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RESEARCH

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Synergistic potential of α-Phellandrene combined with conventional antifungal agents and its mechanism against antibiotic resistant *Candida albicans*

Riya Bhattacharya[1], Prashant Sharma[2], Debajyoti Bose[1*] and Manish Singh[2*]

Abstract

Candida albicans is resistant to various antifungal drugs, this presents a significant problem on a global scale. This study investigates a novel approach on the potential fungicidal effects of α-Phellandrene combinations with fluconazole and amphotericin B against antibiotic resistant *C. albicans*. The agar well diffusion experiment was used to measure the anti-candida activity of α-Phellandrene which exhibited a zone of inhibition of 24 ± 0.5 mm and 22 ± 0.5 mm against the *C. albicans* cells (MTCC277 and ATCC90028), respectively. Additionally, the fungicidal minimum inhibitory concentration (MIC) ranged 0.0312–0.0156 mg/ml (w/v) against *C. albicans* strains. It was determined to have powerful and efficient antifungal action against Candida cells. Further, the synergistic potential was evaluated by employing a time kill assay and a checkerboard technique, respectively, which revealed after 16 h, the colony count of *C. albicans* cells ATCC90028 (2.56 ± 0.33) and MTCC277 (2.53 ± 0.33) dropped by a log10 when treated with a combination of α-Phellandrene and Fluconazole and α-Phellandrene and amphotericin B exhibited synergy against both *C. albicans* strains ATCC90028 and MTCC277 (2.42 ± 0.28 and 2.00 ± 0.21) $\log_{10}$ reduction in colony count, respectively, Additionally, 16–624-fold increase in the antifungal efficacy of clinical medicines, with total cell death occurring after 16 h. α-Phellandrene and antifungal drugs were tested in combination with the osmoprotectant test, ergosterol test and FESEM observations to determine their modes of action. In the era of multidrug-resistant diseases antibiotic resistance can be curtailed in its tracks with the help of combination treatments that allow for lower drug doses.

Keywords α-Phellandrene, Phytocompound, *Candida albicans*, Synergism, Antifungal drugs

Introduction

Fungal infections are responsible for the deaths of over 1.5 million individuals annually (Gonçalves et al. 2022). However, most deaths caused by fungal diseases are preventable, this topic continues to be largely overlooked by public health officials (Rodrigues and Albuquerque 2018). The Candida genus is one of the most prominent opportunistic fungi across the globe, and it is extremely dangerous for those with immunocompromised bodies (Brandt and Lockhart 2012; Dworecka-Kaszak et al. 2020). It is attributed to a high rate of morbidity and mortality. Vaginitis, cutaneous candidiasis, oral candidiasis, candidemia and systemic infection are some of the diseases that can be caused by species of this genus (Vázquez-González et al. 2013). *Candida albicans* is responsible for around 80% of all Candida infections;

*Correspondence:
Debajyoti Bose
debajyoti1024@gmail.com
Manish Singh
manish@inst.ac.in
[1] School of Technology, Woxsen University, Hyderabad, Telangana, India
[2] Chemical Biology Unit, Institute of Nano Science and Technology (INST), Mohali, Knowledge City, Punjab, India

however, there is a gradual trend towards Candida infections that are not caused by *C. albicans*, particularly the ones caused by *C. krusei*, *C. tropicalis*, *C. glabrata* and *C. dubliniensis* (El-Ganiny et al. 2021).

There are four primary antifungal groups that are utilized in the clinical management of candidiasis. These are azoles, polyenes, fluoropyrimidines, and echinocandins (de Oliveira Santos et al. 2018). However, the shortage of available antifungal treatments is one of the main factors contributing to the mortality and morbidity associated with fungal infections across the world (Bhattacharya et al. 2021a, b; Fisher et al. 2022). In addition, the toxicity of existing antifungal drugs and the rising prevalence of Candida infections, both of which have been linked to the emergence of pathogenic organisms that are exceptionally resistant to the majority of antifungal agents, as well as the rise of drug-resistance by other strains of Candida have necessitated the continuous research and development for novel antifungal drugs that have different modes of action and amalgamate this with a low level of toxicity.

Multiple drug resistant (MDR) has been extensively covered under SDG 3, it is defined as the percentage of infections that are caused by certain organisms that are resistant to antimicrobials in the bloodstream. MDR has been referred to as a pandemic that exists in silence. According to estimates provided by the World Bank, if MDR is not addressed, the world economy may have lost ~ 4% of its yearly gross domestic product (GDP) by the year 2050. The losses are expected to be significantly greater in countries with low and intermediate incomes. This circumstance has the potential to force up to 28 million people, the most of whom are in developing countries, into poverty by the year 2050. This is primarily due to the consequences of MDR on economic output, animal production, and the expenses of health treatment (Iqbal et al. 2021). To effectively treat multiple drug resistant (MDR) infections, a new alternative treatment strategy is required (Iqbal et al. 2021).

Combination medication is a novel and expanding treatment option that combines the use of antibiotics and phytocompounds in order to combat multidrug-resistant microbes. Such combinations are effective as well as less toxic than the individual antibiotics, and therefore can be used to treat MDR diseases (Bhattacharya et al. 2022; D'agostino et al. 2019). The discovery of new pharmacological drugs, cosmetics, and pesticides relies heavily on natural compounds derived from plants. Essential oils (EOs) contain wide spectrum of ingredients, some of which have been studied as potential therapies in the treatment of various diseases such as 1,8-cineole for respiratory diseases, cancer, digestive disorders, dysphoria, and cardiovascular illness; eugenol for toxic effects and repellence in insects (Şimşek, et al. 2017). Among various phytocompounds, monoterpenes are the most representative members of the terpenes group because of their widespread distribution and their ability to account for up to 90 percent of the total oil concentration (Amri et al. 2023). Monoterpenes are classified as regular and irregular monoterpenes, as well as iridoids (Kaur et al. 2019). The most prevalent are conventional monoterpenes, and monoterpene hydrocarbons contain various vital compounds (Bhattacharya et al. 2021a, b).

α-Phellandrene is one of a pair of cyclic monoterpene and double-bond isomers which can be identified as phellandrene. It was initially isolated from *Eucalyptus phellandra*, which is now known scientifically as *E. radiata*, and received its name from this plant (Takahasi et al. 2008). It is possible to extract it from the essential oils (EOs) of the, *Eucalyptus elata* (35%), *Boswellia sacra* (42%), dill weed (30%), turmeric leaf (54%), *Aegle marmelos* (44%) and *Eucalyptus dives* (17%) but it is also found in a variety of other plant species, including, *Gossypium hisutum, Heracleum antasiaticum, Cistus ladanifer, E. camaldulensis, E.cryptomeria japonica* and *Cannabis sativa* (Radice et al. 2022; Bhattacharya et al. 2023). Since the 1940s, α-Phellandrene has been used in the wider community, and the Federal Emergency Management Agency (FEMA) has recognized it as an active ingredient that is Generally Recognized as Safe (GRAS) ever since 1965. It was recently reevaluated, and the results showed that it did not pose a threat to the general population's health. According to 21 CFR 172.515, the FDA has given permission for its use in food (Tamburlin et al. 2021). Its biological properties are of significance for agricultural, food, and feed usage, while its pharmacological attributes are of interest to the cosmetics and pharmaceutical sectors. It is additionally utilized to manufacture perfumes, detergents, soaps, moisturizers, and lotions (Kaur and Bhardwaj 2021).

This is the first study that shown that the active phytocompound α-Phellandrene can increase the efficacy of antifungal drugs in combination therapy. The bioactivity and health benefits of this compound has been explored at different capacities but not in the role of synergistic potential with conventional drugs against *Candida* species. In this work, two antifungal drugs from different classes were chosen for combinatorial testing with α-Phellandrene in order to achieve the goals of identifying novel promising combinations that are effective against multidrug-resistant *C. albicans* and confirming the applicability of terpenoid compounds in combined treatments. After conducting some preliminary tests, the most promising combination with a potential partial or

complete synergistic effect was chosen for further study using checkerboard and time-kill experiments. This selection was made after the results of the preliminary tests were analyzed.

Materials and methods

Chemicals used in the study

Phytocompound of interest α-Phellandrene was purchased from Sigma Aldrich Chemicals Pvt Ltd in Bommasandra (Bangalore, India). The growth media that were utilized for the culturing of fungal strains were acquired from Himedia Labs (Mumbai, India). Fluconazole, and amphotericin B were the conventional antifungal drugs availed from Himedia labs. Solvent DMSO were purchased from Loba Chemie Pvt. Ltd. (Mumbai, India). Stock solutions of α-Phellandrene and antifungal drugs were prepared considering the salt concentrations with DMSO up to the following concentrations (mg ml^{-1}): Fluconazole (10 mg ml^{-1}), Amphotericin B (10 mg ml^{-1}), α-Phellandrene (10 mg ml^{-1}) and stored at − 20℃. When used in a laboratory setting, a stock solution is often diluted from a known concentrated solution. Among the many benefits of using a stock solution are: One stock solution can be prepared and kept instead of multiple vials of working solutions, which reduces storage space.

Fungal strains

The two fungal strains used in the study comprise reference strains of drug resistant pathogens (*C. albicans* ATCC 90028 and *C. albicans* MTCC277). There are very few therapeutic options available to patients who have Candida infections that are resistant to both fluconazole and echinocandin drugs. The most common form of treatment is amphotericin B, which is a drug that has the potential to be toxic for patients who are already in a critical condition. A growing body of research suggests that patients who have bloodstream infections caused by Candida that are resistant to antimicrobial agents, often known as candidemia, have a worse chance of survival compared to individuals who have candidemia that can be treated with antifungal medications (El-Ganiny et al. 2021). The Yeast Biology Lab at Shoolini University in Solan, Himachal Pradesh, India provided all the strains that were used in this study. The comprehensive summary of the resistance pattern of some common *Candida* species is presented in Table 1. Each strain was kept at a temperature of 80 °C in cryovials that contained 20% glycerol (Bhattacharya et al. 2023). An overnight culture was kept on YPD-agar plates, and then a fresh fungal suspension was made from that culture before it was used. The inoculum of one colony was added in 5 ml of media, and the mixture was shaken at a rate of 200 revolutions per minute (rpm) while being incubated at 30 °C.

In vitro antifungal sensitivity tests

All solutions were prepared and diluted in accordance with the recommendations of the rules established by the Clinical and Laboratory Standards Institute or CLSI (Xu et al. 2012). Two of the most prevalent drugs used to treat Candida infections—fluconazole and amphotericin B were chosen for the experiments because they employ different modes of action. Antifungal activity of α-Phellandrene was tested with agar well diffusion and broth micro dilution techniques. In order to evaluate the efficacy of the agar well diffusion method, the results were measured in millimeters (mm) and analyzed by zone of inhibition.

The minimal inhibitory concentration (MIC) was measured using the broth dilution method (Wiegand et al. 2008). This was done by monitoring the change in color of resazurin dye as it went from purple to pink or colorless, and the findings were recorded in mg ml^{-1}. The minimum concentration at which there was a noticeable change in color was used in the calculation of the MIC value. The lowest concentration of a drug that would be able to limit the visible growth of a microbe after the microbe has been incubated is referred to as the "minimum inhibitory concentration," also known as "MIC" or

Table 1 Intrinsic susceptibility patterns of the most common wild-type Candida isolates and their origin (McTaggart et al. 2020)

Strain	Origin	Susceptibility Patterns		
		Fluconazole	Echinocandins	Amphotericin B
C. albicans	Human	Vulnerable	Vulnerable	Vulnerable
C. glabrata	Plants and soil	Intermediate	Vulnerable	Vulnerable
C. parapsilosis	Insects and animals	Vulnerable	Vulnerable	Vulnerable
C. krusei	Human	Resistant	Vulnerable	Vulnerable
C. tropicalis	Human	Vulnerable	Vulnerable	Vulnerable
C. dubliniensis	Human	Vulnerable	Vulnerable	Vulnerable

CLSI and EUCAST markers for the common Candida species have been defined, permitting for the categorization of wild-type isolates into vulnerable, intermediate and resistant groups.

