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Myristica fragrans oil as a potent inhibitor of *Candida albicans*: Phase development inhibition and synergistic effect

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ABSTRACT

Increasing reports of *Candida* spp. resistance against azole drugs pressure the need for antifungal discovery with a different mechanism. *Myristica fragrans* oil has been known to have anti-*Candida* activity, but its mechanism of action is unknown. To determine the effect of *M. fragrans* oil on the biofilm development phases of *Candida albicans* and its combination with fluconazole. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and quantitative polymerase chain reaction were used to determine the antifungal activity of *M. fragrans* oil. The time of addition assay was performed at the adhesion, intermediate, and maturation phases. The effect of the combination of *M. fragrans* oil with fluconazole was determined by the interaction index value. Gas chromatography-mass spectroscopy (GC-MS) was performed to identify potential components. *Myristica fragrans* oil showed a 50% inhibitory concentration of 1.76% ± 0.4% against *C. albicans* and also inhibited *Candida krusei* and *Candida glabrata*. The time of addition assay showed *M. fragrans* oil effectively inhibited the adhesion and intermediate phases of biofilm development. The combination with fluconazole produced a synergistic effect. GC-MS indicated the presence of α-copaene (11.47%) and myristicin (11.81%) as the main compounds. *Myristica fragrans* oil acts on different phases of biofilm development and could be used as an antifungal agent in combination with fluconazole.

INTRODUCTION

Candida species are commensal microorganisms in the human body. However, some predisposing factors could lead to *Candida albicans* infection called candidiasis. Not only superficial infection but also candidiasis caused by *C. albicans* is able to cause systemic infection, which contributes to its mortality rate that ranges from 15% to 35% for adults and from 10% to 15% for neonates (Guinea, 2014).

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Fluconazole is the first generation of azole drugs which is widely used for candidiasis treatment because it is affordable and has low toxicity (Murray *et al*., 2015; Paramythiotou *et al*., 2014). Fluconazole inhibits the biosynthesis of ergosterol by interfering with lanosterol 14-α-demethylase activity to produce toxic 14-α-methyl-3,6-diol (Prasad *et al*., 2016). However, *C. albicans* resistance has been reported against fluconazole in recent years (Arora *et al*., 2017). To maintain its existence, *C. albicans* overexpress the ERG11 gene, which will upregulate the targeted enzyme and stimulate the efflux pump. Thus, increasing fluconazole concentration is needed to inhibit *C. albicans* growth. Other than ERG11 gene overexpression, biofilm also plays an important role in *C. albicans* resistance development and infectivity (Corte *et al*., 2016). *Candida albicans* biofilm is an extracellular matrix polymer composed of polysaccharides, amphiphilic chemical

components, and other macromolecules (Ramage *et al*., 2012). The complex structure of this matrix becomes a barrier between *C. albicans* and the extracellular area. The presence of persistent cells also contributes to *C. albicans* drug resistance not only against fluconazole but also against other antifungal drugs, such as polyene (Corte *et al*., 2016).

The emergence of drug resistance necessitates the urgent need for alternative treatment which can inhibit different stages of *C. albicans*. It has been reported that essential oil showed promising activity as a biofilm inhibitor, such as the essential oils of *Cymbopogon citratus*, *Cedrus* sp., and *Syzygium aromaticum* (Manoharan *et al*., 2017). *Myristica fragrans*, an aromatic herbal plant that is traditionally used as a food spice in Southeast Asia, has also been reported to exhibit antimicrobial, antitumor, antioxidant, and antifungal activity (Das *et al*., 2020; Rodianawati *et al*., 2015; Salehi *et al*., 2017). Furthermore, Thileepan *et al*. (2018) reported that *M. fragrans* oil exhibited anticandidal activity; however, its inhibition mechanism and combination effect with azoles have not been reported. In this study, we evaluated the antifungal activity of *M. fragrans* oil against different *Candida* spp., its inhibition in different biofilm development stages, and the effect of the combination with fluconazole and identified its chemical compounds. In addition, an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed for evaluating *M. fragrans* oil activity against different *Candida* spp., followed by a quantitative polymerase chain reaction (qPCR) assay for assessing the relative *C. albicans* cell concentration at biofilm inhibition and combination effect activity.

