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Potential antifungal impact of citral and linalool administered individually or combined with fluconazole against clinical isolates of *Candida krusei*

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ABSTRACT

Introduction: *Candida krusei* is recognized as a major fungal pathogen in patients with immunodeficiency disorders. The present study aimed at investigating the anticandidal activities of citral and linalool combined with fluconazole (FLZ) against FLZ-resistant *C. krusei* strains.

Methods: Antifungal activities were evaluated by the broth microdilution (MD) method to determine the minimum inhibitory and fungicidal concentrations (namely, MICs and MFCs) according to the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document. The interactions were further evaluated using fractional inhibitory concentration indices (FICIs) for combinations of citral+FLZ and linalool+FLZ, calculated from checkerboard MD assays.

Results: The mean ± standard deviation (SD) MIC values of citral, linalool, and FLZ against the *C. krusei* isolates were 70.23 ± 17, 150 ± 38.73, and 74.66 ± 36.95 µg/mL, respectively. Some fungicidal activities were also observed for citral (2.5) and linalool (1.53) against the *C. krusei* isolates. The FICI values of citral+FLZ and linalool+FLZ for the *C. krusei* isolates ranged from 0.4 to 1.00 and 0.19 to 0.63, respectively. The additive and synergistic interactions of linalool + FLZ were further observed in 12 (57.1%) and 9 (42.9%) *C. krusei* isolates. However, there was an additive interaction for citral + FLZ in 17 (80.9%) isolates. They also showed a synergistic interaction in only four (19.1%) isolates. Moreover, linalool and citral plus FLZ did not have any antagonistic effect on any isolates.

Conclusion: The study findings support the possible capabilities of citral and linalool, as anticandidal agents, and FLZ might be supplemented with citral and/or linalool for treating FLZ-resistant *C. krusei* infections.

Implication for health policy/practice/research/medical education:
This research indicated that combining fluconazole and citral and/or linalool might lead to increased efficacy and reductions in the minimum effective doses. These combination therapy may moderate the development of drug toxicity and resistance.

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Introduction

Candida species are among major fungal pathogens that are often isolated from invasive fungal infections. These infections can be formed as deep-seated or superficial diseases. Infections resulting from non-albicans *Candida* (NAC), with high incidence rates, such as *C. krusei*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* have also exposed patients to significant nosocomial risks (1). *C. krusei* is a diploid, dimorphic and with generally elongated cells belonging to ascomycetous yeasts normally found as a commensal and the mucosal membrane inhabitant in healthy individuals (2). Of note, there is an intrinsic resistance in *C. krusei* against fluconazole (FLZ), as a frequent drug used for treating the infections caused by candida species. Thus, nosocomial candidiasis due to *C. krusei* is currently regarded as a therapeutic challenge, particularly in immunocompromised patients. It is also estimated that mortality rates from *C. krusei* fungemia

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are about 60%-80% (3). In addition to a systemic disease, there is an association between *C. krusei* and superficial infections. Vulvovaginal candidiasis and bronchopneumonia can be further induced by this organism; however, it is a rare etiological element in the former, which is isolated merely in 0.1% of cases (4,5). Additionally, evidence shows that *C. krusei* infects the tonsils, where a permanent cure can be achieved just by removing the organ via surgery, causing ulcers, septic arthritis, vasculitis, and urinary tract infections (6).

In this respect, Azoles are typically utilized for treating different lethal fungal diseases (7). Among these agents, FLZ is the most commonly used antifungal drug against candidiasis because it is highly water-soluble, with high bioavailability and little host toxicity. Since FLZ is a fungistatic agent, it does not destroy the fungus. Therefore, it can be partly explained by the emergence of NAC species resistance to antifungal drugs. It has been frequently noticed that FLZ-resistant Candida strains are evolved, particularly within long-term or repeated therapies for recurrent episodes of vulvovaginal/oropharyngeal candidiasis in individuals with acquired immunodeficiency syndrome (AIDS) (2,8). There are also additive impacts for different resistance mechanisms, as revealed by the sequential presentation of resistance mutations in multidrug resistance regulator 1, sterol 14α-demethylase (ERG11), ultra-performance convergence chromatography, and tachykinin precursor 1 (TAC1) in all potential combinations into a drug-susceptible Candida strains (9). Another mechanism of resistance is by the reduction in intracellular azole concentration, due to changes in the cell membrane composition or efflux pump activity (2). This accounts for the selection of strains with multiple resistance mechanisms in long-term FLZ treatment of patients with candidiasis. Hence, there is an urgent need to find a novel efficient therapeutic strategy to control and treat *C. krusei*-caused infections.