"1MIC (dose)" and is described by the term minimum inhibitory concentration (Kowalska-Krochmal et al. 2021). The most typical application for MIC in diagnostic laboratories is as a research tool to investigate the activity of new antimicrobials in vitro. However, MIC is used for a variety of other purposes as well. The data collected from studies like this one is frequently utilized in the process of determining MIC breakpoints. The detection of susceptibility is the primary function of MIC. Antifungal tests utilized Amphotericin B and Fluconazole as positive controls, while the solvent DMSO 10% served as the negative control for both types of tests. Three separate sets of data were collected for each experiment.

Synergistic studies of α-Phellandrene with antifungal drugs by time kill assay

The intricate interactions that form among several distinct fungal strains and the various antimicrobial agents can be better understood with the help of assays that are based on time-kill kinetics (Letrado et al. 2018). The results of the test suggest that the action of antimicrobial medications on different fungal strains may vary with either the dosage of the drug or the duration of time it takes to work. In the time-kill tests, MIC values obtained from broth microdilution were employed. Each strain was tested against fluconazole (1MIC), α-Phellandrene (1MIC) and amphotericin B (1MIC), both by individually and in combination with α-Phellandrene, respectively. *C. albicans* cells (ATCC90028 and MTCC277) were transferred from a 48-h YPD agar plate to two tubes containing sterile broth, and the turbidity of the broth was adjusted so that it had a starting inoculum of 0.5 optical density (OD) at 600 nm. After adding, α-Phellandrene, antifungal drugs and various combinations of the two, each of the different strains was then inoculated into ten flasks that contained 5 ml of SDB broth.

A growth control consisted of two drug-free flasks that were added to each strain. A control group containing only growth medium was also utilized as a negative control. Before transferring a sample for colony counts, each of the tubes containing α-Phellandrene and antifungal drugs individually or in combinations were vortexed and then placed on an orbital shaker for 30 min at a temperature of 30 °C. At predetermined intervals of time, serial aseptic extractions of samples were carried out. Serial dilution was performed in saline according to the guidelines, and the samples were plated using glass beads on Sabouraud agar plates in duplicates. After 48 h of incubation at 30 °C, the plates were scanned and counted using a colony counter made by Spiral Biotech called the QCount. The colony count (in CFU/ml) from duplicate samples was calculated as an average, and the mean was used to calculate synergy.

When compared to the most active compound, synergy effects were defined as a decrease in CFU per ml that was greater than a 100-fold reduction (more than 2 log10 steps). An increase of a $\geq$2 log10 CFU per ml of surviving cells is referred to as antagonism and indifference was a change in colony count that was no greater than 2 log10 in either side (Lady et al. 2023).

Synergistic studies and fractional inhibitory concentration (FIC) determination of α-Phellandrene with antifungal drugs by checkerboard method

The checkerboard microdilution method was used, with few modifications made, in accordance with the Clinical Microbiology Procedures Handbook (Garcia 2010). This allowed for a more in-depth investigation into the potential existences of synergism and antagonism among the most active combinations that were reported from the preliminary testing. Microtiter plates were set up so that the columns along the x-axis had 100 µl of α-Phellandrene that had been diluted two-fold in media, and the rows along the y-axis contained the same quantity of antifungal drugs that had also been diluted two-fold in the same media. After that, 100 µl of media containing 2×10^6 CFU/ml of the fungal strain were placed into each of the wells. Following that, the plates were incubated at a temperature of 30 °C for a period of 48 h. Experiments were performed in triplicates to ensure accurate results. For each combination, the lowest concentration that inhibits noticeable growth was identified.

The FIC index is used to calculate synergistic activity (between the drug and the phytocompound) based on changes in the MIC values of both the α-Phellandrene and the antifungal drugs alone or in combination. FICI (or FIC index) values less than 0.5 to be labeled as synergism, values up to 2.0 to be labeled as indifferent or additive, while values greater than 2.0 are to be labeled as antagonism (Bhattacharya et al. 2021a; Chadha et al. 2023). Fold reduction was then evaluated which indicates how the MIC of antifungal drugs changes from an initial measurement and following one, demonstrating better efficacy although a lower concentration of drug being used.

Mode of action of α-Phellandrene alone and in combination with antifungal drugs

Activity shown by time-kill test and checkerboard method were compared with α-Phellandrene and antifungal drugs and were used in combination during the broth phase compared to when each component was used alone. As mentioned later in "Results and discussion", this would indicate that α-Phellandrene is an aromatic active phytocompound, and that direct contact with it may result in more rapid cellular damage. The

effect of α-Phellandrene and antifungal drugs alone, as well as the combination of the two, on Candida cells was analyzed. Tests were conducted with sorbitol, ergosterol, and a field emission scanning electron microscopy (FESEM).

Assay using an osmoprotectant

The effect of α-Phellandrene and its combination with antifungal drugs on cell wall was assessed in triplicate by microdilution using 96-well microtiter plates with osmoprotectant (sorbitol at 0.8 M final concentration) and without osmoprotectant according to one study (Costa et al. 2021). The determination of the MICs of the samples was carried out in the same manner as detailed above against *C. albicans* ATCC90028 and MTCC277. The plates were kept in an incubator at 30 °C for a week. The variation in the minimal inhibitory concentration (MIC) value of samples grown in medium with osmoprotectant in comparison to samples grown in media without osmoprotectant revealed indication that the cell wall

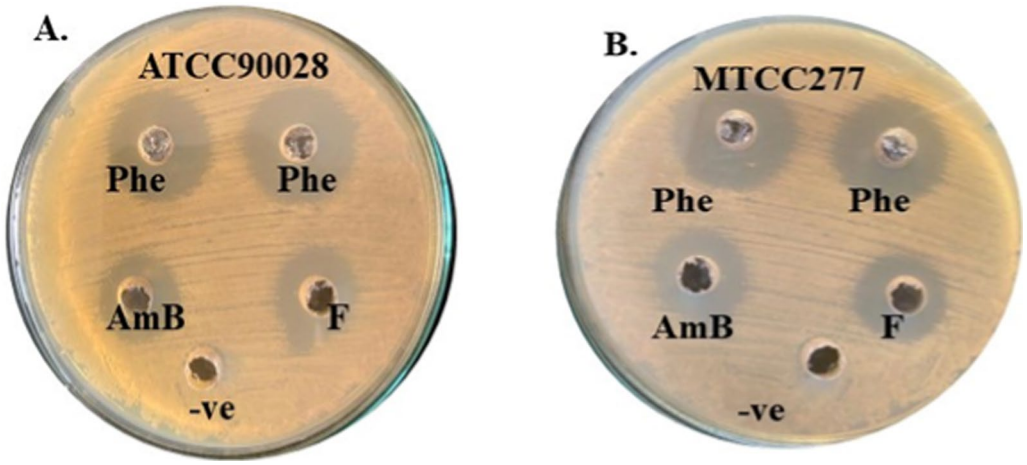

Fig. 1 α-Phellandrene showed antifungal efficacy in agar well diffusion assay against (**A**) *C. albicans* (ATCC90028) and (**B**) *C. albicans* (MTCC277). Each well was loaded with a volume of 50 µl of solution that was 1 mg ml^{-1} (v/v). Annotations: Phe-α-Phellandrene; F: fluconazole; AmB: Amphotericin B; -ve: DMSO/Solvent

Table 2 Antifungal activities of α-Phellandrene against *C. albicans* (ATCC90028 and MTCC277)

Phytocompounds (mg/ml)	Zone of inhibition (mm)	
	***C. albicans* strain (ATCC90028)**	***C. albicans* strain (MTCC277)**
α-Phellandrene	22 ± 0.5 (fungicidal)	24 ± 0.5 (fungicidal)
Fluconazole	19 ± 0.3 (fungistatic)	18 ±0.7(fungistatic)
Amphotericin B	17 ± 0.3 (fungicidal)	18 ±0.5(fungicidal)

The zone of clearance of Candida strains (mm) developed by the indicated compounds are listed

Table 3 Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of α-Phellandrene against *C. albicans* strains ATCC90028 and MTCC277

Samples (mg/ml)	ATCC90028			MTCC277		
	MIC (mg/ml)	MFC (mg/ml)	Nature of inhibition	MIC (mg/ml)	MFC (mg/ml)	Nature of inhibition
α-Phellandrene 1 mg/ml (w/v)	0.0312–0.0156	0.0156	Fungicidal	0.0312–0.0156	0.0156	Fungicidal
Fluconazole 1 mg/ml (w/v)	0.0625–0.0312	0.0625	Fungistatic	0.0625–0.0312	0.125	Fungicidal
Amphotericin B 1 mg/ml (w/v)	0.125–0.0625	0.125	Fungicidal	0.125–0.0625	0.125	Fungicidal

is a potential target for the compound that was evaluated. The drug fluconazole was used in the control group.

Ergosterol binding assay

MIC values of tested samples were determined in 96-well plates in SDB media with and without exogenous ergosterol at a final concentration of 200 µg/ml (de Castro et al. 2015). This was done in the same manner as was stated before. In order to get the experiment started, a total of 100 µl of SDB media were poured into each well of the microtiter plate. After that, 100 µl of the transferring an aliquot of 100 µl from the well that had the highest concentration to the well that contained the concentration that was the next lowest. Following that, 10 µl of fungal cells that had an absorbance < 1 at 600 nm was added to each of the wells.

A positive control was carried out using amphotericin B. The *C. albicans* ATCC90028 and MTCC277 strain was subjected to an incubation period of 48 h at a temperature of 30° C. The ability of the product to bind with ergosterol was represented in the fact that the MIC value decreased when the product being tested was placed in a medium that contained ergosterol.

Field emission scanning electron microscopy analysis

Visualizing minute topography characteristics on the surface of fractionated *C. albicans* cells can be accomplished with the use of Field Emission Scanning Electron Microscopy or FESEM (Zheng et al. 2017). When *C. albicans* cells are subjected to a combination of α-Phellandrene and conventional drugs, this method is applied to investigate structures that are representative of the level of damage caused to the cellular material of the *C. albicans* cells. After being treated with α-Phellandrene alone or in combination with antifungal drugs (MIC values), *C. albicans* cells in liquid phase were fixed with 2.5% glutaraldehyde in phosphate buffer for 30 min and then washed 2–3 times with 0.1 M phosphate buffer solution (pH 7.2). subsequently, ethanol concentrations of 25%, 50%, 75%, 90%, and 100% were applied to each suspension in order to dry it. The process of freeze-drying the cells was completed with the help of a lyophilizer (Gaidhani et al. 2015). On an aluminum rod, the cells were arranged in a thin layer in preparation for the scanning electron microscope. Prior to being examined under a scanning electron microscope, the samples were cathode painted with a gold (Polaron gold) coating. When it comes to electron sources, the FESEM makes use of a field emission gun. With a spatial resolution that is as low as 1.5 nm (Erlandsen et al. 2000), it generates images that have high clarity and are less affected by electrostatic forces as compared to SEM.

