reports point to a wide variation in farnesol activity among different fungal species and in accordance with their different growth stages. In the present study, we observed a limited antifungal activity of farnesol alone only at high concentrations, and we could not detect any synergistic interaction in combination of farnesol with voriconazole or amphotericin B against *A. fumigatus* and *A. flavus* clinical isolates. In contrast, antagonistic interactions were detected. Various mechanisms of action of farnesol have been proposed to explain its activity on fungal cells. However, it is thought that the responses of different fungal species against these effects are different, and combining farnesol with antifungals generates different interactions with different antifungals. Although farnesol appears promising as an alternative antifungal agent, our results have shown otherwise. Therefore, it should be noted that farnesol may have an adverse effect on some fungi or interact negatively with some antifungals used in combination.

**Conflicts of interest**

None.

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