Previous studies have investigated the potential of enhanced antifungal impact of natural products combined with synthetic drugs (10). Using combined antifungal therapies, the killing features of the compounds against microorganisms can be accordingly elevated through synergy and the spectrum effect can be enhanced. Besides, combination therapy can reduce the development of drug toxicity and resistance (11).

Citral (3,7-dimethyl-2-6-octadienal) and linalool (3,7-dimethylocta-1,6-dien-3-ol) are known as monoterpenoids, isolated from various herbal plants. Their anticancer, anti-inflammatory, antimicrobial, antihyperlipidemic, antinoceptive, analgesic, anxiolytic, antidepressive, neuroprotective, and antifungal activities against yeasts and molds have been demonstrated in several studies (12-15). In this research, the *in vitro* antifungal activities of citral and linalool were assessed individually and combined with FLZ against the FLZ-resistant clinical strains of *C. krusei*.

Materials and Methods

Organisms

In total, 20 clinical isolates of *C. krusei* from patients (viz. 10 oral and 10 vaginal strains) were obtained from the Microorganism Bank of Dr. Aghil Sharifzadeh, Department of Microbiology, Mycology Center, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, upon receiving ethical approval according to the national guidelines. In addition, *C. krusei* (ATCC 90030) was used as a control.

Fluconazole (FLC), *citral* natural (≥96%), and linalool (95-97%) were purchased from Sigma-Aldrich. Stock solutions of test compounds were prepared in 10% dimethyl sulfoxide (DMSO) and then further diluted in RPMI 1640 (Thermo Fisher Scientific) to the desired concentration ranges. The final concentration of DMSO was not higher than 1% of the total volume.

Antifungal susceptibility testing

Inoculum preparation

The primary suspensions of each *C. krusei* isolate were prepared by culturing the isolates in Sabouraud Dextrose Broth (SDB, Merck Co., Darmstadt, Germany) for 24 h at 35°C, with 200 rpm agitation. Then, 1 mL of a 24-hour-old culture was centrifuged (3900 × g at 4°C for 1 minute), and the pellets were washed twice with 1 mL of physiological saline. McFarland turbidity of 0.5 at 530 nm was further obtained after adding 5 mL of 0.9% sterile physiological saline, which corresponded with $0.5\text{-}2.5\times10^5$ colony-forming unit (CFU)/mL. Afterward, morpholine propane sulfonic acid (MOPS)-buffered RPMI1640 medium (Sigma-Aldrich, St. Louis, USA) was employed for diluting the suspension and obtaining the final inoculum concentration of $0.5\text{-}2.5\times10^3$ CFU/mL.

MIC and MFC assays

After dissolving of linalool, citral, and FLZ (Sigma-Aldrich, St. Louis, USA) in DMSO, the final serial concentrations of these materials in 96-well plates were obtained with a range of 0 to 256 µg/mL for FLZ and 6.25 to 300 µg/mL for linalool and citral in the RPMI-1640 medium. In this regard, the Clinical and Laboratory Standards Institute (CLSI) M27-A3-S4 document guideline was followed (16). Then, the yeast suspension obtained from the previous step (100 µL) was distributed in all wells from a 96-well micro-TTP.

The negative and positive controls included wells without *C. krusei* and antifungals (citral, linalool, and FLZ), respectively. Then, the plates were incubated at 35°C, followed by reading the endpoints visually after 48 hours. The interpretation of the MICs was as follows: the lowest concentration of antifungal agents preventing the growth of the *C. krusei* strains under test in comparison with the growth control. Each experiment was also carried

out in triplicate. To determine the MFC, SDA-containing plates were used for seeding 10 µL of the volume of each well without microorganism growth (MIC, 2×MIC, and 4×MIC). Then, the plates were incubated for 48 hours at 35°C. The number of colony-forming units (CFU) was further calculated, and the MFCs were recorded as the lowest concentrations of the drugs that led to no visible colonies. Whether citral and linalool had a fungicidal (MFC/MIC<4) or fungistatic activity (MFC/MIC ≥4) was ultimately determined by obtaining the MFC/MIC ratio (17).

