Original Article

Limonene synergistically augments fluconazole susceptibility in clinical *Candida* isolates from cleft lip and palate patients

ABSTRACT

Background: Cleft lip and palate (CLP) patients are prone to Candida infections (oral thrush) mainly due to poor oral hygiene, repetitive surgeries, and orthodontic procedures.

Aim: This study was undertaken to evaluate the antifungal efficacy of limonene against clinical Candida isolates from CLP patients.

Materials and Methods: The antifungal efficacy of limonene was studied alone and in combination with fluconazole (FLC) against six standards, twenty nine FLC sensitive, and three FLC resistant clinical strains using broth dilution, checkerboard microdilution, agar disk diffusion, growth curves, and spot assays.

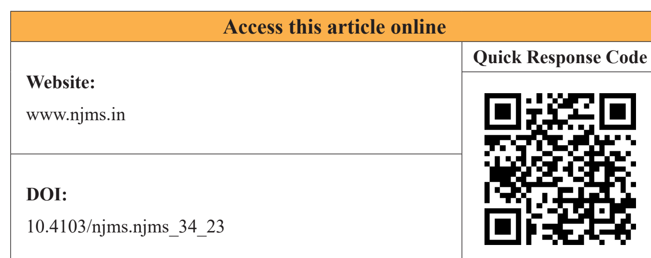
Results: This nontoxic monoterpene gave low minimum inhibitory concentration (MIC) values of 300–375 μg/mL and 500–520 μg/mL for FLC susceptible and FLC resistant strains, respectively. It showed synergistic interaction with FLC in all clinical and standard Candida strains (fractional inhibitory concentration (FIC) index 0.5).

Conclusion: Significant chemosensitization of FLC was observed even against resistant clinical isolates. Complete suppression of fungal growth was observed when using combinations. Negligible toxicity, easy availability, and potent antifungal properties suggest that limonene and FLC combinations in appropriate doses can make excellent antifungal mouthwashes during CLP treatment pre and post surgery. Impending in vivo studies are needed to validate the present data.

Keywords: *Candida*, cleft lip and palate, fluconazole, limonene, synergy

INTRODUCTION

Candida is an opportunistic human fungal pathogen that colonizes the oral cavity, without causing any notable damage in healthy individuals.[1] However, when patient immunity gets compromised, these organisms develop superficial mycoses (oral thrush), which may lead to serious systemic diseases in patients undergoing therapy, surgery, or any other physiological/anatomical alterations. Cleft lip and palate (CLP) is one of the most commonly found deformities of the head and neck.[2] Infants born with CLP can have communication between oral and nasal cavities, extending from the upper lip to the end of the soft palate of the oral cavity. This anatomical malformation can significantly alter the ecological environment of the oral microflora. The problem can be further exaggerated as the infants born with CLP have limited ability to suckle; adults, however, can have impaired swallowing ability. Furthermore, reduction in saliva flow and reduced pH levels seem to favor the adhesion of different microbes.[3,4] Achieving optimal oral health in



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individuals with CLP is challenging due to the anatomy of the cleft area, age of the patient, intraoral prosthetic devices, residual scar tissue, immobility of the lip, and misaligned teeth.[3,5] Extensive dental and orthodontic treatments frequently required in such patients influence the microbial load. The oral cavity, once sterile during fetal development, gets colonized by several microbes, with *Candida* species being among the first inhabitants. The predisposing factors that may alter the microbial colonization of the oral cavity include health status of oral mucosa, craniofacial anatomical alterations, systemic diseases, prolonged use of drugs such as corticosteroids and antibiotics, and smoking/drinking habits.[4-6]

Studies show that the colonization rate of oral *Candida* species is high in CLP patients.[7,8] Children with CLP require several hospital visits and multiple surgeries at different stages of life till adulthood. Poor health status and use of orthodontic appliances and oral prosthetics increase the susceptibility of CLP patients to *Candida*-related infections as a result of poor health status.[7,9] *C. albicans* is the most isolated species, but other non-albicans *Candida* species including *C. glabrata, C. tropicalis, C. parapsilosis,* and *C. krusei* also contribute significantly.[10,11] Recurrent infections and development of resistance toward conventional antifungal drugs such as diflucan or fluconazole (FLC) make treatment of such secondary infections challenging.