Statistical analysis

The results of the statistical analysis offer quantifiable support for the hypothesis that the trials were designed to test. All the trials were carried out three times. Both the SPSS (Statistical Package for Social Sciences, Version

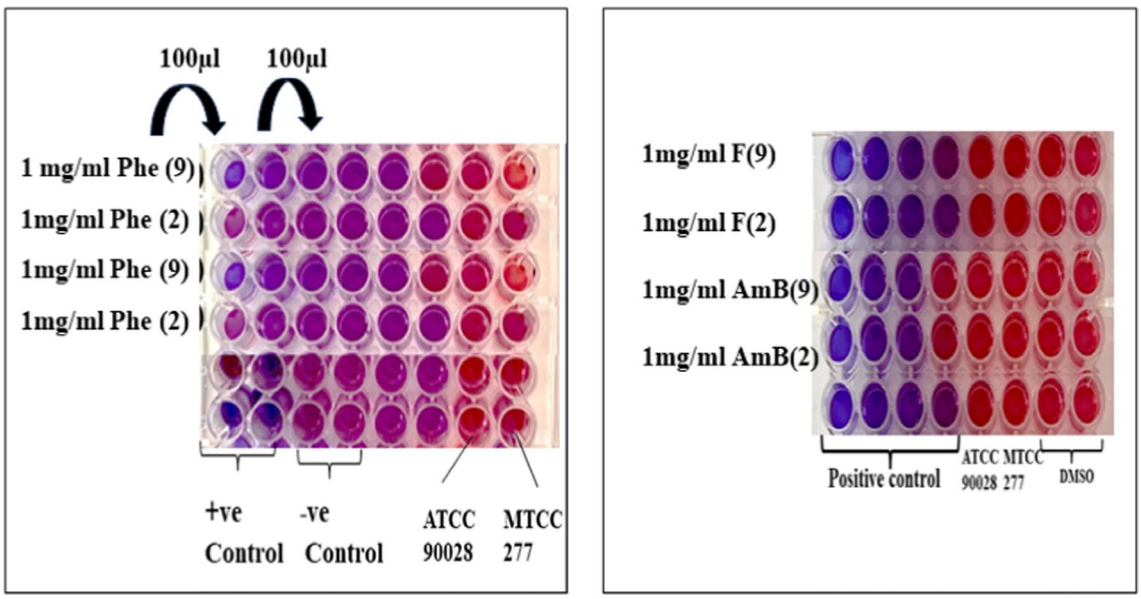

Fig. 2 MIC plate showing minimum inhibitory concentration of α-Phellandrene against *C. albicans* strain ATCC 90028 and MTCC277 by broth dilution method. Labels: Phe: α-Phellandrene, F: Fluconazole, AmB: Amphotericin B, –ve: DMSO, + ve: Antifungal drugs, ATCC90028 and MTCC277- *C. albicans* cells alone

Table 4 Test results for synergism of α-Phellandrene combined with antifungal drugs against *C. albicans* (ATCC90028 and MTCC277) by time-kill assay

Test samples		Time-kill assay log_{10} CFU/ml difference after 16 h (test results for synergism after 16 h) ATCC90028	MTCC277
α-Phellandrene + fluconazole		2.56 ± 0.33, SYN	2.53 ± 0.33, SYN
α-Phellandrene + amphotericin B		2.42 ± 0.28, SYN	2.00 ± 0.21, SYN
Controls	Fluconazole	4.92 ± 0.09	4.48 ± 0.49
	Amphotericin B	4.93 ± 0.08	4.53 ± 0.42

11.0) and GraphPad Prism (Version 9, Edition: 3.1) programs were utilized in order to conduct the analysis on the collected data. The average and standard error of the data were computed with the help of the software packages Prism and SPSS.

Results and discussion

In vitro qualitative antifungal activity of α-Phellandrene and antifungal drugs

Agar well diffusion method was used in order to determine the qualitative antifungal activity of α-Phellandrene. In the current research, it was found that α-Phellandrene demonstrated the strongest antifungal efficacy, with a zone of inhibition that measured 24 ± 0.5 mm and 22 ± 0.5 mm against both MTCC277 and ATCC90028 strains of *C. albicans*, respectively as shown in Fig. 1. On the other hand, fluconazole demonstrated zones of inhibition against *C. albicans* cells ATCC90028 and MTCC277 that measured 19 ± 0.3 mm and 18 ±0.7 mm, respectively. Furthermore, the zone of inhibition of amphotericin B against the *C. albicans* strains ATCC90028 and MTCC277 measured 17 ± 0.3 mm and 18 ±0.5 mm, respectively as shown in Table 2.

In addition to this, the antifungal action of α-Phellandrene was of the fungicidal nature. Research into α-Phellandrene is promising because it effectively eradicated *C. albicans* and blocked its development more effectively than or on par with conventional anti-fungal medications. α-Phellandrene has been shown to be effective in a wide number of therapeutic uses (Takahashi al. 2008); however, there have only been a few studies done to investigate the possibility that it may possess an inhibitory effect against candida.

In vitro quantitative antifungal activity of α-Phellandrene and antifungal drugs

Quantitative analysis of antifungal activity of α-Phellandrene was determined by broth dilution method. In this study, α-Phellandrene had a MIC of 0.0312 mg/ml against both the ATCC90028 and MTCC277 strains of *C. albicans*. Both the *C. albicans* strains were more susceptible to α-Phellandrene than to fluconazole or amphotericin B. This is also demonstrated in Table 3. This shows that α-Phellandrene is more effective at killing fungi than standard medication with antifungal drugs. In the interest of both when tested against two different *C. albicans* strains (ATCC 90028 and MTCC 277), the MIC for α-Phellandrene was equivalent to the MFC (0.0312–0.0156 mg/ml) and Amphotericin B (0.125–0.0625 mg/ml). However, against *C. albicans* MTCC 277, the MFC for fluconazole was greater than the MIC (0.125–0.0625 mg/ml) as shown in Fig. 2. α-Phellandrene against both the *C. albicans* strains was between 2 and 50 folds lower than the MIC of fluconazole and amphotericin B. Based on these findings, it can be determined that α-Phellandrene have an antifungal potency that is better to that of conventional antifungal drugs.

In the reviewed literature, the MIC of a few phytocompounds has been described, but this was done against a variety of *C. albicans* standard strains and clinical isolates. In one study α-Phellandrene and Nonanal inhibited *P. cyclopium* mycelial development in a dose-dependent manner ($P < 0.05$). High doses of α-phellandrene (≥ 1.35 ml/l) or nonanal (≥ 0.35 ml/l) entirely prevented the growth of the mycelium (Zhang et al. 2017). In another study, microdilution method was used to evaluate others active phytocompounds such as cinnamaldehyde, α-pinene, and eugenol for their potential to inhibit the growth of fungi (Tariq et al. 2019). The negative control, which consisted of 1% DMSO, did not hinder the growth of the cells. The MICs and MFCs for cinnamonaldehyde were found to be the lowest. Cinnamaldehyde, α-pinene, and eugenol MICs for *C. albicans* strain were 40.95 mg/l, 268.13 mg/l, and 331.25 mg/l, respectively. Due to the fact that the MFC/MIC ratio was lower than 4, it is possible to classify these compounds as fungicidal (Saracino et al. 2022). There are few literatures available regarding the possible antifungal activity of α-Phellandrene against *C. albicans*, and this work is an extension of the existing supporting the potency of α-Phellandrene.

Table 5 Synergistic potential of α-Phellandrene with antifungal drugs {Fluconazole (FLU) and Amphotericin B (AMP)} against *C. albicans* (ATCC90028)

PHE	AB	MIC (Alone)			Concentration of drug/EO in synergistic combination (%)		FIC in combinations		FICI			
		PHE (w/v)	FLU (w/v)	AMB (w/v)	FLU	AMB	FLU	AMB	FLU	Fold	AMB	Fold
½ MIC	½ MIC	0.0312	0.0312	0.0625	phe:0.002 Ab:0.002	phe:0.002 Ab:0.003	phe:0.06 Ab:0.06	phe:0.06 Ab:0.04	0.12	16	0.10	21
½ MIC	1MIC	0.0312	0.0625	0.125	phe:0.003 Ab:0.007	phe:0.003 Ab:0.002	phe:0.01 Ab:0.05	phe:0.01 Ab:0.01	0.06	16	0.02	62.5
½ MIC	2MIC	0.0312	0.125	0.25	phe:0.003 Ab:0.015	phe:0.003 Ab:0.0312	phe:0.09 Ab:0.12	phe:0.09 Ab:0.12	0.21	8.33	0.21	8.01
1MIC	½ MIC	0.0625	0.0312	0.0625	phe:0.003 Ab:0.002	phe:0.003 Ab:0.003	phe:0.04 Ab:0.06	phe:0.04 Ab:0.04	0.10	15.6	0.08	20.8
1MIC	1MIC	0.0625	0.0625	0.125	phe:0.003 Ab:0.003	phe:0.003 Ab:0.007	phe:0.04 Ab:0.04	phe:0.04 Ab:0.05	0.08	20.8	0.09	17.85
1MIC	2MIC	0.0625	0.125	0.25	phe:0.003 phe:0.007	phe:0.002 phe:0.007	phe:0.04 Ab:0.06	phe:0.03 Ab:0.02	0.10	17.85	0.05	35.71
2MIC	½ MIC	0.125	0.0312	0.0625	phe:0.007 Ab:0.007	phe:0.007 Ab:0.003	phe:0.05 Ab:0.2	phe:0.05 Ab:0.04	0.25	4.45	0.09	20.8
2MIC	1MIC	0.125	0.0625	0.125	phe:0.007 Ab:0.003	phe:0.007 Ab:0.007	phe:0.05 Ab:0.04	phe:0.05 Ab:0.05	0.09	20.8	0.10	17.85
2MIC	2MIC	0.125	0.125	0.25	phe:0.007 Ab:0.007	phe:0.007 Ab:0.003	phe:0.05 Ab:0.05	phe:0.05 Ab:0.01	0.10	17.85	0.06	83.33

Synergistic studies of α-Phellandrene with antifungal drugs by time kill curve assay

Combination therapy is becoming more prevalent as a treatment alternative for drug-resistant fungal infections. When combined with herbal products, it allows for the administration of lower doses of medications, which may result in less undesirable side effects (Cheesman et al. 2017). Combination therapy is additionally growing more

Table 6 Synergistic potential of α-Phellandrene with antifungal drugs {Fluconazole (FLU) and Amphotericin B (AMP)} against *C. albicans* (MTCC277)

PHE	AB	MIC (Alone)			Concentration of drug/EO in synergistic combination (%)		FIC in combinations		FICI			
		PHE (w/v)	FLU (w/v)	AMB (w/v)	FLU	AMB	FLU	AMB	FLU	Fold	AMB	Fold
½ MIC	½ MIC	0.0078	0.0312	0.0625	phe:0.0001 Ab:0.0005	phe:0.00006 Ab:0.0005	phe:0.01 Ab:0.01	phe:0.008 Ab:0.008	0.02	624	0.016	125
½ MIC	1MIC	0.0078	0.0625	0.125	phe:0.00006 Ab:0.0005	phe:0.00006 Ab:0.0009	phe:0.008 Ab:0.008	phe:0.007 Ab:0.007	0.01	125	0.01	139
½ MIC	2MIC	0.0078	0.125	0.25	phe:0.0001 Ab:0.002	phe:0.00006 Ab:0.002	phe:0.01 Ab:0.01	phe:0.007 Ab:0.008	0.01	62.5	0.02	125
1MIC	½ MIC	0.0156	0.0312	0.0625	phe:0.0001 Ab:0.0002	phe:0.0002 Ab:0.0009	phe:0.006 Ab:0.006	phe:0.012 Ab:0.014	0.01	156	0.02	69.44
1MIC	1MIC	0.0156	0.0625	0.125	phe:0.0001 Ab:0.0005	phe:0.0001 Ab:0.0009	phe:0.006 Ab:0.008	phe:0.006 Ab:0.007	0.01	125	0.01	139
1MIC	2MIC	0.0156	0.125	2.5	phe:0.0001 Ab:0.0009	phe:0.0001 Ab:0.002	phe:0.006 Ab:0.007	phe:0.006 Ab:0.008	0.01	139	0.01	125
2MIC	½ MIC	0.0312	0.0312	0.625	phe:0.006 Ab:0.006	phe:0.006 Ab:0.006	phe:0.006 Ab:0.008	phe:0.06 Ab:0.06	0.01	156	0.01	125
2MIC	1MIC	0.0312	0.0625	1.25	phe:0.0002 Ab:0.0005	phe:0.0002 Ab:0.0009	phe:0.006 Ab:0.008	phe:0.006 Ab:0.007	0.01	125	0.01	139
2MIC	2MIC	0.0312	0.125	2.5	phe:0.0002 Ab:0.0009	phe:0.0002 Ab:0.002	phe:0.006 Ab:0.007	phe:0.006 Ab:0.008	0.01	139	0.01	125

*Highlighted boxes represent the combinations of α-Phellandrene and antifungal drugs with showed the best results in terms of synergy.

popular as a bacterial disease therapy option. In addition, the treatment efficacy of a synergistic combination of herbal compound and a conventional medicine is greater than the treatment efficacy obtained by adding up the efficacies of the two constituents separately (Saggar et al. 2022). Table 4 displays the findings that were obtained using the time kill synergy approach.