Checkerboard assay

Fractional inhibitory concentration indices (FICIs) were determined by performing the checkerboard synergy test to express the combined impact of FLZ and terpenoid phenols (18). In brief, the serial dilutions of the antifungal compounds were prepared. For this purpose, 50 µL of FLZ dilutions were mixed with the rows of a 96-well micro-TTP in decreasing concentrations. In addition, linalool and/or citral (50 µL) was mixed with the columns in reducing concentrations. A 100 µL suspension of the *C. krusei* strains was further adjusted to 0.5-2.5×10^3 CFU/mL and added to the wells, followed by incubation for 48 hours at 35°C. Some experiments were also conducted to indicate the interactions between FLZ and citral. The MICs of both combined anticandidal compounds were obtained according to the above-mentioned process. Upon determining the FICs and FICIs, the antagonistic or synergistic activities of the anticandidal combinations were also assessed. In order to calculate the FICs, the MIC of the FLZ-linalool combination was divided by the MIC of FLZ or linalool alone. In addition, the FICs were obtained to indicate interactions between FLZ and citral. For calculating the FICIs, the following relation was used:

Drug A FIC=Drug A MIC in a combined manner/ Drug A MIC individually; Drug B FIC=Drug B MIC in a combined manner/Drug B MIC individually

The FICI is given as the sum of the FIC of drug A and drug B, and its interpretation is as follows:
Additive impact, 0.5<FICI<1.0; indifference, synergy, FICI≤0.5, 1.0<FICI≤ 4.0, antagonism, FICI>4.0

Statistical analysis

One-way analysis of variance (ANOVA) was employed for determining statistical significance (Sigma Stat, version 3.5). *P* values below 0.05 were also regarded statistically significant. The Bliss method (i.e., the dose-effect based multiplicative impact of a single drug as if they served independently) was applied for combination therapies to determine the visualization of the synergistic score and the interactive analysis of linalool + FLZ and citral+FLZ profiling data against the *C. krusei* isolates. Moreover, the SynergyFinder was used for generating the plots, which was a stand-alone web application. The synergy score for a drug combination was further averaged over all the dose combination measurements. In the SynergyFinder, the two-dimensional (2D) and three-dimensional (3D) synergy maps were presented, highlighting antagonistic and synergistic dose regions in green and red. According to the SynergyFinder, if the synergy score was:

- below -10, it was likely that the two drugs had an antagonistic interaction.
- between -10 and 10, it was likely that the two drugs have an additive interaction
- above 10, it was likely that the two drugs have a synergistic interaction (19).

Results

The results showed that 16 (76.2%) *C. krusei* strains were resistant to FLZ with the MICs ranging from 64 to 128 µg/mL, except for five (23.8%) clinical isolates of *C. krusei*, indicating the MIC of 32 µg/mL (susceptible-dose-dependent [SDD]) (Table 1). The mean ± SD MIC value of FLZ was 74.66 ± 36.95 µg/mL.

Based on M27-A3 broth MD assay, the test compounds, citral and linalool, were active against all the strains tested (Table 1). The MICs of citral also ranged from 50 to 100 µg/

Table 1. The *in vitro* susceptibilities of clinical and standard strains of *Candida krusei* (C. k1-C. k20) to linalool, citral and fluconazole

Broth MD (µg/mL)							
C. krusei strains	Linalool			Citral			FLZ
	MIC	MFC	MFC/MIC	MIC	MFC	MFC/MIC	MIC
C. k1	100	150	1.5	50	150	3.0	64
C. k2	100	200	2	50	100	2.0	64
C. k3	100	200	2	75	150	2.0	64
C. k4	150	200	1.33	100	300	3.0	64
C. k5	150	200	1.33	75	150	2.0	128
C. k6	150	150	1	75	100	1.3	128
C. k7	100	150	1.5	75	150	2.0	32
C. k8	200	300	1.5	75	150	2.0	32
C. k9	200	300	1.5	75	150	2.0	32
C. k10	200	200	1	75	150	2.0	64
C. k11	200	200	1	75	200	2.7	128
C. k12	150	300	2	75	150	2.0	128
C. k13	100	200	2	100	300	3.0	64
C. k14	150	150	1	50	150	3.0	64
C. k15	200	300	1.5	50	150	3.0	32
C. k16	200	300	1.5	75	200	2.7	128
C. k17	100	200	2	50	150	3.0	32
C. k18	150	300	2	75	200	2.7	64
C. k19	150	200	1.33	100	300	3.0	64
C. k20	150	200	1.33	50	150	3.0	128
C. krusei ATCC 90030	150	300	2	50	200	4.0	64