A first-generation triazole, FLC, is the most prescribed antifungal drug. Unfortunately, its prolonged usage, especially for the treatment of systemic infections, has resulted in the evolution of resistant *Candida* species. Besides being fungistatic, FLC displays several adverse side effects including hepatotoxicity in some patients.[11] Other drawbacks of azole therapy include high drug doses, recurring infections, and longer hospital stays. More efficacious therapeutic strategies are required to overcome the weaknesses of current therapies, mainly resistance and drug toxicity. Combination therapy with nontoxic natural compounds has shown promising results. Plant phytochemicals possess multiple biological applications including antimicrobial properties. Limonene, commonly found in citrus fruits, is a cyclic monoterpene that possesses various pharmacological properties, namely antimicrobial, antioxidant, insecticidal, and anticancer properties.[12] It has shown excellent potential in reducing *Candida* virulence traits both *in vitro* and *in vivo*.[13-15]

This study was undertaken to evaluate the antifungal efficacy of limonene, alone and in combination with FLC against both FLC-susceptible and FLC-resistant clinical *Candida* isolates from CLP patients. The study was performed using checkerboard microdilution, agar disk diffusion, growth curves, and spot assays to show the chemosensitizing potential of limonene, hence reducing the drug doses of the fungistatic and toxic FLC.

MATERIAL AND METHODS

Strains, media, and culture conditions

Thirty-eight *Candida* strains including six standard, twenty-nine FLC-sensitive, and three FLC-resistant clinical strains were studied here [Table 1]. The clinical strains were isolated from patients visiting the Department of Oral and Maxillofacial Surgery, Strains were identified and maintained in the Department of Biosciences, Medical Mycology Lab, The patient details were collected and recorded. Institutional biosafety clearance (Ref. No. PI/44-21.12.20) was taken before performing the study as per the Department of Biotechnology (DBT), Govt. of India guidelines. All the strains were identified based on colony color and morphology on HiCrome agar.[16] All the *Candida* cells were maintained on yeast extract–peptone–dextrose (YEPD) in the ratio of 1:2:2 along with 2.5% agar at 4°C. For experimental purposes, *Candida* cells were subcultured for 24 h at 37°C and inoculated into fresh YEPD media. Limonene, media components, and other chemicals were obtained from HiMedia (India). FLC and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Germany). All the chemicals were of analytical grade. Ethical clearance was obtained from Institutional Ethical Committee, with Ref no 1/10/293/JMI/IEC/2020 dated 27.10.2020.

Antifungal susceptibility assays

Minimum inhibitory concentration (MIC)

The MIC of limonene and FLC was determined using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[17] Stock solutions of limonene and FLC were prepared in DMSO ($<1\%$). MIC was defined as the lowest concentration of test compound that prevents visible growth causing 90% decrease in absorbance in comparison with that of the control.[18] The concentration of limonene was taken in the range of 50–1500 μg/mL, while that of FLC was in the range of 0.125–128 μg/mL. The cell suspension (1×10^3 cfu/mL) was serially diluted in 96-well flat-bottom microtitration plates, which were incubated for 48 h at 37°C. Absorbance was recorded at 595 nm for each well using a microplate reader (Bio-Rad, USA).[19]

Checkerboard microdilution assay

Drug interaction studies were performed in 96-well flat-bottomed microtitration plates according to CLSI guidelines.[17] The cell suspension (1×10^3 cfu/mL) was serially diluted with media, and final concentrations of

Table 1: *In vitro* susceptibility of FLC-sensitive and FLC-resistant *Candida* strains to limonene alone and in combination with fluconazole (FLC). The MIC and FICI values are shown as the mean of three independent experiments. Combination studies showed synergistic interaction against all *Candida* strains (FICI≤0.5)