MATERIALS AND METHODS

Candida spp., *M. fragrans* oil, and control drug

Candida albicans UICC Y-29, *Candida glabrata* ATCC 66032, and *Candida krusei* ATCC 6258 cells were grown and maintained in a 90 mm Petri dish containing potato dextrose agar (Difco, USA) at 28°C. For experimental purposes, *Candida* spp. from the Petri dish were further transferred into slant potato dextrose agar and incubated at a 28°C for 24 hours. Fluconazole (Sigma-Aldrich, Germany) was dissolved in 1% dimethyl sulfoxide (DMSO) (Sibelco, Germany) prior to use. *Myristica fragrans* oil with a certificate of analysis was purchased from Costumessentialoil, Jakarta, Indonesia. *Myristica fragrans* oil was diluted in Tween 80 (Sigma-Aldrich®, Germany) to a final tested concentration of 0.2% prior to use.

A 50% inhibitory concentration (IC_{50}) was achieved using a 96-well culture plate coated with 50% fetal bovine serum (FBS) (Sigma-Aldrich, USA). A volume of 100 µl/well of twofold dilution of fluconazole (0.098–50 µg/ml) or *M. fragrans* oil (0.009%–5% v/v) diluted in Sabouraud dextrose broth (SDB) (Millipore, Germany) was added to a 96-well culture plate, followed by the addition of 100 µl/well of *C. albicans* (3×10^4 cells) in SDB. The mixture was then incubated at 37°C for 48 hours. The supernatant was carefully aspirated, and then 100 µl of phosphate-buffered saline (PBS) containing 5 µg/µl MTT (Sigma, USA) was added and mixed. After 3 hours of incubation, 100 µl of DMSO was added and the absorbance was measured at 570 nm using a microplate reader (VersaMax, USA).

Inhibition phase activity was evaluated using 96-well culture plates coated with 5% FBS. *Candida albicans* cell viability was measured using a tetrazolium-based MTT assay, as performed in our previous work (Rahmasari *et al*., 2020). The evaluation for inhibition at different stages was performed as mentioned below.

Adhesion stage (0–2 hours)

100 µl/well of fluconazole or *M. fragrans* oil and 100 µl/well of *C. albicans* (1×10^7 cells) in SDB were added to a 96-well plate coated with 50% FBS. The plate was incubated for 2 hours at 37°C, followed by cell viability measurement by the MTT assay.

Intermediate stage (2–18 hours)

100 µl/well of *C. albicans* (1×10^7 cells) in SDB was added to a 96-well plate coated with 50% FBS. The plate was incubated for 2 hours at 37°C. The supernatant was then aspirated, followed by the addition of fresh SDB and 100 µl/well fluconazole or *M. fragrans* oil and 100 µl/well SDB. The plate was incubated for 16 hours at 37°C, followed by the MTT assay.

Maturation stage (24–48 hours)

100 µl/well of *C. albicans* (1×10^7 cells) in SDB was added to a 96-well plate coated with 50% FBS. The plate was incubated for 24 hours at 37°C, followed by the addition of 100 µl/well fluconazole or *M. fragrans* oil. The plate was further incubated for 24 hours at 37°C, followed by the MTT assay.

MTT assay

After incubation, the supernatant was carefully aspirated, followed by the addition of 100 µl PBS containing 5 µg/µl of MTT, and incubated for 3 hours at room temperature. After incubation, 100 µl DMSO was added and absorbance was measured at 570 nm using a microplate reader.

Drug combination

These effects were further determined by the interaction index value, as described by Tallarida (2002) and as performed in our previous work (Makau *et al*., 2018). In order to calculate the interaction index for the combination of drugs A and B, the following equation was used: $Ac/Ae + Bc/Be = \gamma$, where Ac and Bc correspond to the concentrations of A and B when used in combination; Ae and Be correspond to the concentrations able to produce an effect of the same magnitude if used alone; and γ corresponds to the interaction index value. If γ is <1, the effect of the combination is synergistic, whereas if γ = or >1, the effect is additive or antagonistic, respectively. The evaluation was carried out using a 96-well culture plate coated with 50% FBS. In brief, 100 µl/well of 3×10^4 *C. albicans* cells in SDB was added to 100 µl/well of a mixture containing twofold dilution of fluconazole (0.098–50 µg/ml) and *M. fragrans* oil (0.047%–3% v/v) diluted in SDB. The mixture was then incubated at 37°C for 48 hours. The supernatant was aspirated, and then 50 µl of MTT was added and mixed. After 3 hours of incubation, 100 µl of DMSO was added and the absorbance was measured at 570 nm using a microplate reader.

