Indian Journal of Pharmaceutical Education and Research., 2023; 57(1):113-119.
https://www.ijper.org

Original Article

Antifungal Activity of *Cymbopogon flexuosus* Essential Oil and its Effect on Biofilm Formed by *Candida parapsilosis* and *Candida tropicalis* on Polystyrene and Polyvinyl Plastic Surfaces

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ABSTRACT

Aim: To determine the antifungal and antibiofilm activity of *Cymbopogon flexuosus* (Lemongrass oil) against *C. parapsilosis* and *C. tropicalis.* **Materials and Methods:** Lemongrass oil, was analyzed by GC-MS. Antifungal activities, minimum inhibitory concentration, and minimum fungicidal concentration against *C. parapsilosis* and *C. tropicalis* were determined. The effect of LGO inhibiting biofilm formation by these *Candida* species on different surfaces was evaluated. Cytotoxicity of *Cymbopogon flexuosus* (Lemongrass oil) on human keratinocytes (HaCaT) cells was evaluated under *in-vitro* conditions. **Results:** The major compounds found in LGO were 4-tert-butylcalix 4 arene (58.52%), diethyl 3, 4-dihydro-1-naphthalenylester (8.13%), 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2, 8-dione (8.84%) and hexadecanoic acid (4.83%). The essential oil had a considerable antifungal activity against both the *Candida* species. Further, a sub MIC concentration (0.5%) in the medium inhibited the biofilm formation on polystyrene and polyvinyl plastic surfaces. The *in-vitro* cell viability of HaCaT keratinocytes in presence of LGO was studied and the IC_{50} value was observed at 1250 µg/ml concentration. **Conclusion:** The active components of LGO with antifungal and biofilm activity can be effectively used for controlling the biofilm formation by *C. parapsilosis* and *C. tropicalis* on polystyrene and polyvinyl plastic surfaces. Further investigations on the bioactive components present in LGO may be useful to control biofilm-related infection.

Keywords: Antifungal, Antibiofilm, *Candida tropicalis*, *Candida parapsilosis, Cymbopogon flexuosus*.

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Received: 23-03-2022;
Revised: 30-08-2022;
Accepted: 11-10-2022

INTRODUCTION

Pathogens are capable of transmitting through health workers, forming biofilms on medical devices that increase the nosocomial outbreaks and mortality rates.[1] Biofilm formation on temporary medical devices made up of polystyrene, polyvinyl plastic, and other implants is life-threatening, and finally ends up in device failure or medical issues. Therefore, different strategies have been developed to reduce infections and biofilms by preventing microbial adhesion, inhibiting the growth, interrupting the quorum sensing, and disrupting the early biofilm formation.[2] The usage of anti-adhesive materials and biomaterials that can inhibit biofilm formation is gaining importance. However, the availability of anti-adhesive biomaterials, their production cost, and the effective functioning of the medical implants or devices are challenging.[3] Additionally, the cell wall adhesins of *Candida* and the nature of medical implants facilitate biofilm formation by increasing the interaction between *Candida* cells and host cells. Yeast cells are osmotolerant, able to survive in high salt concentration that contributes to high virulence and resistance to antifungal drugs.[4] *Candida parapsilosis* and *C. tropicalis* causing candidemia[5] have been reported for resistance development against fluconazole in many countries.[6,7] *C. tropicalis* has been reported for resistance towards the available antifungal drugs such as azole derivatives, echinocandins, and amphotericin B.[8] To overcome these issues and to reduce resistance towards conventional antimicrobials, searching for novel and cost -effective natural products have been increased.[9] Plant products such as extracts, essential oils, and different phytochemical formulations have been used to treat a wide range of infections and to control microbial infections.[10] *Cymbopogon flexuosus*, (lemongrass) a tropical grass, with long leaves belonging to the Poaceae family, has been used for the production of essential oil. Lemongrass oil (LGO) has been reported for anti-inflammatory, analgesic, diuretic, antipyretic, antispasmodic, sedative

DOI: 10.5530/001954641705

properties, and is even used in aromatherapy.[11] Antimicrobial and antibiofilm activities of *Cymbopogon citratus* have been reported.[12] The presence of monoterpenes, citral compounds, neral (citral B), geranial, geraninol, geranyl acetate, and monoterpene olefins (myrcene) was reported earlier for their medicinal properties.[13] *Cymbopogon flexuosus* has been studied for the antibiofilm activity in *Candida albicans*.[14] To date, there are no reports available on *C. flexuosus* for anti-candidal activity against *C. parapsilosis* and *C. tropicalis* and biofilm formation. Therefore, the present study was to determine the antimicrobial and antibiofilm activity against *C. parapsilosis* and *C. tropicalis* that form biofilms in medical devices and implants. The cytotoxic effect of LGO on HaCatT keratinocytes cell line and the phytochemical components were also analyzed by GC-MS.