As can be shown in Tables 5 and 6, the effectiveness of fluconazole and amphotericin B is enhanced by 624 and 139 folds, respectively, when combined withMTCC277.

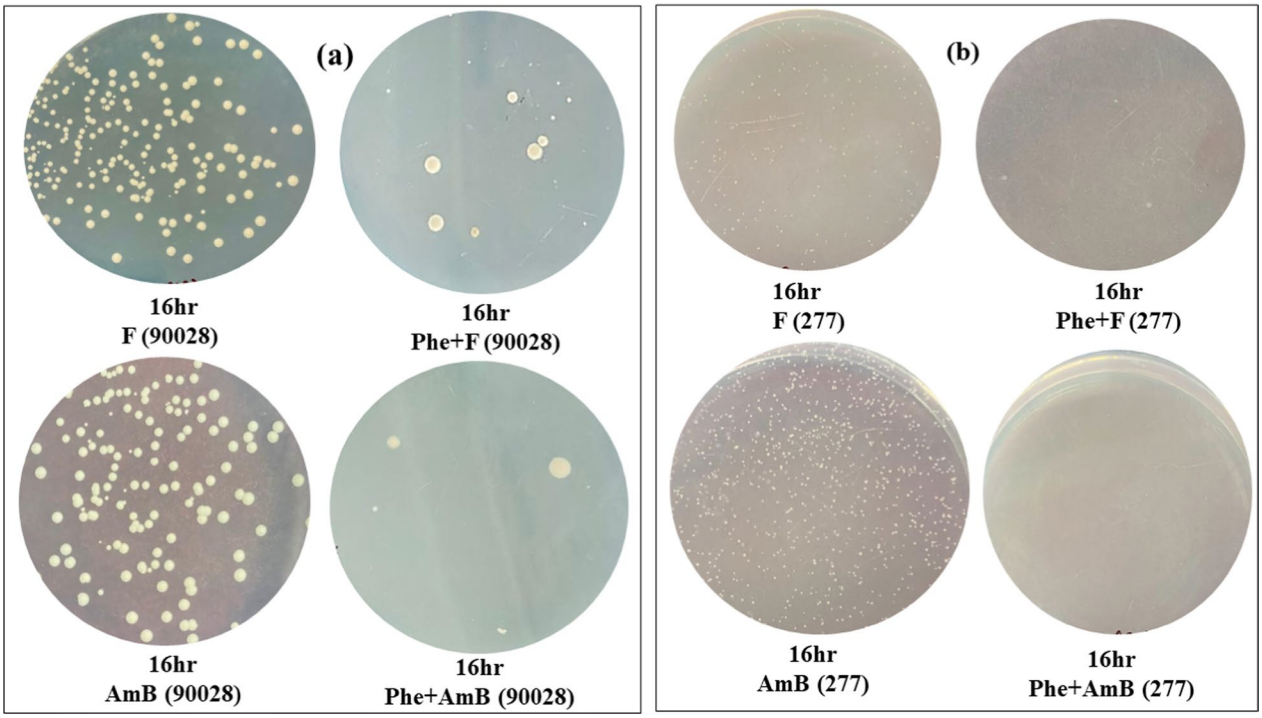

Fig. 3 **a** CFU/ml of antifungal drugs alone and its combination with α-Phellandrene against *C. albicans* strain ATCC90028 after 16 h. Labels: AmB- Amphotericin B, Phe- α-Phellandrene, F- Fluconazole, 90028- ATCC90028. **b** CFU/ml of antifungal drugs alone and its combination with α-Phellandrene against *C. albicans* strain MTCC277 after 16 h. Labels: AmB- Amphotericin B, Phe- α-Phellandrene, F- Fluconazole, 277- MTCC277

This is a novel study of bio-enhancer potential of α-Phellandrene when combined with antifungal drugs against C. albicans. Other phytocompoundscombined with antifungal drugs have shown synergistic potential against C. albicans. In one study, the checkerboard method was used to determine the combinedeffect of the two drugs (nystatin and thymol), which allowed for the FIC index to be calculated. When thymol and nystatin were used together, there was a discernibledrop in the values of the minimum inhibitory concentration (MIC). The MIC reduction for all three Candida strains that were examined was 87.4% when using either ofthese medicines. The FIC index value was 0.25, which indicates that this connection had a synergistic effect (FIC < 0.5) in relation to the growth inhibition of the testedstrains (De Castro et al. 2015).

The time kill assay method showed synergy for the combination of α-Phellandrene (1MIC) with Fluconazole (1MIC) against both the *C. albicans* strains ATCC90028 (2.56 ± 0.33) and MTCC277 (2.53 ± 0.33) − $\log_{10}$ decrease in colony count after 16 h. In comparison, the α-Phellandrene and amphotericin B exhibited synergy against both *C. albicans* strains (2.42 ± 0.28 and 2.00 ± 0.21)-$\log_{10}$ reduction in colony count respectively, after 16 h with the combination versus the most active single drug alone. α-Phellandrene combined with antibiotics displayed synergy against both strains of *C. albicans* (a 2-log10 drop in colony count after 16 h with the combination as compared to the most active single medication alone).

After 16 h, the colony count of *C. albicans* cellsATCC90028 (2.56 ± 0.33) and MTCC277 (2.53 ± 0.33) dropped by a $\log_{10}$ when treated with a combination of α-Phellandrene (1MIC) and Fluconazole (1MIC), as determined by the time kill assay method. As can be seen in Fig. 3a, b, after 16 h of treatment with the combination, *C. albicans* colony counts were reduced by 2.42 ± 0.28 and 2.00 ± 0.21 log10, respectively, compared to the most active single medication alone.

When α-phellandrene was combined with antibiotics, the results were synergistic (a 2-log10 reduction in colony count after 16 h with the combination, compared to the most active medicine alone) against both strains of *C. albicans*. α-Phellandrene and the drugs, on their own were shown to have no effect. There was little evidence to suggest that there was any antagonism. When used in combination, however, α-Phellandrene and fluconazole had a fungicidal effect against both strains of candida, resulting in a reduction of 99% in the log10 of the number of CFU/ml compared to the initial inoculum. This is a measure of fungicidal activity. After 12–16 h, the combination of α-Phellandrene with amphotericin

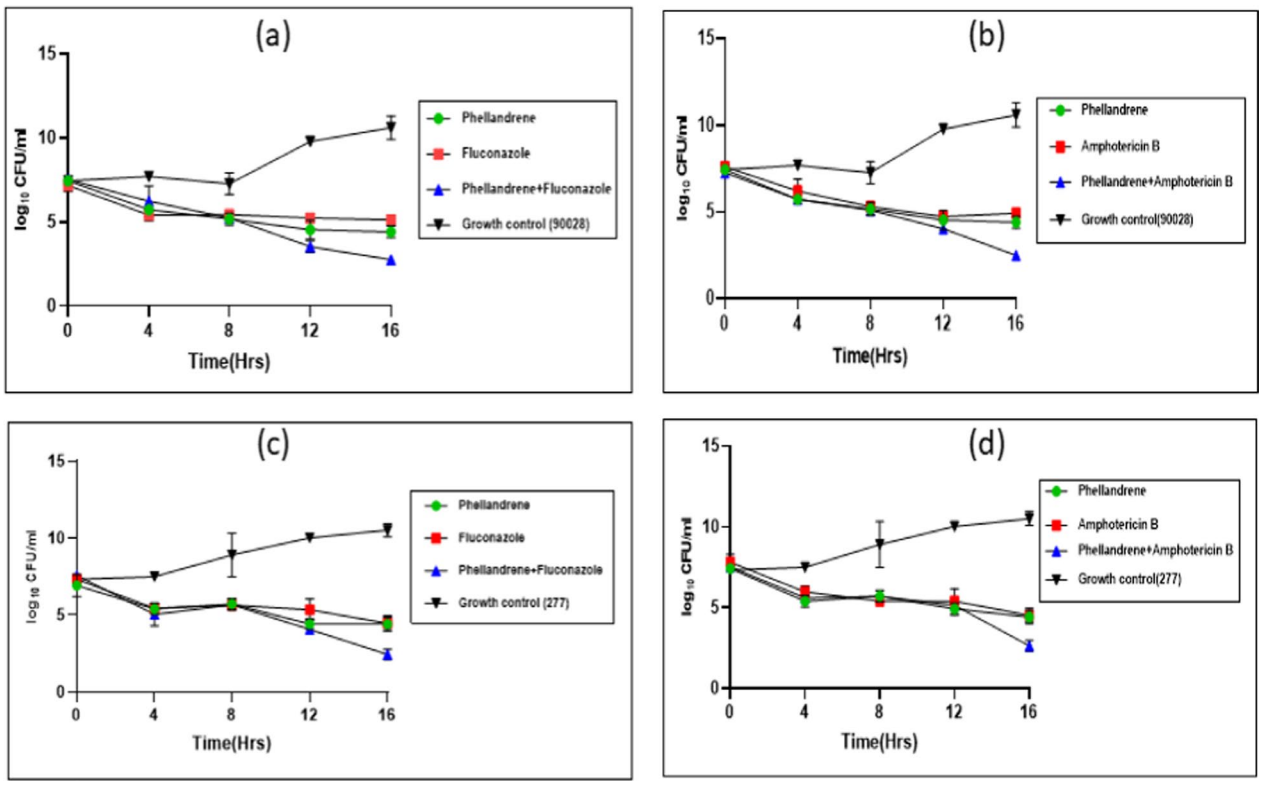

Fig. 4 Time-kill graphs to show synergistic potential in which flasks containing SDB broth were added with α-Phellandrene, antifungal drugs and their combinations and then inoculated with the respective fungal cells. **a** Plots of mean values for $\log_{10}$ of the numbers of CFU/ml versus time tested against *C. albicans* ATCC90028 at the concentrations: 1MIC α-Phellandrene, 1 MIC Fluconazole and 1MIC α-Phellandrene + 1 MIC fluconazole. α-phellandrene was combined with fluconazole, the results were synergistic (a 2-log10 reduction in colony count after 16 h with the combination, compared to the most active medicine alone. **b** The same limiting fungal growth pattern observed in plots of mean values for $\log_{10}$ of the numbers of CFU/ml versus time tested against *C. albicans* ATCC90028 at the concentrations: 1MIC α-Phellandrene, 1 MIC Amphotericin B and 1MIC α-Phellandrene + 1 MIC Amphotericin B. **c** Plots of mean values for $\log_{10}$ of the numbers of CFU/ml versus time tested against *C. albicans* MTCC277 at the concentrations: 1MIC α-Phellandrene, 1 MIC Fluconazole and 1MIC α-Phellandrene + 1 MIC fluconazole. α-Phellandrene and fluconazole, alone were shown to have no effect. α-phellandrene was combined with fluconazole, the results were synergistic. **d** Plots of mean values for $\log_{10}$ of the numbers of CFU/ml versus time tested against *C. albicans* MTCC277 at the concentrations: 1MIC α-Phellandrene, 1 MIC Amphotericin B and 1MIC α-Phellandrene + 1 MIC Amphotericin B shows similar limiting fungal growth pattern in respect of combination

B showed an inhibitory action against ATCC90028 and MTCC277. Figure 4 depicts time-kill studies that were conducted with both the strains in order to demonstrate this synergistic effect with antifungal drugs and their combinations.