Quantitative real time polymerase chain reaction

Candida albicans cultures from the evaluation on 96-well plates were collected into microcentrifuge tubes and centrifuged (Sorvall® Fresco, USA) at 4,000 rpm for 10 minutes. The supernatants were discarded and the pellets that formed were extracted using the YeaStar Genomic DNA Kit™ (Zymo Research, USA). The extracted DNAs were used as DNA templates. Two pairs of specific primers from the internally transcribed spacer

(ITS) SACALF (ITS-1) 5′-TTTATCAACTTGTCACACCAGA-3′ and SACALR (ITS-2) 5′-GGTCAAAGTTTGAAGATATACGT-3′ were used based on research conducted by Asadzadeh *et al*. (2018). The DNA samples were then amplified using THUNDERBIRD™ SYBR™ qPCR Mix (Toyobo, Japan) with the MA-600 Real-Time Quantitative Thermal Cycler (Molarray Biotech, China). The qPCR cycle settings were predenaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. All samples were run in duplicate. A standard curve was made using standard solutions from serial dilutions (10^2–10^6 CFU/ml) to obtain a linear regression equation from plotting the cycle threshold (Ct) value against a predetermined CFU value (cell concentration). The results were presented as relative cell concentration, which was determined as (CFU value of treated sample divided by CFU value of untreated sample) × 100%.

Chemical analysis of *M. fragrans* oil

This was performed by gas chromatography-mass spectroscopy (GC-MS) using TRACE GC Ultra with flame ionization detector (FID), MS DSQ II detector, and MS-FID splitter with an Rtx-1 MS column (Restek, 60 m × 0.25 mm ID, film thickness 0.25 µm) using helium as a carrier gas and an electron impact ionization mode at 70 eV. The temperature of the injector was kept at 280°C, ion source temperature at 200°C, FID temperature at 300°C, and temperature programming at 2°C/minute from 50°C to 310°C. The identification of the compounds was based on the comparison of their retention times and mass spectra with those stored in the GC/MS data system and National Institute of Standards and Technology. The compound percentages were computed from the total area of the peak by a GC/MS software apparatus.

Statistical analysis

Data are represented as the mean ± standard error from two independent experiments (each in duplicate). Data were analyzed using GraphPad Prism 9.1.0.

RESULTS

M. fragrans activity against *C. albicans*

We used fluconazole as a positive control and determined the inhibitory activity of *M. fragrans* oil against various *Candida* species using the MTT assay. The comparison of treated *C. albicans* viability to untreated *C. albicans* was analyzed using GraphPad Prism 9.1.0. The results showed that the IC_{50} of fluconazole was 5.05 ± 2.22 µg/ml and *M. fragrans* oil showed an IC_{50} of 1.76% ± 0.44% (v/v) against *C. albicans,* as shown in Table 1, respectively. In order to check the spectrum activities of fluconazole and *M. fragrans* oil against *Candida* spp., their inhibitory activity was tested against *C. glabrata* and *C. krusei*. All *Candida* spp. tested were sensitive to fluconazole, while *M. fragrans* oil was more effective against *C. albicans* and *C. krusei* than *C. glabrata* (Table 1).

Furthermore, the inhibitory activity was also evaluated by quantifying the relative cell concentration of *C. albicans* using the qPCR method. The cell concentration was obtained from the standard curve (Supplementary Data). The inhibitory activity was evaluated by comparing the cell concentration of the treated *C. albicans* to that of the untreated *C. albicans* (Liu *et al*., 2011). *Candida albicans* was treated with fluconazole at concentrations of 12.5, 0.4, and 0 µg/ml and *M. fragrans* oil at concentrations of 2.5%, 0.6%, and 0% v/v. Based on the qPCR results, it was found that fluconazole with concentrations of 0.4 and 12.5 µg/ml inhibited the growth of *C. albicans* up to 40.6% and 72.2% with percentages of relative cell concentration at 59.4% ± 6.6% and 27.8% ± 2.4%, as shown in Figure 1A. Meanwhile, in *M. fragrans* oil, concentrations of 0.6% and 2.5% v/v could inhibit the growth of *C. albicans* up to 23.9% and 67.8% with percentages of relative cell concentration at 76.1% ± 1.1% and 32.2% ± 4.2%, as shown in Figure 1B, respectively.

Inhibitory activity of *M. fragrans* oil against *C. albicans* biofilm formation

The inhibitory activity of fluconazole and *M. fragrans* oil against *C. albicans* biofilm formation is shown in Figure 2. Analysis using the MTT assay showed that the addition of fluconazole 12.56 µg/ml decreased the viability of *C. albicans* to 86.77% at the cell adhesion, 79.36% at intermediate, and 76.59% at maturation phase compared to the untreated cells. Meanwhile, the addition of 3.1% (v/v) *M. fragrans* oil decreased the viability of *C. albicans* to 13.08% at the cell adhesion phase and to 19.84% at the intermediate phase and did not inhibit the maturation phase compared to the untreated cells (Fig. 2A).