MIC, Minimum inhibitory concentration; MFC, Minimum fungicidal concentration; FLZ, fluconazole.

mL, with a mean ± SD value of 70.23 ± 17 µg/mL. As well, linalool was the weaker antifungal compared with citral, with the MIC values of 100 to 200 µg/mL (mean ± SD: 150 ± 38.73 µg/mL). Increasing the concentrations of both citral and linalool in the media also led to a dose-dependent progressive and significant ($P < 0.05$) reduction in growth for all *C. krusei* strains (Figure 1).

The mean ± SD MFC values of citral and linalool were obtained as 176.2 ± 58.35 µg/mL and 223.8 ± 8.35 µg/mL for the *C. krusei* strains, respectively. According to these results, the MFC values for both compounds were 1-2 folds higher than their respective MIC values. As depicted in Figure 2, the mean MFC/MIC ratios of citral and linalool were 2.5 and 1.53 for the *C. krusei* strains, respectively. Based on the MFC/MIC ratios, which were lower than 4, it was suggested that both compounds had fungicidal rather than fungistatic activity.

As listed in Table 2, the results of the chequerboard microtiter assay indicated significant combined effects between citral/linalool and FLZ calculated for the FLZ-resistant *C. krusei* isolates ($P < 0.05$). The FICI values for the citral ± FLZ and linalool ± FLZ combinations against all *Candida* isolates studied here also ranged from 0.40 to 1.00 and 0.19 to 0.63, respectively.

Out of 21 *C. krusei* isolates tested (namely, 20 clinical isolates and 1 ATCC strain), the interaction between linalool and FLZ was synergistic in 12 and additive in nine cases, whereas four isolates showed a synergy for citral and FLZ, and 17 cases had an additive interaction based on the FICIs. Linalool and citral also decreased the mean MICs of FLZ from 74.66 µg/mL to 9.81 and 9.9 µg/mL for the *C. krusei* strains (>75%), respectively. Moreover, the FICI values and the Bliss synergy score indicated that both terpenoids under test exhibited excellent synergistic activities with FLZ (Figure 3). Of note, linalool and citral did not show any antagonistic activities with FLZ.

Discussion

The high incidence rates of candidiasis among immunocompromised patients due to the emergence of drug resistance in clinical *Candida* isolates is a matter of concern. The increasing frequency of non-*C. albicans* species in clinical situations, like *C. krusei*, which often respond differentially to FLZ, has further worsened the situation (2,20). The present study investigated the antifungal activity of citral and linalool against the clinical *C. krusei* strains, and their synergistic interactions in combination with FLZ.

In this study, most of the clinical isolates and the reference strain (76.2%, 16/21) were resistant to FLZ, except five isolates that were considered as dose-dependent (23.8%, 5/21). The MICs for FLZ also ranged from 32 to 128 µg/mL