Growth profile curves further verified the fungicidal action displayed by α-Phellandrene both on their own and in combination with antibiotics against both strains of *C. albicans*, and these curves may be found in Fig. 5. *C. albicans* ATCC90028 and MTCC277 were actively developing from 4 to 20 h, as seen by the plotted growth curve, which indicated an increase in OD value for the control groups. *C. albicans* that was treated with fluconazole at a concentration of 1MIC exhibited rapid growth from 0 to 4 h, then reduced at 16 h, and then continued to grow until 20 h. The cells were subjected to a modest antifungal impact from the amphotericin B, which resulted in a growth delay of between 16 and 20 h. After 12 h, the α-Phellandrene demonstrated fungicidal action at 1 MIC, as evidenced by a considerable decrease in OD values, in comparison to both the control and beginning inoculums.

After a period of 12 h, there was no evidence of cell development, which may be a consequence of the α-Phellandrene ability to cause cell death. After 4 h, the OD value of α-Phellandrene mixed with fluconazole and amphotericin B began a rapid decline, which was followed by the onset of the death phase after 16 h. After 20 h, the growth curve showed no signs of improvement. After a period of 16 h, the combination had fully destroyed all the cells. When coupled with antibiotics, α-Phellandrene tested showed antifungal action against

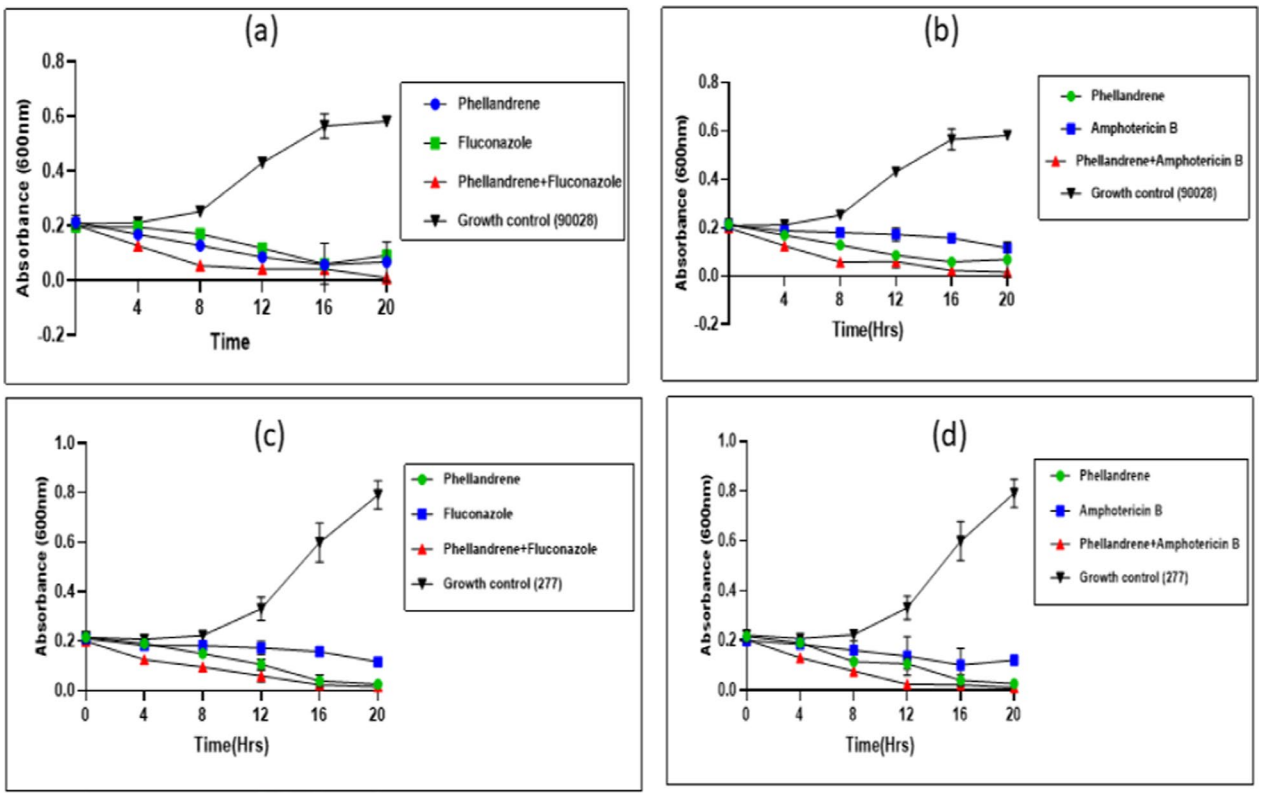

Fig. 5 Curves of growth profile to demonstrate synergy. **a**, **c** Representative growth curve plots for *C. albicans* ATCC90028 and MTCC277 at the concentrations: Growth Control, 1MIC α-Phellandrene, 1 MIC Fluconazole and 1MIC α-Phellandrene + 1 MIC fluconazole. **b, d** Representative growth curve plots for *C. albicans* ATCC90028 and MTCC277 at the concentrations: Growth Control, 1MIC α-Phellandrene, 1 MIC Amphotericin B and 1MIC α-Phellandrene + 1 MIC Amphotericin B

C. albicans ATCC90028 and MTCC277, as shown by the results of a time kill.

Synergistic studies of α-Phellandrene with antifungal drugs by checkerboard method

There is evidence in the scientific literature that supports the use of fluconazole and amphotericin B for the treatment of fungal infections (Wolff et al. 2000, Saad et al. 2010, Soulaimani et al. 2021). Additionally, it has been stated that fluconazole and amphotericin B have advantages over other antifungals, including the ability to be applied topically and fewer negative effects (Mahdy et al. 2010). However, fungal resistance has been reported, and the combination of natural and synthetic antifungal agents is an alternative to decreasing the amount necessary for the impact. It also has the potential to reduce undesirable side effects and avoid the emergence of resistance to antifungals.

One of the most common and well-known methods used in laboratories to examine the antimicrobial properties of synthetic or natural compounds is the checkerboard test (Sardana et al. 2021). To determine if a combination displays synergism, additivity, indifference, or antagonism, the FIC must be calculated, and this test offers a two-dimensional layout of different concentrations of compounds to facilitate this calculation.

As a result of this, the checkerboard approach was utilized to study the synergistic potential of α-Phellandrene that possessed anti-fungal potential when combined with fluconazole and amphotericin B. The information that was provided in "Synergistic studies and Fractional Inhibitory Concentration (FIC) determination of α-Phellandrene with antifungal drugs by Checkerboard method" served as the framework for the results that were drawn from this investigation. It was determined that there was a possibility of synergy by analysing FICI values that were either lower than 0.5 or equal to 0.5. The FICI values of the different combinations ranged somewhere between 0.06 and 0.24 on the scale. This suggests that α-Phellandrene can display synergistic potential when taken with fluconazole and amphotericin B against *C. albicans* strains MTCC277 and ATCC90028 as shown in Fig. 6.

Both antifungal medications are currently used in clinical practice. The fact that minimum inhibitory concentrations (MIC) of both antifungal drugs dropped

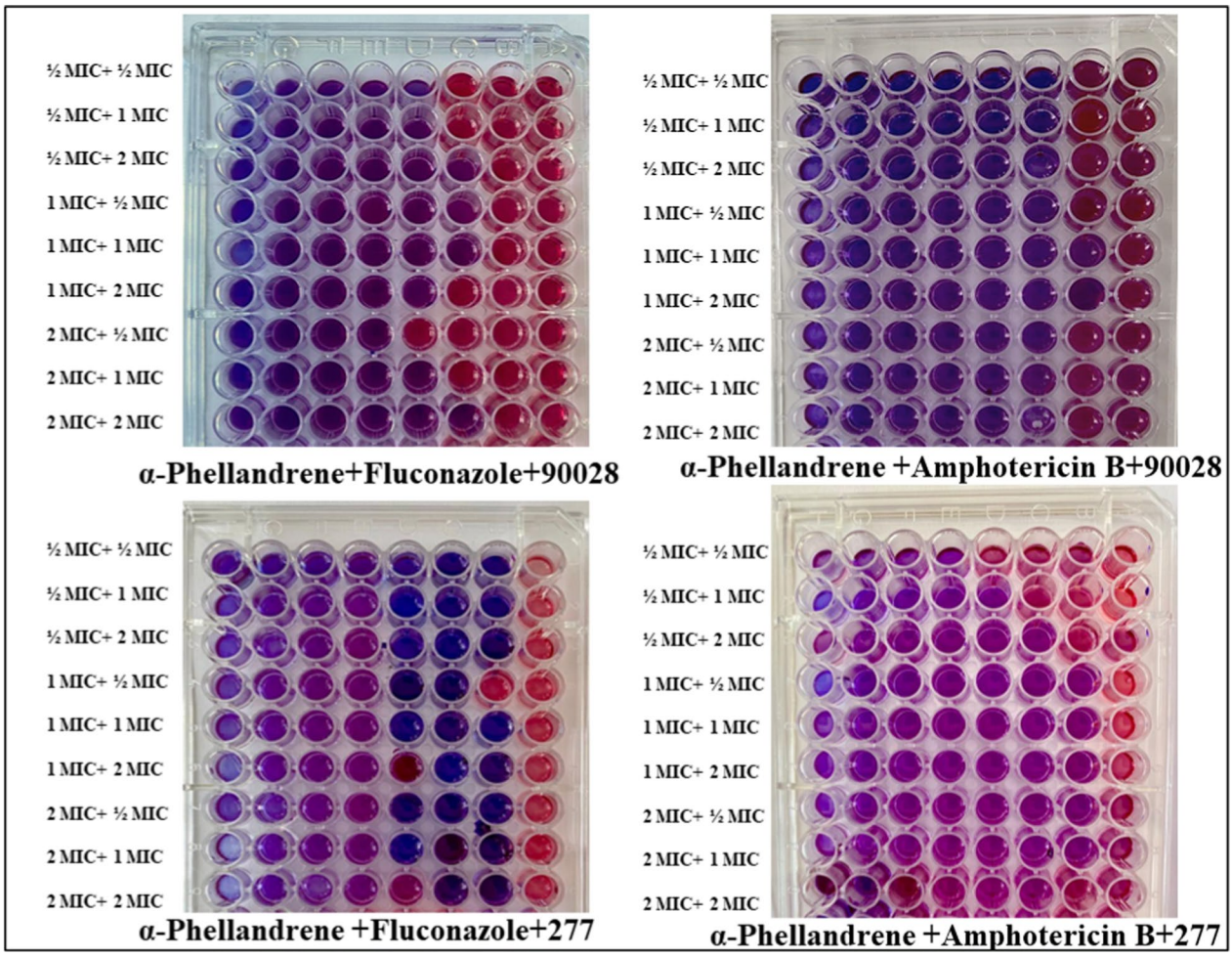

Fig. 6 A schematic representation of the 96-well resazurin broth microdilution model for α-Phellandrene in combination with fluconazole and amphotericin B against *C. albicans* strains (ATCC90028 and MTCC277). Labels: Violet/blue colour indicates growth inhibition; pink/orange indicates that organisms are live in the respective combinations of α-Phellandrene with antifungal drugs

by a factor of when they were tested in combination with one another offered further proof of the synergistic effect that α-Phellandrene possessed. It was discovered that the combination of 1MIC of α-Phellandrene (0.0625 mg/ml) and 1MIC of fluconazole (0.0625 mg/ml); 2MIC of α-Phellandrene (0.125 mg/ml) and 2MIC of amphotericin B (0.25%) was the most effective combination against *C. albicans* ATCC90028. This combination also boosts the potency of fluconazole and amphotericin B by 18 and 83 folds, respectively. Combinations of ½ MIC of α-Phellandrene (0.0078 mg/ml) and ½ MIC of fluconazole (0.0312 mg/ml); ½ MIC of α-Phellandrene (0.0078 mg/ml) and 1MIC of amphotericin B (0.125 mg/ml) were the most effective treatments for *C. albicans* MTCC277 when used in combination.