Type of strains		MIC (μg/mL) Alone		MIC (μg/mL) In combination		FICI
		FLC	Limonene	FLC	Limonene	FLC + limonene
Standard strains	*C. albicans* ATCC 90028	10	300	2	75	0.45
	C. albicans ATCC 5314	10	300	2	75	0.45
	C. glabrata ATCC 90030	10	300	2	80	0.46
	C. tropicalis ATCC 750	10	320	2	70	0.41
	C. krusei ATCC 14243	12	350	2.5	80	0.43
	C. parapsilosis ATCC 22019	10	350	2.5	80	0.47
FLC-sensitive *Candida* isolates FLC-sensitive strains	*C. tropicalis* 1901	12	325	1.5	80	0.37
	C. albicans 1903	10	320	2	75	0.43
	C. albicans 1904	9.5	320	1	80	0.35
	C. glabrata 1904	10	300	1.5	90	0.45
	C. albicans 1905	10	325	2	85	0.46
	C. parapsilosis 1905	12	355	2.5	75	0.42
	C. dubliniensis 1905	12	350	2	75	0.38
	C. glabrata 1906	10	320	1	65	0.30
	C. dubliniensis 1907	12	350	1.5	80	0.35
	C. parapsilosis 1907	10	360	1	95	0.36
	C. albicans 1908	10	375	1.5	75	0.35
	C. dubliniensis 1908	11	325	2	70	0.38
	C. parapsilosis 1908	12	345	3	75	0.47
	C. albicans 1910	10	325	1.5	70	0.36
	C. parapsilosis 1910	11	320	2.5	65	0.43
	C. albicans 1911	12	330	3	75	0.48
	C. glabrata 1912	12	345	3	80	0.48
	C. albicans 1912	11	325	1.5	70	0.35
	C. albicans 1913	10	330	1	70	0.31
	C. parapsilosis 1913	12	320	2	65	0.37
	C. utilis 1913	13	365	2.5	90	0.44
	C. parapsilosis 1915	12	350	2	75	0.38
	C. utilis 1915	12.5	360	2	80	0.38
	C. dubliniensis 1919	10.5	335	1.5	75	0.37
	C. albicans 1919	10	325	2	60	0.38
	C. utilis 1919	12	355	2.5	80	0.43
	C. tropicalis 1921	12	320	2.5	65	0.41
	C. parapsilosis 1921	12	350	2.5	75	0.42
	C. utilis 1921	12	365	2.5	85	0.44
FLC -resistant	*C. krusei* 1902	90	500	20	125	0.47
	C. krusei 1904	100	520	22	130	0.47
	C. parapsilosis 1916	110	520	25	135	0.48

limonene and FLC were taken between 50–1500 μg/mL and 0.125–128 μg/mL, respectively.[19] The compounds were serially diluted (horizontally and vertically for each compound). The plates were incubated for 48 h at 37°C. Fractional inhibitory concentration index (FICI) values were calculated to study the interaction of drug combinations (limonene + FLC) based on the Loewe additivity zero-interaction theory.[20]

$$FICI = FIC_A + FIC_{B,} \text{ where } FIC_A = MIC_A \text{ in combination}/MIC_A \text{ alone}$$

$$FIC_B = MIC_B \text{ in combination}/MIC_B \text{ alone}$$

MIC_A and MIC_B are the MIC values of FLC and limonene, respectively. The FICI values were interpreted as follows: synergy when FICI ≤ 0.5; additive effect when $0.5 < \text{FICI} \leq 1$; indifferent effect when $1 < \text{FICI} < 2$; and antagonistic effect when FICI ≥ 2.[21]

Agar disk diffusion assay

Candida cells were grown overnight in YEPD media at 37°C. An inoculum size of 1×10^5 cells/mL was taken in molten

YEPD agar and poured into 90-mm petri plates. Sterile filter disks (4 mm) were placed on agar plates after loading with test compounds alone and in combination with their respective MIC values. For loading higher concentrations (>500 μg/mL), wells were made in the agar with the help of a sterile syringe. Plates were incubated at 37°C for 48 h, and the diameter of the zones of inhibition (ZOIs) was recorded in each case.[19]

Growth curves`

Candida cells were subcultured for 24 h at 37°C on YEPD agar plates. The inoculum size was adjusted to 1 × 10^6 cells (A_{595} = 0.1) in 50 ml fresh YEPD media along with the required concentrations of FLC and limonene. To determine antifungal efficacies in combination, both the test compounds (limonene + FLC) were added together at their respective MIC values. All the culture flasks were incubated at 37°C with constant agitation at 200 rpm. Growth was followed at 595 nm using Labomed Inc. spectrophotometer (USA) every 2 h for a period of 24 h.[12]

Spot assay

Overnight-grown *Candida* cells were suspended in 0.9% saline to achieve an absorbance of 0.1 at 595 nm.[12] FLC and limonene were added at their respective MIC values alone and in combination with molten YEPD agar in petri plates. After solidification, 5 μL of five times serially diluted *Candida* cells were spotted at equidistant points on agar plates containing the test compounds (FLC, limonene, and limonene + FLC) and incubated for 48 h at 37°C.