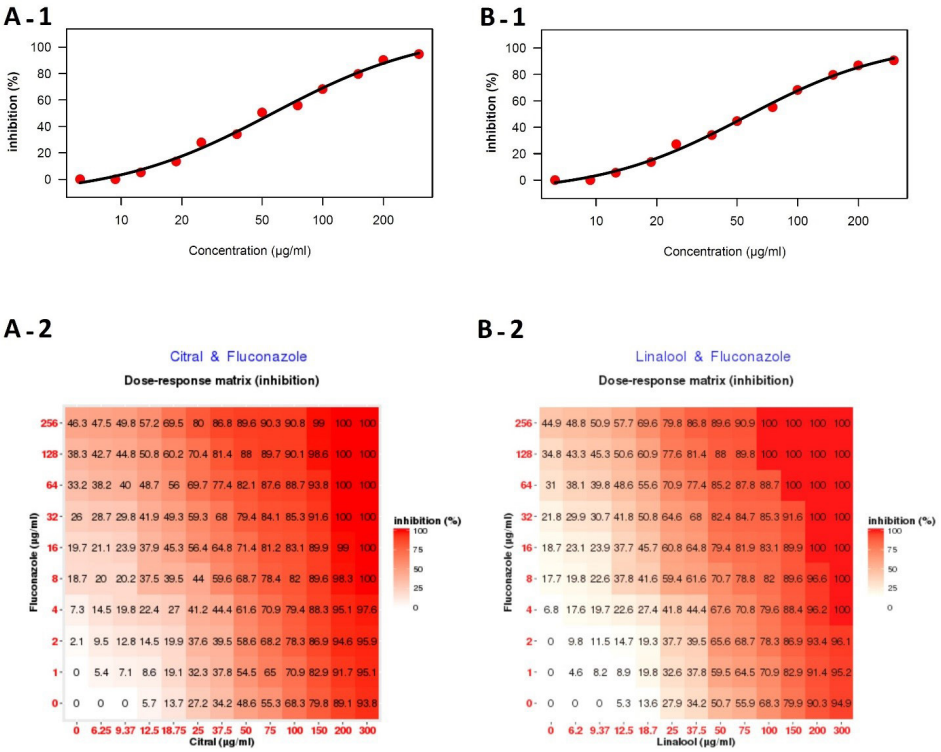

Figure 1. A-B, the visualization of input dose-response data. The pictures show an overview of the full dose-responses for the single drug against the *C. krusei* standard strain; A-1: linalool and B-1: citral (the percentage of growth inhibition that depends on increasing drug concentration). The matrix format, which was supported by two drug combinations illustrates the dose-response interactions in a matrix form, using the cNMF algorithm. A-2: linalool+FLZ against the *C. krusei* standard strain; B-2: citral+FLZ against the *C. krusei* standard strain.

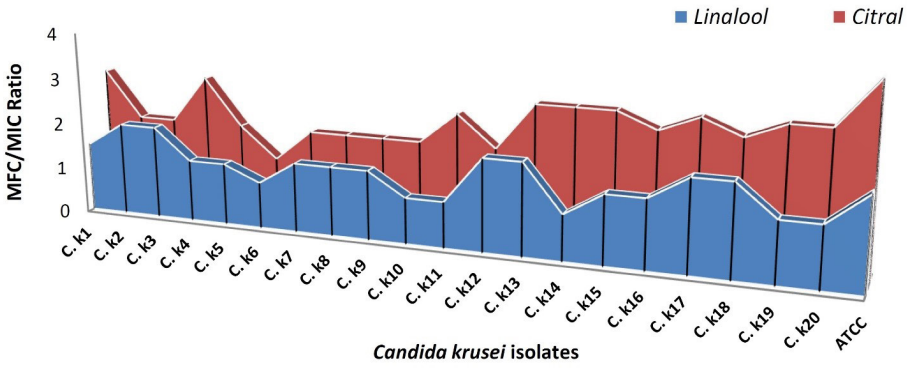

Figure 2. The fungicidal vs. fungistatic activity of linalool and citral against the *C. krusei* isolates. MFC/MIC<4: fungicidal activity; MFC/MIC≥4: fungistatic activity.

(mean ± SD: 74.66 ± 36.95 µg/mL). Consistent with these findings, previous reports have demonstrated the FLZ MIC values of 64 µg/mL for *C. krusei* (21,22). *C. krusei* has recently emerged as an important nosocomial pathogen. Currently available triazoles, such as FLZ, have also limited or no activity on this organism, because *C. krusei* is assumed to be intrinsically resistant to this compound (2,20). Thus, it is more difficult to treat *Candida* infections via FLZ monotherapy. The scientific community has thus paid attention to the potential antifungal impacts of some bioactive compounds from plants.