Synergistic combinations of α-Phellandrene and antibiotics: elucidating their underlying mechanisms

It turns out that when α-Phellandrene and antifungal drugs were combined, the results exhibited by time-kill assay and checkerboard method were much better than when each component was used separately during the broth phase. This was the case irrespective of whether method was used. Since α-phellandrene is an aromatic compound, and considering the manner of contact may induce more rapid cell damage. The mode of action of the *C. albicans* cells (untreated, treated with α-Phellandrene and antifungal drugs individually, and treated with α-Phellandrene + antifungal drugs) was investigated with the use of sorbitol, ergosterol, and a field emission scanning electron microscopy (FESEM).

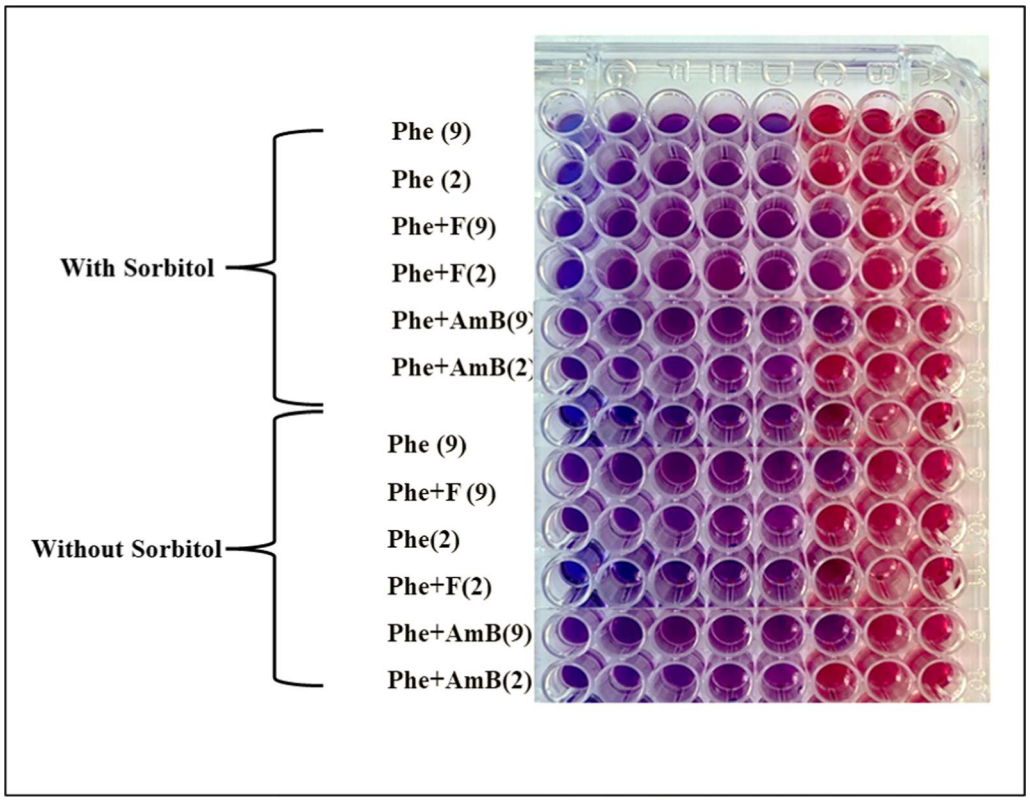

Fig. 7 MIC of α-Phellandrene was determined using a broth dilution assay against *C. albicans* strains ATCC 90028 and MTCC277 in the presence and absence of sorbitol

Table 7 MIC of α-Phellandrene alone and its combinations with antifungal drugs in absence and presence of sorbitol against *C. albicans* strains ATCC90028 and MTCC277

Samples (%)	ATCC90028 MIC	MTCC277 MIC
Without sorbitol		
α-Phellandrene	0.0312	0.0312
α-Phellandrene + Fluconazole	0.0156	0.0156
α-Phellandrene + Amphotericin B	0.0156	0.0312
Fluconazole	0.0625	0.125
With sorbitol		
α-Phellandrene	0.0312	0.0312
α-Phellandrene + Fluconazole	0.0156	0.0156
α-Phellandrene + Amphotericin B	0.0156	0.0312
Fluconazole	0.0625	0.125

Effect of sorbitol assay of α-Phellandrene on the cell wall of C. albicans

In order to study the activity of the compound on the fungal cell wall, we carried out an assay with sorbitol as shown in Fig. 7, which is an ingredient that functions as an osmoprotectant. An osmotic protector, sorbitol is utilized in the process of stabilizing fungal protoplasts (Tariq et al. 2019). Specific fungal cell wall inhibitors all have a particular property in which their antifungal effects are neutralized in mediums containing sorbitol. This is the case for all these inhibitors (Jang et al. 2013). In the presence of fungal cell wall inhibitors, growth can occur in cells that have been protected with sorbitol; however, growth cannot occur in cells that do not have sorbitol protection. Increases in the minimal inhibitory concentration (MIC) value are what make this impact observable. When comparing the MIC value in sorbitol-containing medium to the MIC value in regular medium, similar results are observed. Rearrangement of the cell wall which is essential for the survival of fungal cells is triggered by exposure to osmotic destabilizing chemicals or by disruption of the cell wall.

In this study, the MIC values of α-Phellandrene individually and in combination with antifungal drugs were the same in both assays, regardless of whether the medium contained sorbitol or not as shown in Table 7. This finding suggests that α-Phellandrene does not hinder fungal cell wall formation as one of its mechanisms of action; rather, it most likely affects another target.

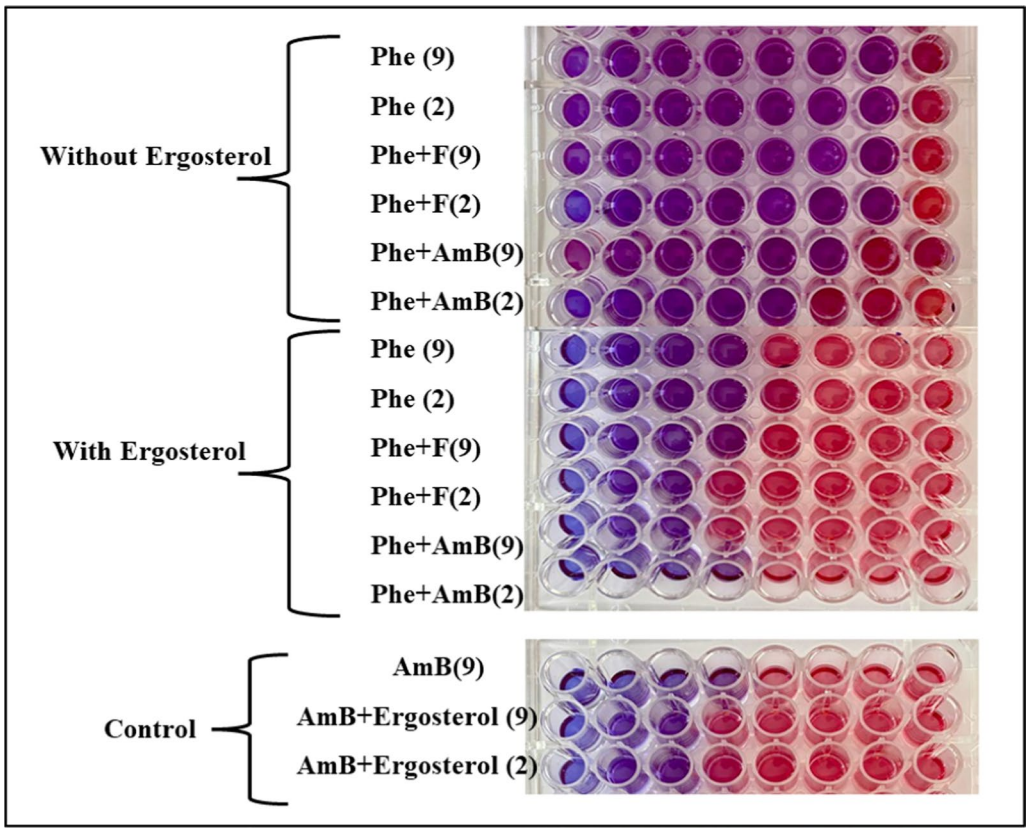

Fig. 8 The minimum inhibitory concentration (MIC) of α-Phellandrene against *C. albicans* strains ATCC90028 and MTCC277 was determined using a broth dilution assay with and without the addition of ergosterol

Table 8 MIC and MFC of -Phellandrene alone and in combination with antifungal medications against *C. albicans* strains ATCC90028 and MTCC277 in the presence and absence of ergosterol

Samples (%)	ATCC90028 MIC	MTCC277 MIC
Without ergosterol		
α-Phellandrene	0.0078	0.0078
α-Phellandrene + fluconazole	0.0078	0.0078
α-Phellandrene + amphotericin B	0.0156	0.0312
Amphotericin B	0.0625	0.0625
With ergosterol		
α-Phellandrene	0.0625	0.0625
α-Phellandrene + fluconazole	0.0625	0.125
α-Phellandrene + amphotericin B	0.125	0.0625
Amphotericin B	0.125	0.125

Effect of MIC value in presence of ergosterol on the cell membrane of C. albicans

α-Phellandrene was put through a battery of tests to see if it could form complexes with ergosterol because of concerns that it would interfere with the membranes of fungal cells both on its own and when combined with antifungal medications. Ergosterol, the most common sterol in fungal plasma membranes, serves the same purpose as cholesterol in mammalian cell membranes (Jordá and Puig 2020). Therefore, the qualities exhibited by these two sterols are qualitatively comparable. Drugs may exert their effects by competing with endogenous ergosterol in fungal cell membranes, thus adding exogenous ergosterol to the mix would be counterproductive.

Therefore, the presence of exogenous ergosterol will result in an increase in the minimum inhibitory concentration (MIC) for both -Phellandrene and α-Phellandrene + drugs compared to the control test. Because interaction with ergosterol in fungal membranes requires a high concentration of product in the growing media, this will be the case. Therefore, researchers investigated how exogenous ergosterol affected the MIC of -Phellandrene alone and in combination with other medicines (Behbehani et al. 2023). α-Phellandrene did exhibit a difference in MIC values, as can be shown; the values changed with the medium contained more ergosterol. The same was observed when the compound was

combined with drugs as shown in Fig. 8. This suggests that complexation of α-Phellandrene + antifungal drugs with ergosterol are involved in the process by which it exerts its effects.

Even when combined with ergosterol, fluconazole (Control) creates an interaction that results in a rise in the MIC of the drug as mentioned in Table 8. These findings are in accordance with those found in earlier research on *C. albicans*, which found that the minimum inhibitory concentration (MIC) value of amphotericin B increased by 32 times in the presence of ergosterol. Interestingly, the same finding was true for the MIC of α-Phellandrene. In 2015, researchers used an antifungal test to determine that thymol's antifungal properties have nothing to do with the metabolic pathways of the cell wall. This was shown by the fact that the MIC in the presence of an osmotic protector (sorbitol) was not affected by its presence in the test. However, in the presence of exogenous ergosterol, the MIC of thymol against C. albicans increased from 39.0 to 312.5 g/ml, an increase of an average of eight. De Castro et al. (2015) found that thymol increases ion permeability via binding to ergosterol in the membrane, leading to cell death.