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation. Student's *t*-test was used to determine the significance of differences between treated and untreated samples. A statistical significance was accepted for $P < 0.05$.

RESULTS

MIC and FIC index of FLC and limonene alone and in combination

Table 1 shows the MIC values of FLC and limonene, alone and in combination for 32 clinical isolates (29 FLC-susceptible and three FLC-resistant strains). All the standard *Candida* strains (*C. albicans* ATCC 90028, *C. albicans* ATCC 5314, *C. glabrata* ATCC 90030, *C. tropicalis* ATCC 750, and *C. parapsilosis* ATCC 22019) gave an MIC value of 10 μg/mL for FLC, except *C. krusei* ATCC 14243, which gave an MIC of 12 μg/mL. For limonene, the MIC was 300 μg/mL in the case of both *C. albicans* ATCC strains and *C. glabrata* ATCC 90030. MIC was 320 μg/mL for *C. tropicalis* ATCC 750 and 350 μg/mL for both *C. krusei* ATCC 14243 and *C. parapsilosis* ATCC 22019.

The twenty-nine clinical *Candida* isolates gave MIC values in the range of 9.5–12.5 μg/mL for FLC, indicating their susceptibility to this conventional antifungal drug, while three clinical strains gave an MIC of 90–110 μg/mL showing that these strains were FLC-resistant. An MIC ≥ 64 μg/mL is the interpretive breakpoint for FLC resistance.[4,17] The MIC of limonene for clinical FLC-susceptible strains was in the range of 300–375 μg/mL, while that for FLC-resistant strains it was slightly higher at 500–520 μg/mL [Table 1]. The toxicity of this natural compound toward host cells is very low in comparison with FLC.[22]

The combined antifungal effect of limonene and FLC was studied to investigate the type of interaction based on the FICI values. Interestingly, besides the susceptible strains, the three FLC-resistant strains also showed significant synergy. The FICI values were between ≤ 0.5 and 4.0≥, which showed significant synergistic interaction between the tested natural compound and the conventional antifungal drug. The FICI values for limonene and FLC in combination were in the range of 0.34–0.48 against all tested FLC-sensitive strains, while the values for the three FLC-resistant isolates ranged between 0.47 and 0.48.

Agar disk diffusion alone and in combination

All FLC-susceptible strains (both standard and clinical) showed large ZOIs on agar disk diffusion. At their respective MIC values, limonene and FLC formed ZOIs with diameters in the range of 15.75–20.65 mm and 18.75–22.75 mm, respectively. At the same concentrations, when given in combination, they formed even larger ZOIs with diameters in the range of 23–26.7 mm. A significant synergistic increase in ZOI diameters was also observed in FLC-resistant *Candida* isolates on YEPD media. The ZOIs formed in the presence of FLC and limonene were ∼8.5 mm and ∼10.5 mm, respectively, while in combination, the diameters were in the range of 18–18.5 mm [Table 2, Figure 1].

Growth curves alone and in combination

The growth pattern of all susceptible *Candida* strains (both standard and clinical isolates) after treatment with limonene and FLC, alone and in combination, showed significant alteration in the growth pattern. In FLC-resistant clinical isolates, the drug and the natural antifungal showed significant suppression in growth-related activity. The untreated control cells showed a lag phase of 6 h followed by a log phase of ∼16 h and then a stationary phase [Figure 2]. As expected, the growth pattern of FLC-resistant *Candida* strains was similar to the control cells, with both FLC and limonene showing reduced efficacy against these three isolates. Interestingly, limonene at its MIC value was more inhibitory in comparison with FLC, and when given in combination, the synergistic inhibitory effect increased further.