Here, the M27-A3 broth MD assay indicated that citral had a good inhibitory effect on all 20 *C. krusei* specimens, and the MICs ranged from 50 to 100 µg/mL, with the mean ± SD value of 70.23 ± 17 µg/mL. In addition, all clinical strains were inhibited by linalool with the MICs ranging from 100 to 200 µg/mL (mean ± SD: 150 ± 38.73 µg/mL). Increasing the concentrations of both citral and linalool in the media also led to a dose-dependent progressive and significant reduction in growth for all *Candida* strains. The biological activity of the natural products was further interpreted using the MIC values, according to the criteria adopted by Sartoratto et al (23): viz. the biological activity of a product may be interpreted as excellent/good if the MIC value ranges from 50 to 500 µg/mL. As well, if compounds show a variation in the MIC value from 600 to 1500 µg/mL, they are considered to have moderate activity, and in cases when the MIC is greater than 1500, it has weak activity. Thus, according to the findings in the present study, linalool and citral presented good antifungal activity for the *C. krusei* strains. High anti-*Candida* efficacy by linalool and citral had also been reported in other studies. In agreement with these results, Ferreira et al (24) exhibited the very strong antifungal activity of citral with MIC values lower than 100 µg/ml. In another study by Mesa-Arango et al (25), citral presented an antifungal effect, with MICs of 39.7 µg/mL for *C. krusei*. As well, Leite et al (15) found the MFC and MIC of citral against *Candida* species as 256 and 64 µg/mL, respectively. In a study by Dias et al. (26), linalool had the MIC values of 2 mg/mL against *C. krusei*. Moreover, Hsu et al (27) demonstrated the antifungal activity of linalool against *Candida* species, with an MIC of 8 mM.

In this research, the mean MFC values of citral and linalool were obtained as 176.2 ± 58.35 µg/mL and 223.8 ± 8.35 µg/mL for the *C. krusei* strains, respectively. The mean MFC/MIC ratios of citral and linalool were also equal to 2.5 and 1.53 for the *C. krusei* strains, respectively. The results correspondingly revealed the potent fungicidal activity of linalool and citral against the clinical *Candida* strains. Accordingly, the MFC/MIC ratio of linalool outperformed that of citral, in line with the study by Dias et al (26). The findings outlined herein were also consistent with the results presented by Zore et al (28), in which linalool and citral have been assumed effective in all isolates of *Candida* strains (namely, dose-dependent and resistant to FLZ), and showing fungicidal activity. Previous studies have also shown that linalool was able to significantly interfere in the biosynthesis of the cell wall and/or increase the ionic permeability of the fungal cell membrane (29,30). Citral additionally demonstrated fungicidal activity against the *Candida* strains isolated after 2 hours of exposure and caused major morphological changes via mechanisms other than cell wall biosynthesis or ergosterol complexation (15). As documented, there is a relationship between the fungicidal activity of antifungals and higher therapeutic success and less persistent and lower recurrent infection rates, particularly in candidemia and invasive candidiasis (31).

The synergistic effects of various components of plant extracts are also supposed to contribute to the effectiveness of herbal preparations used in traditional medicine. Synergy research in phytomedicine is thus really needed to address this issue. The present study investigated the FICI values for linalool and citral in combination with antifungal agent, FLZ. The potential antifungal activities of linalool and citral alone or in combination with FLZ against the clinical *C. krusei*

Table 2. The checkerboard assay of linalool, citral, and FLZ against the *C. krusei* strains