MATERIALS AND METHODS

Materials

The yeast cultures *C. parapsilosis* MTCC998 and *C. tropicalis* MTCC1000 (maintained at Aakaar Biotechnologies Pvt. Ltd., Lucknow, India) stored at -80°C, were inoculated into brain heart infusion broth (Himedia Laboratories, Mumbai, India), incubated for 24 hr at 36±1°C. These cultures were transferred to BHI agar media and single colonies were further inoculated to 10 ml BHI broth for inoculating into 200 ml broth. The flasks were incubated and used for harvesting the required number of cells. Lemongrass oil (*Cymbopogon flexuosus*) was purchased from the local market. As per the manufacturer's claim on the label, the essential oil was isolated by steam distillation process from leaves, and the product was tested for 100% purity and certified (Bloomingdale IL60108 USA).

GC-MS analysis of lemongrass oil

Gas chromatograph-mass spectrometric analysis of LGO was carried out using GCMS-QP2010 Ultra gas chromatograph (Shimadzu) following the method of Zhao *et al*.[15] with slight modification. Briefly, in a separating funnel, 100 µl of LGO was added with a mixture of water and ethyl acetate, 250 and 750 µl respectively. The upper layer was collected and concentrated. To this a mixture, N,O-Bis(trimethylsilyl) trifluoroacetamide and trimethylchlorosilane (50 µl) were added (prepared by mixing BSTFA-99 µl + TMCS-1 µl) and then 10 µl pyridine. The sample was maintained at 60°C for 30 min and transferred to GC vials. Before analysis, the preparation was dried using liquid nitrogen and dissolved in methanol. The following conditions were maintained for GC-MS analysis. Injection temperature 250°C; column temperature 120°C; total flow rate 16.3 ml/min and column flow rate at 1.21 ml/min with a linear velocity of 41.3 cm/sec and purge flow of 3.0 ml/min with an ion source temperature of 220°C and inlet pressure of 100 kPa. The mass spectral data were compared with NIST17 and Wiley8 libraries.

Antifungal activity

The fungal culture of *C. parapsilosis* MTCC998 and *C. tropicalis* MTCC1000 (100 µl; 1.5×10^8 CFU/ml) was inoculated by swabbing into Sabouraud dextrose agar. Different concentrations of LGO (10 – 50 µl/ml) diluted in dimethyl sulfoxide (DMSO) were loaded in filter paper discs placed on the surface of the agar. Amphotericin B (100 units) disc was placed as a positive control. The inhibitory zones were measured after incubation at 36±1°C for 24 hr.

Minimum inhibitory concentration

The lowest concentration of LGO inhibiting the growth of *Candida* species was determined by the broth dilution method.[16] The minimum concentration of LGO in which no visible growth was taken as MIC. A loop of culture from the MIC wells was inoculated into Sabouraud dextrose agar and incubated at 36±1°C for 24 hr. The minimum concentration at which no fungal growth in agar media was taken as the minimum fungicidal concentration. Wells without LGO was taken as positive control and wells without yeast cells were taken as a negative control.

Biofilm formation on polystyrene microtiter plates

Candida cells of two strains were prepared separately and the cell density was adjusted to $3x10^6$ CFU/ml in brain heart infusion broth. Different concentrations (0.03-0.5 µl/ml) of LGO were incorporated in 100 µl of broth medium in a microtiter plate, inoculated with the prepared cells, and incubated for 48 hr at 36±1°C. Biofilm formation was determined by the crystal violet binding assay method.[17] Briefly, filtered (0.44 µm filter) 0.1% crystal violet (20 µl) was added after incubation into each well and allowed to stain for 10 min. Planktonic cells were removed using a micropipette from the comer and the wells were rinsed with sterile distilled water three times successively. The wells were again rinsed with 10 mM potassium phosphate buffer and air-dried for 15 min. The dye in the wells was solubilized by adding 96% v/v ethanol (100 µl). After 15 min the contents were mixed well and the absorbance was measured at 560 nm using the Micro ELISA auto reader (Biotek, US).