Table 2: *In vitro* susceptibility of FLC-sensitive and FLC-resistant *Candida* strains to limonene alone and in combination with FLC measured in terms of diameter of zone of inhibition (ZOI). Each isolate was tested in duplicate. ZOI was measured and expressed as mean±SD

Strains		ZOI (mm) Alone		ZOI (mm) In combination
		FLC	Limonene	FLC+limonene
Standard strains	*C. albicans* ATCC 90028	20.5±0.70	18.25±0.35	24.75±0.35
	C. albicans ATCC 5314	21.25±0.35	19.25±0.35	25.75±0.35
	C. glabrata ATCC 90030	22.75±0.35	20.65±0.91	25.65±0.91
	C. tropicalis ATCC 750	21.5±0.70	18.5±0	24.5±0.70
	C. krusei ATCC 14243	22.25±0.35	18.25±0.35	24.5±0.70
	C. parapsilosis ATCC 22019	21.25±0.35	19.15±0.21	24.35±0.21
FLC-sensitive *Candida* isolates	*C. tropicalis* 1901	19.25±0.35	19.75±0.35	23±0.70
	C. albicans 1903	19.25±0.35	16.5±0.70	25.75±0.35
	C. albicans 1904	18.25±0.35	16.75±0.35	25.25±0.35
	C. glabrata 1904	19.25±0.35	17±0.70	26±0.70
	C. albicans 1905	20.65±0.91	18.9±0.56	26.05±0.63
	C. parapsilosis 1905	18.25±0.35	18.4±0.14	23.9±0.56
	C. dubliniensis 1905	20±0.70	17.25±0.35	25.5±0.70
	C. glabrata 1906	17.9±0.84	15.75±0.35	25.6±0.84
	C. dubliniensis 1907	19±0.70	17±0.70	25.85±0.91
	C. parapsilosis 1907	18.25±0.35	16.75±0.35	26±0.70
	C. albicans 1908	19.25±0.35	17±0.70	24.9±0.84
	C. dubliniensis 1908	20.65±0.91	18.9±0.56	25.25±0.35
	C. parapsilosis 1908	20.65±0.91	17.85±0.91	23.8±0.28
	C. albicans 1910	18.25±0.35	17.25±0.35	26.25±0.35
	C. parapsilosis 1910	20.25±1.06	15.75±0.35	24.5±0.70
	C. albicans 1911	17.9±0.84	17±0.70	24.5±0.70
	C. glabrata 1912	19±0.70	17±0.70	24.75±0.35
	C. albicans 1912	19.25±0.35	16.75±0.35	26.25±0.35
	C. albicans 1913	18.25±0.35	17±0.70	24.9±0.84
	C. parapsilosis 1913	19.9±0.56	18.9±0.56	25.25±0.35
	C. utilis 1913	17.9±0.84	18±0.70	25.5±0.70
	C. parapsilosis 1915	19±0.70	17.25±0.35	24.35±0.21
	C. utilis 1915	19.25±0.35	15.75±0.35	23±0.70
	C. dubliniensis 1919	18.25±0.35	17.25±0.35	24.3±0.98
	C. albicans 1919	21.25±0.35	15.75±0.35	24±0.70
	C. utilis 1919	17.9±0.84	15.75±0.35	25.5±0.70
	C. tropicalis 1921	19±0.70	16.75±0.35	24.5±0.70
	C. parapsilosis 1921	19.25±0.35	16.25±0.35	26.7±0.14
	C. utilis 1921	18.5±0.70	15.75±0.35	25.75±0.35
FLC -resistant	*C. krusei* 1902	8.5±0.707	10.25±0.35	18.25±0.35
	C. krusei 1904	8.25±0.35	10.5±0.70	18.5±0.70
	C. parapsilosis 1916	8.25±0.35	10.5±0.70	18±0.70

Spot assays alone and in combination

The synergistic antifungal susceptibility of FLC and limonene, alone and in combination, was further studied by performing spot assays. In the presence of test compounds at their respective MICs, no growth was observed in the last spotted dilution, while untreated control cells showed growth till the last spotted dilution. In FLC-resistant strains, growth was observed in the first and second diluted spots for both the test compounds. However, in combination, there was no visible growth in all the tested dilutions [Figure 3].