The qPCR quantification showed that fluconazole (Fig. 2B) mainly showed an inhibition effect at the *C. albicans* biofilm maturation stage with relative cell concentration decrease to 39.7% ± 0.4% compared to the untreated *C. albicans*. Meanwhile, *M. fragrans* oil (Fig. 2B) exhibited the pronounced activity at the adhesion and intermediate stages with relative cell concentrations at 39.2% ± 12.4% and 11.8% ± 3.6% compared to the untreated cells. Mild activity was observed at the maturation phase (76% ± 17.4% relative cell concentration). The MTT assay and cell quantification by qPCR suggest that *M. fragrans* oil targets the adhesion and biofilm intermediate stages.

Combination effect of *M. fragrans* oil and fluconazole against *C. albicans*

Myristica fragrans oil and fluconazole exhibited activity towards different stages of biofilm maturation. We therefore performed combination studies to determine the effect of *M. fragrans* oil and fluconazole cotreatment on *C. albicans*. As shown in Table 2, the addition of *M. fragrans* oil potentiates the inhibitory effect of fluconazole, as determined by the MTT assay. The IC_{50} of fluconazole and *M. fragrans* oil alone is 5.05 µg/ml and 1.76% (v/v), respectively. Therefore, the isobole method was used to calculate the interaction index at 50% inhibitory activity to determine whether the cotreatment resulted in a synergistic effect or not. The addition of 0.047% of *M. fragrans* oil could reduce the IC_{50} of fluconazole against *C. albicans* by more than half to 2.82 µg/ml, resulting in an interaction index of 0.49, which

Table 1. Activity of *M. fragrans* oil against *Candida* spp.

Candida spp.	IC_{50} fluconazole (µg/ml)	IC_{50} *M. fragrans* oil (%)
C. albicans	5.05 ± 2.22	1.76 ± 0.44
C. glabrata	5.24 ± 1.27	>5
C. krusei	24.1 ± 0.14	1.52 ± 0.02

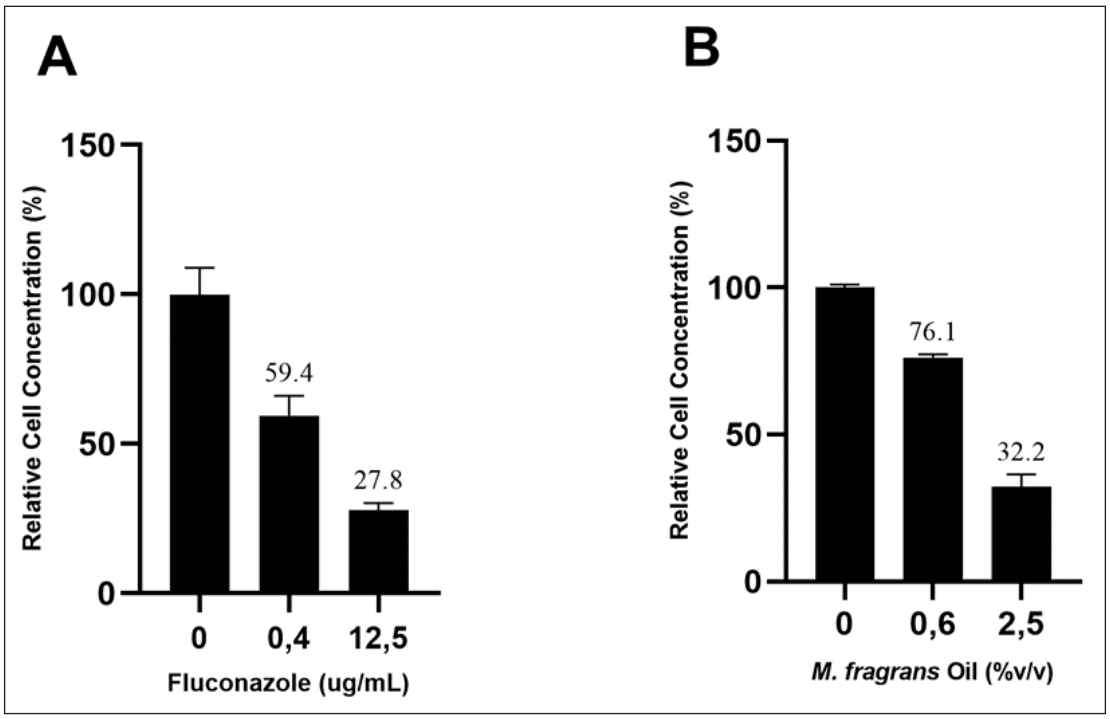

Figure 1. Relative *C. albicans* cell concentration (quantified by qPCR) after treatment with fluconazole and *M. fragrans* oil incubated in 37°C incubator for 48 hours. The percentages of cell concentration after treatment with (A) fluconazole and (B) *M. fragrans* oil were obtained by comparing the treated to untreated *C. albicans*. Data represented as mean ± SD from two independent experiments, each done in duplicate.