FESEM imaging for the study of the morphology

Field emission scanning electron microscopy (FESEM) was applied in order to investigate the profound impact that the presence of test compounds had on the morphology of Candida cells. FESEM micrographs of *C. albicans* ATCC90028 and MTCC277 cells following exposure to test compounds for 40 h at their respective minimum inhibitory concentration values (MIC). The untreated *C. albicans* ATCC90028 cells are depicted in Fig. 9a and the untreated MTCC277 *C. albicans* cells are showed in Fig. 9b as having a clearly defined round shape and normal smooth surface areas. Figure 9c illustrates the effect of α-Phellandrene on ATCC90028 Candida cells, which can be seen to have aggregated and distorted structures as a result. The micrographs of fluconazole and amphotericin B shown in further Fig. 9d, e, respectively, emphasize the deformation and creation of pits in ATCC90028 candida strain cells. In addition, substantial breakdown of the fungal membrane was detected when these antibiotics were combined with α-Phellandrene. This was accompanied by the production of a sludge-like substance, which was attributed to the leakage of intracellular contents from ATCC90028 fungal cells. Figure 9f, g illustrate this detail. This shows the efficacy of synergism between α-Phellandrene with fluconazole and with amphotericin B, respectively.

Now with the MTCC277 strain, the same process of analytical observations was repeated, where the effect of α-Phellandrene on MTCC277 are shown in Fig. 9h, where the deep wrinkles and shrinkge flattening of the cells were visible. The effect of fluconazole and amphotericin B are shown in Fig. 9i, j, respectively which exhibited aberrations, including the appearance of pits and flattening of the fungal cells. Further when both the antibiotics (fluconazole and amphotericin B) were combined with α-Phellandrene Fig. 9k, 10l, respectively shows the extensive cell damage and only the footprints of lysed fungus cells were observed which confirmed that the fungal membrane got completely ruptured.

The class of chemical molecules known as terpenes and the essential oils that comprise them are both extensively used and researched due to the pharmacological qualities that they possess and their capacity to increase penetration (D'agostino et al. 2019). Due to their extremely lipophilic nature and low molecular weight, terpenes and terpenoids can disrupt the cell membrane, inducing cell death, or restricting the sporulation and germination of fungus (Sil et al. 2020). Terpenes found in EOs such α-phellandrene, eucalyptol, D-limonene, α-pinene, and related compounds are thought to have a role in the mechanism. In one study with *C. albicans* 1601, researchers looked at the synergistic effect of 8 g/mL fluconazole

(See figure on next page.)

Fig. 9 Micrographs taken with a FESEM of untreated and treated Candida cells after 48 h: **a** untreated *C. albicans* (ATCC90028) cells with typical round smooth surfaces (×12,000), **b** untreated *C. albicans* (MTCC277) cells with usual oval-shaped and smooth outer layers (×4,000) (**c**) clustered and damaged ATCC90028 Candida strain cells that had been treated with α-Phellandrene (×12,000), (**d**) inflated and distorted *C. albicans* cells ATCC90028 that had been treated with fluconazole (×6000), (**e**) creation of pits and deformity in amphotericin B treated ATCC90028 candida strain cells (×12,000), (**f**) rupture of the fungal membrane and a total breakdown of the integrity of the membrane in α-Phellandrene + fluconazole treated ATCC90028 candida strain cells (×12,000), (**g**) intrinsic material of the fungal cells released and the aggregate cells showed as sludge when treated with α-Phellandrene and amphotericin B against ATCC90028 Candida strain cells (×6,000), (**h**) contraction and deformation in α-Phellandrene treated *C. albicans* MTCC277 cells (×6000), (**i**) fluconazole-treated MTCC277 Candida strain cells displaying substantial folds and deformities (×6000), (**j**) pits like framework formed in amphotericin B treated MTCC277 Candida strain cells (×6000), (**k**) lysed cells and only the traces of lysed fungus in α-Phellandrene + amphotericin B treated MTCC277 Candida strain cells (×6000), (**l**) extensive cell damage in fluconazole treated MTCC277 Candida strain cells (×12,000). Arrows in red show any specific observations that have been found. The resolutions/magnifications for the topographical information and the elemental information are represented as ×4000, ×6000 ×12,000 (the reader is directed to the web version of this article for an interpretation of the symbols to color that are contained within this figure legend).

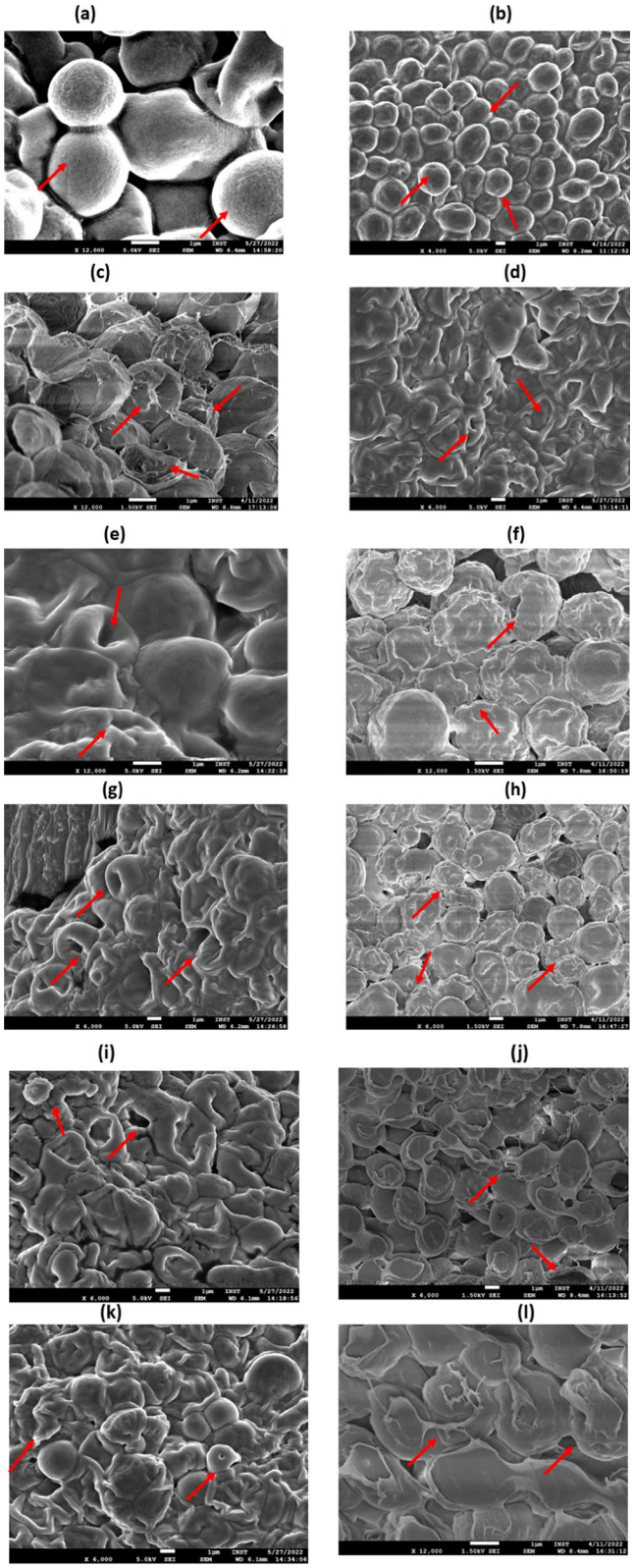

Fig. 9 (See legend on previous page.)

and 16 g/mL sodium houttuyfonate (Shao et al. 2017). When compared to the control group, it was obvious that only yeast-like cells remained after the fungal cells were eliminated.

FESEM micrographs of α-Phellandrene in combination with antifungal medications against *C. albicans* have not before been reported. Conventional antibiotics have been shown to be ineffective due to antimicrobial resistance, hence alternatives have been investigated. One such alternative is combination therapy, which integrates the synergistic advantages of phytocompounds. These results will illuminate the approaches to targeting fungal infections, making the drug discovery process more manageable. Researching synergistic combinations may lead to the repurposing of existing medicinal drugs or the discovery of entirely new therapeutic treatments. An approach that shows promise in combating the growing problem of drug-resistant fungal infections.

To combat antimicrobial resistance (AMR), it was determined that the search for of alternatives to antibiotics (ATA) and the study of adjuvants could be a solution. This was due to the fact that there were no new medications in the pipeline at the time, and time was also a constraint. Some of these possibilities are the enhancement of the bioavailability of drugs that are not widely available and the combination therapy with multiple drugs or drugs that contain small molecules (Sharifi-Rad et al. 2020).

When it comes to the development of medications that have a novel mechanism of action, the synergistic drug combination strategy has proven to be a viable and practical choice. The idea of synergism is emerging as a new alternative therapy, which occurs when the synergistic effect of two or more components in a combination is significantly higher than the cumulative effect of the individual constituents (Shao et a. 2017). The combination of these compounds has been thoroughly investigated in the context of antibacterial drugs and phytocompounds derived from plants (Moussaoui et al. 2016; Cheesman et al. 2017). The research indicates the utilization of phytocompounds can decrease the dosage of a single drug while simultaneously increasing the drug's efficacy, which in turn leads to a reduction in the drug's toxicity. Present work is consistent with this finding showing the efficacy as an antifungal agent as part of the combination therapy.

Conclusion

This study proved that α-Phellandrene possesses strong antifungal activity against *C. albicans*. The anti-candida activity of α-Phellandrene was measured using the agar well diffusion experiment. It resulted in a zone of inhibition of 24 ± 0.5 mm and 22 ± 0.5 mm against the *C. albicans* cells (MTCC277 and ATCC90028), respectively. In addition, the minimum inhibitory concentration (MIC) of the fungicide ranged from 0.0312 to 0.0156 mg/ml (w/v) against strains of *C. albicans*. Additionally, the investigation revealed that the drug's dosages that restrict growth are the same as those that cause death. It was observed that the death kinetics accelerated for the fungal cells with this compound, both independently and in combination with antifungal drugs. The synergism was assessed using a time kill assay and the checkerboard method. The results showed that after 16 h, the number of *C. albicans* cells ATCC90028 (2.56 ± 0.33) and MTCC277 (2.53 ± 0.33) decreased by a log10 when treated with a combination of α-Phellandrene and Fluconazole. Additionally, α-Phellandrene and amphotericin B exhibited synergy against both *C. albicans* strains ATCC90028 and MTCC277, resulting in a log10 reduction in colony count of 2.42 ± 0.28 and 2.00 ± 0.21 respectively.

Furthermore, the combination therapy shows an increase of 16 to 624 folds in the antifungal activity of conventional drugs, with complete cell death observed after 16 h. The research also studies the mechanisms of action of α-Phellandrene. This was done through the sorbitol test, ergosterol test, and FESEM analysis. The antifungal effect of oil alone and in conjunction with antibiotics has the capacity to affect the morphology of the *Candida* species, and its mode of action is demonstrated through the rupturing of the cell membrane leading to severe damage of the fungal cells. As a result, the combination of α-Phellandrene with conventional drugs is presented as a significant and viable antifungal that has the potential to be investigated as an ideal drug candidate for the manufacture of new and future antifungals. Investigations of this sort are crucial because they provide more precise targets for future pharmacological research. These studies attempt improved comprehension of the mode of action of phytocompounds such as α-Phellandrene with its potential therapeutic applications.

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Author contributions

RB performed the experiments and wrote the paper, PS and MS helped with the experiments, DB supervised the work.

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References

Amri I, Khammassi M, Ben Ayed R, Khedhri S, Mansour MB, Kochti O, Pieracci Y, Flamini G, Mabrouk Y, Gargouri S, Hanana M. Essential oils and biological activities of *Eucalyptus falcata*, *E. sideroxylon* and *E. citriodora* growing in Tunisia. Plants. 2023;12(4):816.

Behbehani JM, Irshad M, Shreaz S, Karched M. Anticandidal activity of capsaicin and its effect on ergosterol biosynthesis and membrane integrity of *Candida albicans*. Int J Mol Sci. 2023;24(2):1046.