C. krusei strains	MIC combination (µg/mL) (linalool/FLZ)	FICL- FICF index (linalool/FLZ)	FICI index (combination)	Interpretation	MIC combination (µg/mL) (citral/FLZ)	FICL- FICF index (citral/FLZ)	FICI index (combination)	Interpretation
C. k1	37.5-8	0.38-0.125	0.50	ADD	18.75-4	0.38-0.06	0.44	SYN
C. k2	37.5-8	0.38-0.125	0.50	ADD	25-8	0.50-0.13	0.63	ADD
C. k3	50-8	0.50-0.125	0.63	ADD	37.5-16	0.50-0.25	0.75	ADD
C. k4	25-4	0.17-0.062	0.23	SYN	50-8	0.50-0.13	0.63	ADD
C. k5	50-32	0.33-0.25	0.58	ADD	25-8	0.33-0.06	0.40	SYN
C. k6	25-8	0.17-0.062	0.23	SYN	25-16	0.33-0.13	0.46	SYN
C. k7	37.5-4	0.38 -0.125	0.50	ADD	37.5-8	0.50-0.25	0.75	ADD
C. k8	25-2	0.13-0.062	0.19	SYN	25-4	0.33-0.13	0.46	SYN
C. k9	37.5-4	0.19-0.125	0.31	SYN	37.5-8	0.50-0.25	0.75	ADD
C. k10	25-8	0.13-0.125	0.25	SYN	50-4	0.67-0.06	0.73	ADD
C. k11	37.5-16	0.19-0.125	0.31	SYN	37.5-8	0.50-0.06	0.56	ADD
C. k12	37.5-8	0.25-0.062	0.31	SYN	37.5-16	0.50-0.13	0.63	ADD
C. k13	37.5-16	0.38- 0.25	0.63	ADD	50-16	0.50-0.25	0.75	ADD
C. k14	37.5-16	0.25-0.25	0.50	ADD	25-8	0.50-0.13	0.63	ADD
C. k15	25-4	0.13-0.125	0.25	SYN	37.5-8	0.75-0.25	1.00	ADD
C. k16	37.5-16	0.19-0.125	0.31	SYN	37.5-4	0.50-0.03	0.53	ADD
C. k17	37.5-4	0.38-0.125	0.50	ADD	25-8	0.50-0.25	0.75	ADD
C. k18	50-8	0.33-0.125	0.46	SYN	37.5-16	0.50-0.25	0.75	ADD
C. k19	50-8	0.33-0.125	0.46	SYN	50-16	0.50-0.25	0.75	ADD
C. k20	75-16	0.50-0.125	0.63	ADD	25-8	0.50-0.06	0.56	ADD
C. krusei ATCC 90030	25-8	0.17-0.125	0.29	SYN	25-16	0.50-0.25	0.75	ADD

Abbreviations: C. K, *Candida krusei*; ADD, additive; SYN, synergistic; MIC, Minimum inhibitory concentration; MFC, Minimum fungicidal concentration; FICIs, Fractional inhibitory concentration indices.

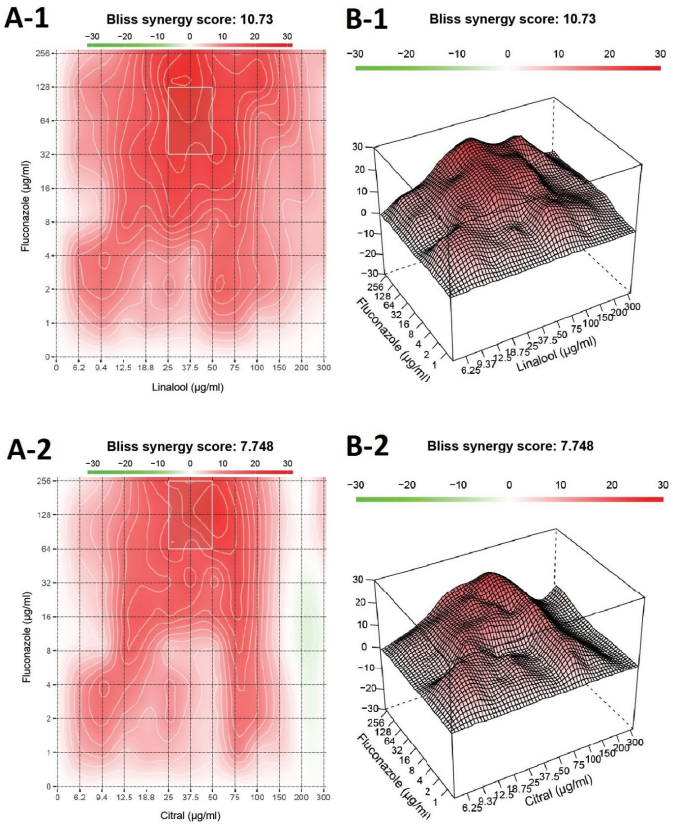

Figure 3. A-B: The interactive analysis and visualization of the synergistic score of the linalool+FLZ (A-1 and B-1) and citral+FLZ profiling data against the *C. krusei* isolates and their statistical significance. The level of antagonism or synergism was represented by a colored scale bar (from white to dark red). The plots were generated using the SynergyFinder, as a stand-alone web application, by applying the Bliss method (the dose-effect based multiplicative impact of a single drug as if they served independently) for combined treatments. The synergy score for a drug combination was further averaged over all the dose combination measurements. The 2D (A-1 and A-2) and 3D (B-1 and B-2) synergy maps highlight synergistic dose regions in red (There is no antagonistic point).