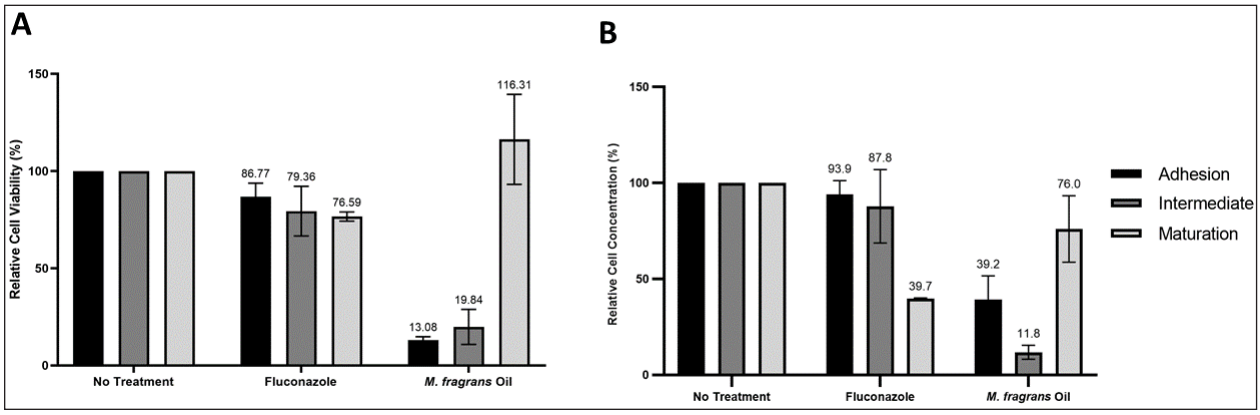

Figure 2. Inhibitory activity of 12.56 µg/ml fluconazole and 3.1% (v/v) *M. fragrans* oil against *C. albicans* biofilm formation at adhesion, intermediate, and maturation stages as evaluated by (A) MTT assay and (B) qPCR. Adhesion indicated by black color, intermediate indicated by dark-gray color, and maturation phase indicated by light-gray color. Data represented as mean ± SD from two independent experiments, each in duplicate.

Table 2. Combination effect of fluconazole with *M. fragrans* oil.

M. fragrans oil (% v/v)	IC_{50} fluconazole (µg/ml)	Interaction index	Effect
0	5.05	—	—
0.047	2.82	0.58	Synergistic
0.094	0.79	0.21	Synergistic
0.188	0.42	0.19	Synergistic
0.375	<0.39	<1	Synergistic
0.75			Synergistic

IC_{50} of *M. fragrans* oil alone = 1.76%.

is synergistic. As indicated in Table 2, other combinations also showed an interaction index of <1, denoting a synergistic effect.

In addition, we used qPCR to determine the relative concentration of the *C. albicans* cells in a combined treatment of 0.39 µg/ml fluconazole and 0.8%, 0.4%, and 0.2% (v/v) of *M. fragrans* oil. The IC_{50} of fluconazole is 5.05 µg/ml. Therefore, we used 0.39 µg/ml, a much lower concentration, to show the synergistic effect of *M. fragrans* oil. As shown in Figure 3, the addition of various concentrations of *M. fragrans* oil greatly reduced the cell concentration of *C. albicans*, which could be detected by qPCR.

Chemical analysis of *M. fragrans* oil

Identification of potential active components in *M. fragrans* oil was performed by gas chromatography. Based on the GC-MS analysis, shown in Table 3, respectively, myristicin (11.81%) and α-copaene (11.47%) existed as major compounds, followed by caryophyllene (6.84%), α-pinene (6.15%), and sabinene (5.98%).