DISCUSSION

Increasing resistance toward available antifungal drugs and recurrent infections has become a major problem in the treatment of fungal infections worldwide. Due to high drug toxicity and reduced efficacy, monotherapy frequently fails. Safer and more effective antifungal therapeutic approaches are urgently needed. Plant-based phytochemical limonene has shown immense antifungal potential against *C. albicans*.[22] Previous studies have shown that treatment with this monoterpene leads

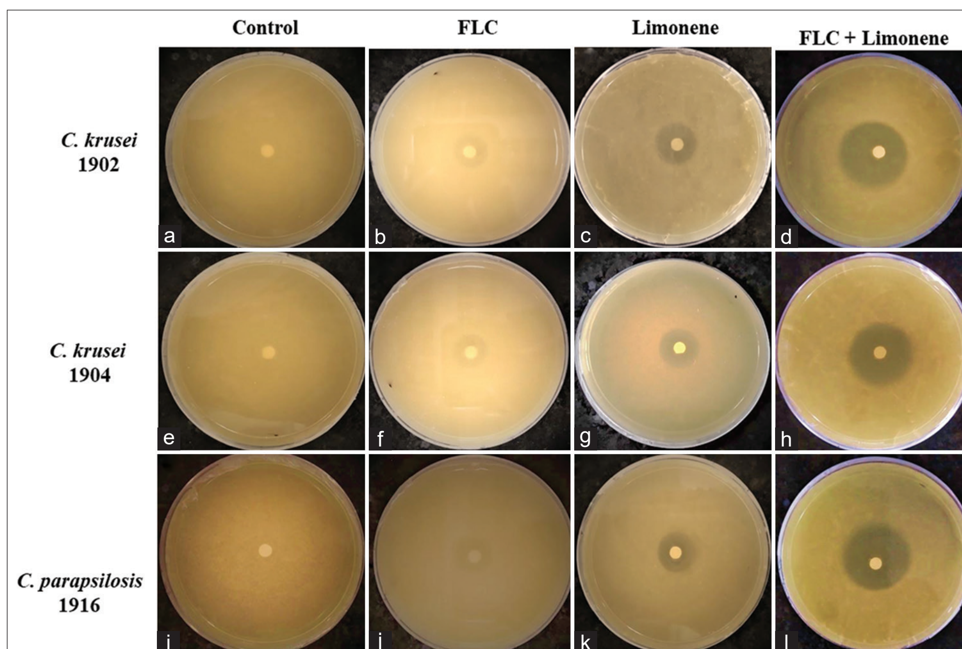

Figure 1: Representative pictures showing agar disk diffusion assay of FLC-resistant *Candida* strains in the presence of FLC (b, f, and j) and limonene (c, g, and k) at their respective MIC values. When FLC was given in combination with limonene (d, h, and l), a synergistic interaction was observed in the form of larger and clearer ZOIs in all tested strains. No ZOIs were observed in untreated control *Candida* cells (a, e, and i)

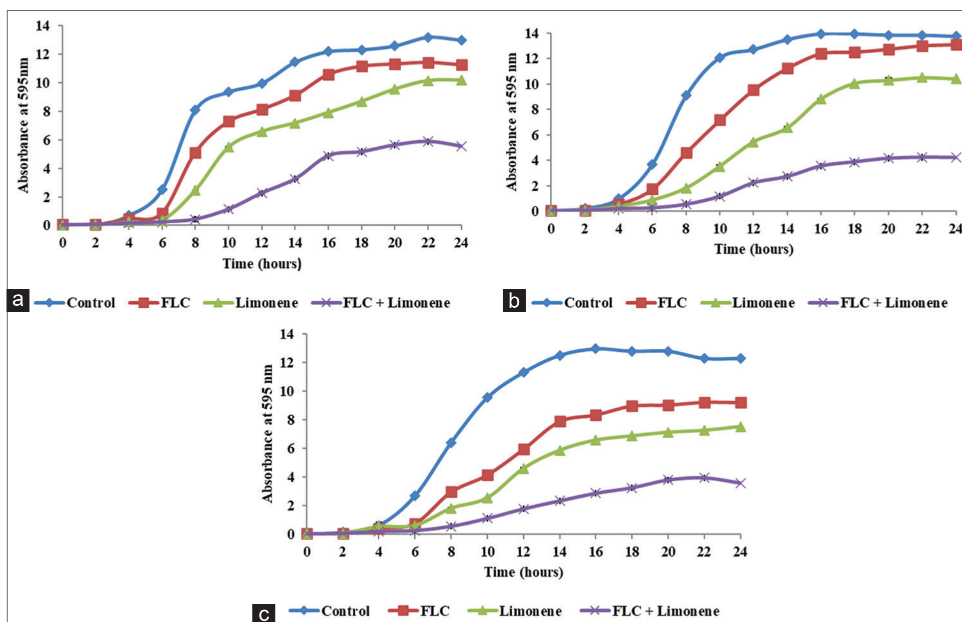

Figure 2: Growth pattern of FLC-resistant *C. krusei* 1902 (a), *C. krusei* 1904 (b), and *C. parapsilosis* 1916 (c) in the presence of FLC and limonene alone and in combination with their respective MIC values