were accordingly approved. The FICI values for linalool and citral were, respectively, in the range of 0.19 to 0.63 and 0.40 to 1.00 against all the fungal strains tested. The highest synergistic interaction based on the MIC values was also observed for linalool+FLZ (57.1%), followed by citral+FLZ (19%). Besides, the checkerboard assay outcomes showed that all terpenoids under test sensitized and significantly reduced the MICs of FLZ to very low concentrations in the FLZ-resistant strains of *C. krusei*. Linalool and citral additionally decreased the mean MICs of FLZ from 74.66 µg/mL to 9.81 and 9.9 µg/mL for the *C. krusei* strains, respectively. The FLZ concentrations were diminished by citral and linalool (>75%) in order to inhibit the *C. krusei* growth. The FICI values and the Bliss synergy score also showed that both terpenoids under test exhibited excellent synergistic interactions with FLZ. A similar study describing the synergistic effect of essential oils with antifungals against *Candida* species has also been reported elsewhere (32). To the best of the authors' knowledge, there is little information about the anti-*Candida* efficacy of citral and linalool in combination with FLZ. The results of the present study were consistent with the reports by Zore et al (28) suggesting that linalool could minimize the MIC of FLZ by 64 folds (i.e. from 64 µg/mL to 1 µg /mL) at 0.008% (v/v) concentration while citral could bring down the MIC of FLZ by 32 folds (i.e. from 64 µg/mL to 2 µg/mL) at 0.016% (v/v) concentration. Some of the plant extracts and essential oils have further shown excellent synergistic activities and have moderated the MICs of conventional antibiotics in recent studies. For example, thymol acted synergistically with FLZ against candidiasis (33). Eugenol showed a synergistic interaction with voriconazole against *C. krusei* (34), and terpenoids were reported to arrest the eukaryotic cell cycle (35). Moreover, Zore et al (28) demonstrated that terpenoids could arrest *Candida* cells at different phases of cell cycle, i.e., G1 arrest by linalool and S phase arrest by citral. In addition, terpenoids could induce membrane fluidization, modulating the functions of membrane bound proteins involved in signaling and transport (28). A hypothetical model was proposed for the anti-*Candida* activity of citral and linalool terpenoids as well as their synergies with FLZ. In this context, the increased cellular concentration of FLZ could inhibit ergosterol biosynthesis, while the terpenoid-mediated impairment of membrane integrity could modulate signaling pathways and transport. Thus,

the synergistic effect exhibited by terpenoids with FLZ is a multifactorial phenomenon involving membrane destabilization, cell signaling modulation, and cell cycle arrest. It is noteworthy that this *in vitro* study is the first attempt that reports the synergistic interactions of citral+FLZ and linalool+FLZ and their potential uses to treat superficial infections in the oral/vaginal mucosa caused by FLZ-resistant *C. krusei* strains. Hence, the study findings are encouraging for designing clinical trials to evaluate the effectiveness of these combination therapies.

Conclusion

By combining a synthetic drug (FLZ) and citral/linalool, an increased efficacy was found, and the minimum effective dose was reduced. The study findings encouraged the growing treatment failures and antibiotic resistance in *Candida* and proposed one for *Candida* infections via a drug combination approach. Clinical evidence or *in vivo* studies are thus required to confirm these *in vitro* findings.

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Authors' contributions

MA, AS, AK, and HS researched and contributed to data collection and manuscript preparation. The first draft was prepared by MA and AS. All authors read the final version and confirmed it for publication.

Conflict of interests

There was not any conflict of interests to declare by the authors.

Ethical considerations

Ethical issues (including plagiarism, data fabrication, double publication, etc) have been completely observed by the authors. The Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, guidelines confirmed the ethical issues by 6/7/300511 Ethical Code. The protocol was fulfilled in accordance with the CLSI.

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