DISCUSSION

The incidence of candidiasis has increased in the last few decades (de Oliveira Santos *et al*., 2018). Since late 1990, the emergence of resistance among *Candida* species has been reported, especially against azole drugs (Orozco *et al*., 1998). *Candida albicans*, the main cause of candidiasis, is able to adapt to environmental changes by polymorphism, proteolytic enzyme production, phenotype switch, and biofilm formation (Clark-Ordóñez *et al*., 2017). It was reported that some essential oils, as mentioned above, show potential inhibitory effects against *Candida* spp. biofilm by inhibiting hyphal formation, altering genes related to biofilm formation, or inhibiting adhesive proteins (Arora *et al*., 2017; Clark-Ordóñez *et al*., 2017; El-Baz *et al*., 2021). In the present research, we used relative cell viability and cell quantification by the MTT assay and qPCR, respectively, to demonstrate that *M. fragrans* oil has potent inhibition activity against *C. albicans*, which is in line with a previous report (Thileepan *et al*., 2017). In addition, we showed that *M. fragrans* oil exhibits activity against other *Candida* species, *C. krusei* and *C. glabrata* (Table 1), indicating its broad spectrum of activity. The IC_{50} of fluconazole against *C. krusei* was higher than those of *C. albicans* and *C. glabrata*. It has previously been reported that the activity of 14α-demethylase in *C. krusei* is more resistant to inhibition by fluconazole than in *C. albicans* strains (Orozco *et al*., 1998). However, *M. fragrans* oil showed potent activity against *C. albicans* and *C. krusei* more than against *C. glabrata*. *Candida albicans* and *C. krusei* were found to have the ability to produce extracellular DNase (DNA cutting enzyme), but no such ability in *C. glabrata* (Riceto *et al*., 2015). Extracellular DNA in *Candida* spp. is known to have an important role in the integrity and maintenance of the biofilm structure (Martins *et al*., 2010; Sapaar *et al*., 2014). Therefore, the sensitivity of *C. albicans* and *C. krusei* to *M. fragrans* oil compared to *C. glabrata* may be due to its components that affect the extracellular DNase activity so that it disrupts biofilm integrity, but further research is needed to prove this.

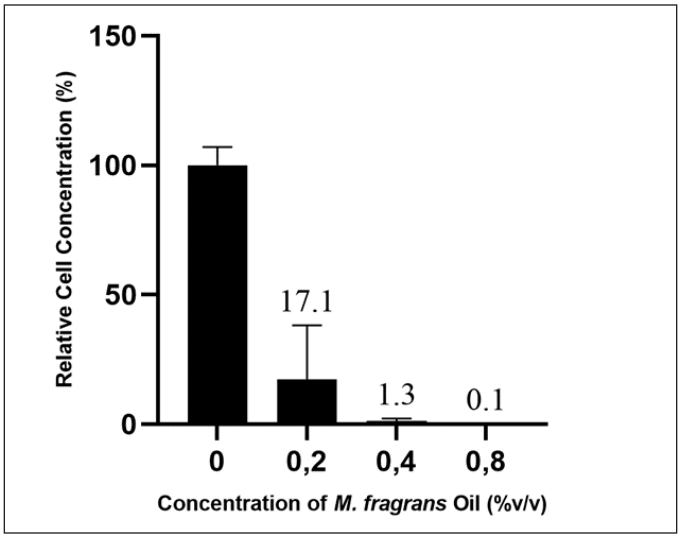

Figure 3. Relative *C. albicans* cell concentration (quantified by qPCR) after treatment with 0.39 µg/ml fluconazole and various concentrations of *M. fragrans* oil. Data represented as mean ± SD from two independent experiments.

Candida albicans biofilm is formed in three phases, which are the adhesion, intermediate, and maturation phases. In the adhesion phase, *C. albicans* cells adhere to the surface of the host during the first 1-2 hours. The intermediate or biofilm development phase occurs for up to approximately 24 hours, followed by the maturation phase in which biofilms with complex structures and compositions exist. The maturation phase occurs between 24 and 72 hours after the attachment of the *C. albicans* cells (Cavalheiro and Teixeira, 2018; Mayer *et al*., 2013). In the current research, fluconazole, as a control drug, mainly inhibited the intermediate and maturation phases of *C. albicans* biofilm more than the adhesion phase (Fig. 2A), as confirmed by qPCR (Fig. 2B). According to research by Khodavandi *et al*. (2011), a significant decrease in *C. albicans* cells treated with fluconazole was observed only after 4 hours incubation compared to the untreated controls, which is in accordance with the results of experiments carried out previously. This phenomenon is related to the mechanism of the azole class to inhibit lanosterol demethylase (14-α-sterol demethylase). When this enzyme is inhibited, other enzymes in the ergosterol biosynthetic pathway will synthesize toxic sterols, namely, 14α-methylergosta-8-24-(28)-dinol, which is fungistatic; therefore, it takes a longer time for fluconazole to

Table 3. Phytochemical compositions of *M. fragrans* oil by GC/MS analysis.