Bhattacharya R, Dev K, Sourirajan A. Antiviral activity of bioactive phytocompounds against coronavirus: an update. J Virol Methods. 2021a;290: 114070.

Bhattacharya R, Rolta R, Dev K, Sourirajan A. Synergistic potential of essential oils with antibiotics to combat fungal pathogens: present status and future perspectives. Phytother Res. 2021b;35(11):6089–100.

Bhattacharya R, Bose D, Maity S, Prasanna SS, Nagireddi S. Phytocompounds analysis and antimicrobial potential of callistemon viminalis essential oil from North-East India. In: North-East research conclave. Singapore: Springer Nature Singapore; 2022. p. 209–26.

Bhattacharya R, Sourirajan A, Sharma P, Kumar A, Upadhyay NK, Shukla RK, Dev K, Krishnakumar B, Singh M, Bose D. Bioenhancer potential of *Aegle marmelos* (L.) Corrêa essential oil with antifungal drugs and its mode of action against *Candida albicans*. Biocatal Agric Biotechnol. 2023;48:102647.

Brandt ME, Lockhart SR. Recent taxonomic developments with Candida and other opportunistic yeasts. Curr Fungal Infect Rep. 2012;6:170–7.

Chadha J, Singh J, Harjai K. α-Terpineol synergizes with gentamicin to rescue *Caenorhabditis elegans* from *Pseudomonas aeruginosa* infection by attenuating quorum sensing-regulated virulence. Life Sci. 2023;313: 121267.

Cheesman MJ, Ilanko A, Blonk B, Cock IE. Developing new antimicrobial therapies: are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? Pharmacogn Rev. 2017;11(22):57.

Costa AA, Paiva EP, Torres SB, SouzaNeta ML, Pereira KTO, Leite MS, Sá FVS, Benedito CP. Osmoprotection in *Salvia hispanica* L. seeds under water stress attenuators. Brazil J Biol. 2021;82:e233547.

D'agostino M, Tesse N, Frippiat JP, Machouart M, Debourgogne A. Essential oils and their natural active compounds presenting antifungal properties. Molecules. 2019;24(20):3713.

De Castro RD, de Souza TMPA, Bezerra LMD, Ferreira GLS, de Brito Costa EMM, Cavalcanti AL. Antifungal activity and mode of action of thymol and its synergism with nystatin against Candida species involved with infections in the oral cavity: an in vitro study. BMC Complement Altern Med. 2015;15(1):1–7.

de OliveiraSantos GC, Vasconcelos CC, Lopes AJ, de Sousa Cartágenes MDS, Filho AK, do Nascimento FR, Ramos RM, Pires ER, de Andrade MS, Rocha FM, de AndradeMonteiro C. Candida infections and therapeutic strategies: mechanisms of action for traditional and alternative agents. Front Microbiol. 2018;9:1351.

Dworecka-Kaszak B, Biegańska MJ, Dąbrowska I. Occurrence of various pathogenic and opportunistic fungi in skin diseases of domestic animals: a retrospective study. BMC Vet Res. 2020;16(1):1–8.

El-Ganiny AM, Yossef NE, Kamel HA. Prevalence and antifungal drug resistance of nosocomial Candida species isolated from two university hospitals in Egypt. Curr Med Mycol. 2021;7(1):31.

Erlandsen SL, Frethem C, Chen Y. Field emission scanning electron microscopy (FESEM) entering the 21st century: nanometer resolution and molecular topography of cell structure. J Histotechnol. 2000;23(3):249–59.

Fisher MC, Alastruey-Izquierdo A, Berman J, Bicanic T, Bignell EM, Bowyer P, Bromley M, Brüggemann R, Garber G, Cornely OA, Gurr SJ. Tackling the emerging threat of antifungal resistance to human health. Nat Rev Microbiol. 2022;20(9):557–71.

Gaidhani KA, Harwalkar M, Bhambere D, Nirgude PS. Lyophilization/freeze drying—a review. World J Pharmaceut Res. 2015;4(8):516–43.

Garcia LS, editor. Clinical microbiology procedures handbook. USA: American Society for Microbiology Press; 2010.

Gonçalves SM, Ferreira AV, Cunha C, Carvalho A. Targeting immunometabolism in host-directed therapies to fungal disease. Clin Exp Immunol. 2022;208(2):158–66.

Iqbal Z, Ahmed S, Tabassum N, Bhattacharya R, Bose D. Role of probiotics in prevention and treatment of enteric infections: a comprehensive review. 3 Biotech. 2021;11(5):242.

Jang JH, You SY, Oh TJ. Antimicrobial activity of jaborandi extract and sorbitol to oral microbes. J Korean Soc Dental Hyg. 2013;13(3):517–23.

Jordá T, Puig S. Regulation of ergosterol biosynthesis in *Saccharomyces cerevisiae*. Genes. 2020;11(7):795.

Kaur KK, Allahbadia GN, Singh M. Iridoids. Some monotepenes and other natural products for development of potential therapiesin Alzheimer's disease—a review. Nutr Food Toxicol. 2019;3(5):741–56.

Kaur V, Kaur R, Bhardwaj U. A review on dill essential oil and its chief compounds as natural biocide. Flavour Fragr J. 2021;36(3):412–31.

Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: methods, interpretation, clinical relevance. Pathogens. 2021;10(2):165.

Lady J, Nurcahyanti AD, Tjoa E. Synergistic effect and time-kill evaluation of eugenol combined with cefotaxime against *Staphylococcus aureus*. Curr Microbiol. 2023;80(8):244.

Letrado P, Corsini B, Díez-Martínez R, Bustamante N, Yuste JE, García P. Bactericidal synergism between antibiotics and phage endolysin Cpl-711 to kill multidrug-resistant pneumococcus. Future Microbiol. 2018;13(11):1215–23.

Mahdy RA, Nada WM, Wageh MM, Kader MA, Saleh MM, Alswad MM. Assessment safety and efficacy of a combination therapy of topical amphotericin B and subconjunctival fluconazole for the treatment of fungal keratitis. Cutaneous Ocular Toxicol. 2010;29(3):193–7.

McTaggart LR, Cabrera A, Cronin K, Kus JV. Antifungal susceptibility of clinical yeast isolates from a large Canadian reference laboratory and application of whole-genome sequence analysis to elucidate mechanisms of acquired resistance. Antimicrob Agents Chemother. 2020;64(9):10–1128.

Moussaoui F, Alaoui T. Evaluation of antibacterial activity and synergistic effect between antibiotic and the essential oils of some medicinal plants. Asian Pac J Trop Biomed. 2016;6(1):32–7.

Radice M, Durofil A, Buzzi R, Baldini E, Martínez AP, Scalvenzi L, Manfredini S. Alpha-phellandrene and alpha-phellandrene-rich essential oils: a systematic review of biological activities. Pharmaceut Food Appl Life. 2022;12(10):1602.

Rodrigues ML, Albuquerque PC. Searching for a change: the need for increased support for public health and research on fungal diseases. PLoS Negl Trop Dis. 2018;12(6): e0006479.

Saad A, Fadli M, Bouaziz M, Benharref A, Mezrioui NE, Hassani L. Anticandidal activity of the essential oils of *Thymus maroccanus* and *Thymus broussonetii* and their synergism with amphotericin B and fluconazol. Phytomedicine. 2010;17(13):1057–60.

Saggar S, Mir PA, Kumar N, Chawla A, Uppal J, Kaur A. Traditional and herbal medicines: Opportunities and challenges. Pharmacognosy Research. 2022;14(2):107–14.

Saracino IM, Foschi C, Pavoni M, Spigarelli R, Valerii MC, Spisni E. Antifungal activity of natural compounds vs. candida spp.: a mixture of cinnamaldehyde and eugenol shows promising in vitro results. Antibiotics. 2022;11(1):73.

Sardana K, Gupta A, Sadhasivam S, Gautam RK, Khurana A, Saini S, Gupta S, Ghosh S. Checkerboard analysis to evaluate synergistic combinations of existing antifungal drugs and propylene glycol monocaprylate in isolates from recalcitrant tinea corporis and cruris patients harboring squalene epoxidase gene mutation. Antimicrob Agents Chemother. 2021;65(8):10–1128.

Shao J, Cui Y, Zhang M, Wang T, Wu D, Wang C. Synergistic in vitro activity of sodium houttuyfonate with fluconazole against clinical *Candida albicans* strains under planktonic growing conditions. Pharm Biol. 2017;55(1):355–9.

Sharifi-Rad J, Kamiloglu S, Yeskaliyeva B, Beyatli A, Alfred MA, Salehi B, Calina D, Docea AO, Imran M, Anil Kumar NV, Romero-Román ME. Pharmacological activities of psoralidin: a comprehensive review of the molecular mechanisms of action. Front Pharmacol. 2020;11:571459.

Sil A, Pramanik K, Samantaray P, Firoz M, Yadav V. Essential oils: a boon towards eco-friendly management of phytopathogenic fungi. J Entomol Zool Stud. 2020;8:1884–91.

Şimşek M, Duman R. Investigation of effect of 1,8-cineole on antimicrobial activity of chlorhexidine gluconate. Pharmacogn Res. 2017;9(3):234.

Soulaimani B, Varoni E, Iriti M, Mezrioui NE, Hassani L, Abbad A. Synergistic anticandidal effects of six essential oils in combination with fluconazole or amphotericin B against four clinically isolated Candida strains. Antibiotics. 2021;10(9):1049.

Takahashi O, Yamasaki K, Kohno Y, Ueda K, Suezawa H, Nishio M. Origin of the axial-alkyl preference of (R)-α-phellandrene and related compounds investigated by high-level ab initio MO calculations. Importance of the CH/π hydrogen bond. Tetrahedron. 2008;64(24):5773–8.

Tamburlin IS, Roux E, Feuillée M, Labbe J, Aussagues Y, El Fadle FE, Fraboul F, Bouvier G. Toxicological safety assessment of essential oils used as food supplements to establish safe oral recommended doses. Food Chem Toxicol. 2021;157: 112603.

Tariq S, Wani S, Rasool W, Shafi K, Bhat MA, Prabhakar A, Shalla AH, Rather MA. A comprehensive review of the antibacterial, antifungal and antiviral potential of essential oils and their chemical constituents against drug-resistant microbial pathogens. Microb Pathog. 2019;134: 103580.

Vázquez-González D, Perusquía-Ortiz AM, Hundeiker M, Bonifaz A. Opportunistic yeast infections: candidiasis, cryptococcosis, trichosporonosis and geotrichosis. JDDG J Deut Dermatol Gesellsch. 2013;11(5):381–94.

Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc. 2008;3(2):163–75.

Wolff SN, Fay J, Stevens DE, Herzig RH, Pohlman B, Bolwell B, Lynch J, Ericson S, Freytes CO, LeMaistre F, Collins R. Fluconazole vs low-dose amphotericin B for the prevention of fungal infections in patients undergoing bone marrow transplantation: a study of the North American Marrow Transplant Group. Bone Marrow Transplant. 2000;25(8):853–9.

Xu R, Polk RE, Stencel L, Lowe DK, Guharoy R, Duggal RW, Wiest M, Putney KS, Flint NB. Antibiogram compliance in University HealthSystem Consortium participating hospitals with Clinical and Laboratory Standards Institute guidelines. Am J Health Syst Pharm. 2012;69(7):598–606.

Zhang JH, Sun HL, Chen SY, Zeng LI, Wang TT. Anti-fungal activity, mechanism studies on α-Phellandrene and Nonanal against *Penicillium cyclopium*. Bot Stud. 2017;58:1–9.

Zheng Y, Cosgrove DJ, Ning G. High-resolution field emission scanning electron microscopy (FESEM) imaging of cellulose microfibril organization in plant primary cell walls. Microsc Microanal. 2017;23(5):1048–54.

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