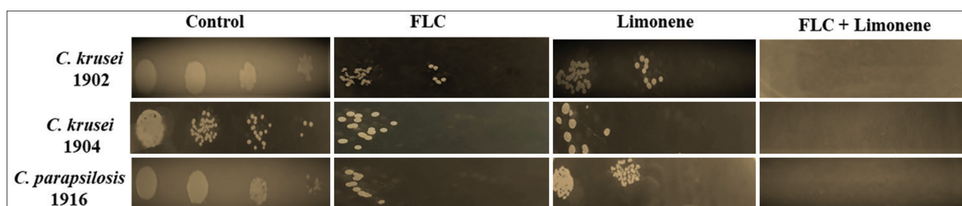

Figure 3: Representative spot assays of FLC-resistant *Candida* strains on YEPD agar in the presence of FLC and limonene alone and in combination with their respective MIC values. The initial inoculum was 10-fold diluted in a range of 1×10^2–1×10^4 cfu/mL. The control was strain growth in the absence of the test compounds

to the formation of defective biofilms and apoptotic cell death in *Candida*.[15] Limonene was also found effective against clinical isolates from patients with recurrent vulvovaginal Candidiasis. *In vivo* studies with mouse model have also shown that treatment with an ointment containing 10% limonene significantly reduces colonization in vulvovaginal Candidiasis.[23] This study was conducted to estimate the antifungal efficacy of limonene in combination with the fungistatic conventional FLC against clinical *Candida* strains isolated from CLP patients.

The rate of *Candida* colonization in CLP patients is high due to poor oral hygiene.[7] The deformity in these patients requires the fixation of orthodontic appliances as part of dental procedures and hence provides a surface for *Candida* colonization. Limonene showed significant synergy when used in combination with FLC against all tested strains including the resistant strains. Synergistic activity was shown by agar disk diffusion, growth pattern, and spot assays.

Natural antifungals, such as limonene, can be used for the treatment of systemic and invasive Candidiasis as they are nontoxic and much cheaper than fungistatic azoles, the first-line antifungal drugs. The fungicidal limonene[22] can kill both FLC-susceptible and FLC-resistant oral *Candida* isolates. Limonene has great potential to be used in therapeutic mouthwashes. Appropriate standardized formulations that contain both compounds can be included during the CLP treatment, before and after surgery, to avoid Candida-related complications. Further *in vivo* studies are required to authenticate the chemosensitizing effect of limonene on FLC and other conventional antifungal drugs.

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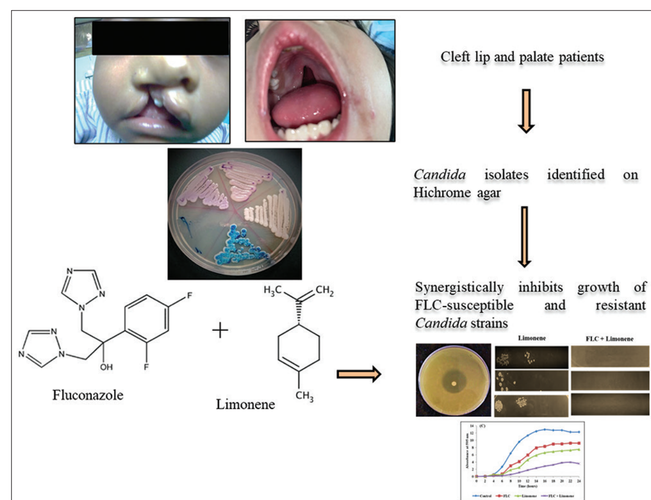
Graphical Abstract



Abbreviations

CLP, cleft lip and palate; FLC, fluconazole; FICI, fractional inhibitory concentration index; CLSI, Clinical and Laboratory Standards Institute; DMSO, dimethyl sulfoxide; MIC, minimum inhibitory concentration; cfu, colony-forming unit; ZOI, zone of inhibition; YEPD, yeast extract–peptone–dextrose

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Nil.

Conflicts of interest

There are no conflicts of interest.

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