No.	Retention time (minute)	% area	Compounds
1	6.239	1.36	α-Thujene
2	6.390	6.15	α-Pinene
3	6.667	0.16	Camphene
4	7.058	5.98	Sabinene
5	7.159	3.85	β-Pinene
6	7.259	0.82	β-Myrcene
7	7.587	0.87	α-Phellandrene
8	7.764	2.22	(+)-4-Carene
9	7.890	1.00	*p*-Cymene
10	8.016	3.49	β-Phellandrene
11	8.431	2.88	γ-Terpinene
12	8.633	0.11	4-Thujanol
13	8.860	0.95	Terpinolene
14	9.062	0.10	Linalool
15	9.137	0.10	*trans*-4-Thujanol
16	10.423	3.81	*L*-4-Terpineol
17	10.599	0.62	α-Terpineol
18	11.872	0.21	Bornyl acetate
19	11.998	1.79	Safrole
20	12.099	0.76	4-Propyloxyacetophenone
21	12.389	0.31	Carvacrol
22	12.553	0.59	γ-Pyronene
23	12.754	1.41	α-Cubebene
24	13.107	1.09	Cyclosativene
25	13.233	11.47	α-Copaene
26	13.334	2.05	Bicyclosesquiphellandrene
27	13.435	3.64	Methyleugenol
28	13.611	1.66	α-Gurjunene
29	13.838	6.84	Caryophyllene
30	14.065	0.67	(E)-β-Farnesene
31	14.141	0.51	Isoledene
32	14.267	2.63	1,1,4,8-Tetramethyl-4,7,10-cycloundecatriene
33	14.317	1.08	Alloaromadendrene
34	14.456	1.27	γ-Muurolene
35	14.569	0.92	Germacrene D
36	14.670	1.16	Methylisoeugenol
37	14.746	1.13	α-Muurolene
38	14.821	0.41	β-Bisabolene
39	14.897	0.24	Isoeugenol
40	15.086	11.81	Myristicin
41	15.262	2.14	Elemicin
42	15.325	0.23	β-Calacorene
43	15.502	1.40	Isoeugenol
44	15.779	0.48	Spathulenol
45	15.867	0.93	Caryophyllene oxide
46	16.0815	0.8429	3,4-Dimethyl-2,5-diphenyl-1,3,2-oxazaborolidine
47	18.602	2.46	Myristic acid

have the effect of reducing fungal cell viability (Bhattacharya *et al*., 2020; Ellepola *et al*., 2015; Lu *et al*., 2021). On the other hand, *M. fragrans* oil was confirmed to mainly inhibit the adhesion and intermediate phases (Fig. 2A and B). In addition, the inhibitory activity of fluconazole and *M. fragrans* oil against *C. albicans* biofilm formation at the adhesion to intermediate phases (0–18 hours) was also evaluated. The continuous incubation of fluconazole or *M. fragrans* oil showed better activity than when the drug or oil was added at each stage only (Supplementary Data).

GC-MS demonstrated the presence of sesquiterpenes, especially α-copaene (11.47%) and caryophyllene (6.84%), as shown in Table 3, respectively. Those *M. fragrans* oil sesquiterpenes may contribute to adhesion phase inhibition of *C. albicans*, as the sesquiterpenes from *Carpesium macrocephalum* have been reported to inhibit the morphogenetic transformation of yeast to hyphae form (Xie *et al*., 2015). In addition, *M. fragrans* oil is known to contain α-pinene, β-pinene, sabinene, and myristicin (Table 3), which possibly inhibited the adhesion and intermediate phases (de Macêdo Andrade *et al*., 2018; Hammer *et al*., 2003; Moreira Valente *et al*., 2015; Thakre *et al*., 2018). α-Pinene compounds were found to inhibit DNA replication, RNA synthesis, polysaccharides in cell walls, and ergosterol in the cytoplasmic membrane and have inhibitory activity on biofilm formation of *C. albicans*. The compound (+)-β-pinene most likely acts by disrupting cell walls through molecular interactions with delta-14-sterol reductase and, to a lesser extent, with 1,3-β-glucan synthase (de Macêdo Andrade *et al*., 2018). In addition, myristicin and sabinene, which are the main compounds in *M. fragrans* oil, are fungicidal with unknown mechanisms (Moreira Valente *et al*., 2015; Zhou *et al*., 2019). However, *M. fragrans* oil did not exhibit activity at the maturation phase of *C. albicans*, possibly because the biofilm matrix had already formed, thus blocking or slowing the rate of the diffusion of the external factors (Limoli *et al*., 2015; Pinto *et al*., 2020). In addition, hyphal cells in *C. albicans* biofilms express antioxidant defense proteins (e.g., superoxide dismutase 5) that play a role in the detoxification of reactive oxygen species. These proteins are able to block off limonene activity to produce oxidative stress-induced cell apoptosis (Noble *et al*., 2017; Thakre *et al*., 2018).

The combination of antifungal therapies, which has different targets and works synergistically, is one of the strategies to overcome drug resistance (Pai *et al*., 2018). The combination of fluconazole at lower concentrations with *M. fragrans* oil resulted in a synergistic effect (Table 2). Quantification of the *C. albicans* genetic material in a combined treatment of *M. fragrans* oil and fluconazole (Fig. 3) showed a strong synergistic effect, which could result from different mechanisms of action of both drugs (Pai *et al*., 2018). In Figure 2B, *M. fragrans* oil exhibited a strong inhibitory effect on the adhesion and intermediate phases; meanwhile, fluconazole exhibited an inhibitory effect mainly at the maturation phase. Therefore, a combination of both could produce a strong synergy against *C. albicans*. The concomitant use of fluconazole and *M. fragrans* oil for candidiasis treatment can reduce *C. albicans* resistance against fluconazole due to their different mechanism of action. Further, *M. fragrans* oil can be used topically as an adjunctive therapy in mucocutaneous and oropharyngeal candidiasis treated with oral fluconazole.

CONCLUSION

The result presented here demonstrated clearly that *M. fragrans* oil possesses potent activity against *C. albicans* by inhibiting biofilm formation and could be used as an antifungal agent in combination with fluconazole, as emphasized by its synergistic effect. Further research on its potential active compound activity against *C. albicans* infection, safety, and effectiveness *in vivo* is needed.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ETHICAL APPROVAL

No informed consent or animal ethical statement was needed for this research.

DISCLOSURE

All authors certify that they have no financial or proprietary interests in any material discussed in this article.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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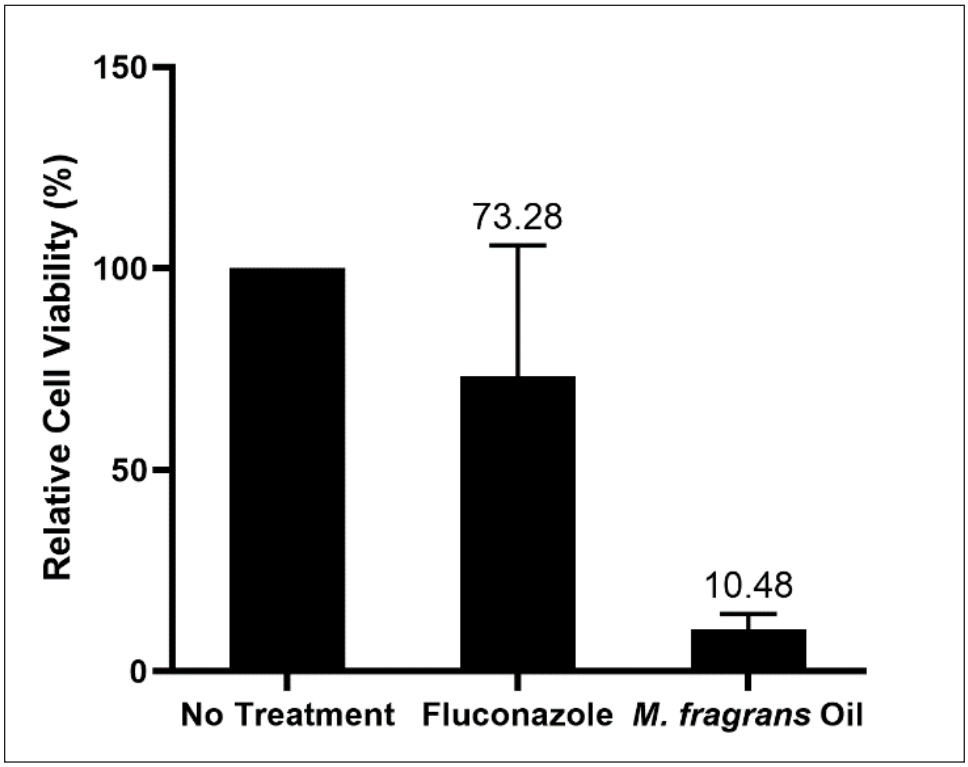
SUPPLEMENTARY DATA



Supplementary Data. Inhibitory activity of 12.56 µg/ml fluconazole and 3.1% (v/v) *M. fragrans* oil against *C. albicans* biofilm formation at the adhesion to intermediate stages (0–18 hours) as evaluated by MTT assay. The experiment was performed as follows: 100 µl/well of fluconazole or *M. fragrans* oil and 100 µl/well of *C. albicans* (1×10^7 cells) in SDB were added into a 96-well plate coated with 50% FBS. The plate was incubated for 18 hours at 37°C, followed by cell viability measurement by the MTT assay. Data represented as mean ± SD from two independent experiments, each in duplicate.