Biofilm formation on polyvinyl plastic coverslips

Anti-biofilm activity of LGO against *C. parapsilosis* and *C. tropicalis* on polyvinyl plastic coverslips (Fisher Scientific, UK) were determined.[18] The Sterile polyvinyl coverslip was placed to attain a 90° angle in the culture tubes relative to the bottom of the test tubes and inoculated with culture. LGO was added to each tube at different concentrations (0.03-0.5 µl/ml). The tubes were incubated at 36±1°C for 48 hr. The coverslips were taken out and rinsed with 10mM potassium phosphate buffer, and the remaining dye was solubilized for 20 min with 96% v/v ethanol. The quantification of biofilm formed on the surface was calculated by measuring the absorbance at 560 nm. The tubes

without LGO were used as positive control and set as 100%. The decrease in biofilm formation at different concentrations of LGO was calculated relative to the positive control.

Cytotoxicity assay

The normal human epidermal keratinocyte HaCaT cells were obtained from the National Centre for Cell Sciences (NCCS), Pune, maintained at Aakaar biotechnologies Pvt. Ltd., Lucknow, India, were used in the present study. Cell viability studies on HaCatT keratinocytes cells were studied by using 3-(4, 5-dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT assay).[19] Approximately, 1000 cells were grown in each well of the microtitre plate in RPMI medium supplemented with 10% FBS and 1% antibiotic solution in a 96 well plate at 37°C for 24 hr with 5% CO_2. Different concentration of lemongrass oil was added along with the medium (20-2560 µg/ml) in different wells. After 24 hr of incubation MTT 25 µg/ml (final concentration) was added and incubated for 2 hr. The culture supernatant was removed and the cell layer matrix was dissolved in 100 µl DMSO (dimethyl sulfoxide) and the results were recorded by measuring the absorbance using an ELISA plate reader (iMark, Biorad, USA) at 540 nm.

Statistical analysis

All experiments were conducted in triplicate on separate occasions. The data obtained in the present study were summarised by percentage and presented in mean ± standard error.

RESULTS

Several bioactive compounds were reported in other species of lemongrass for biological activities. In the present study, the ability of *C. flexuosus* (LGO) to prevent biofilm formation on polystyrene and the polyvinyl plastic surface has been determined. The components present in LGO used in the study have been identified and tabulated (Table 1). The primary compound was 4-tert-butylcalix 4 arene (58.52%) and components such as diethyl 3, 4-dihydro-1-naphthalenyl ester (8.13%), and 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione (8.84%) were also detected in the *C. flexuosus* oil. Additionally, hexadecanoic acid was also detected in essential oil.

The antifungal activity of LGO against *C. parapsilosis* and *C. tropicalis* is shown in Figure 1. A concentration above 20 µl/ml showed an inhibition zone and LGO at 50 µl/ml inhibited the growth of *Candida* species tested. An increase in inhibitory activity was observed with the increase in the concentration of LGO. *C. tropicalis* showed resistance towards the tested antifungal drug Amphotericin B (100 U) when compared to *C. parapsilosis*. However, *C. tropicalis* was found more susceptible to LGO.

The minimum inhibitory concentration of LGO for *C. parapsilosis* was 2.5 µl/ml and the minimum fungicidal concentration was 5.0 µl/ml (Table 2). A low concentration of the essential oil was able to inhibit the multiplication of the fungal pathogen. On the other hand, *C. tropicalis* growth was inhibited by a minimum concentration of 1.25 µl/ml and a minimum fungicidal concentration was 2.5 µl/ml. The ability of lemongrass oil to inhibit biofilm formation in *C. parapsilosis* and *C. tropicalis* were

Table 1: Chemical composition of main constituents presents in *Cymbopogon flexuosus* oil (LGO).

Name	Percentage	Retention Time
Phytol	1.23	6.301
(E)-11 tertradecen-1-ol	1.29	6.515
3, 5-di-tert butylphenol	1.21	6.954
1,2-Dimethylpropylethyl ketone	0.55	7.807
Panaquinquecol 7	8.84	11.382
Hexadecanoic-d31 acid	4.83	12.071
(E,2R,3R)-2-aminoheptadec-4-ene-1,3-diol	4.55	12.772
9,12-Octadecadienoic acid	1.66	13.153
17-Octadecynoic acid, methyl ester	0.46	13.201
Diethyl-, 3,4-dihydro-1-naphthalenyl ester	8.13	13.253
1-ethynyl-4-octyl benzene	1.19	14.190
Ditridecyl phthalate	0.64	16.896
(Z)-N-ethyloctadec-9-enamide	1.22	18.939
Ioxynil octanoate	2.03	22.075
tris(2,4-ditert-butylphenyl)phosphite	3.63	23.826
4-tert-butylcalix	58.52	26.441

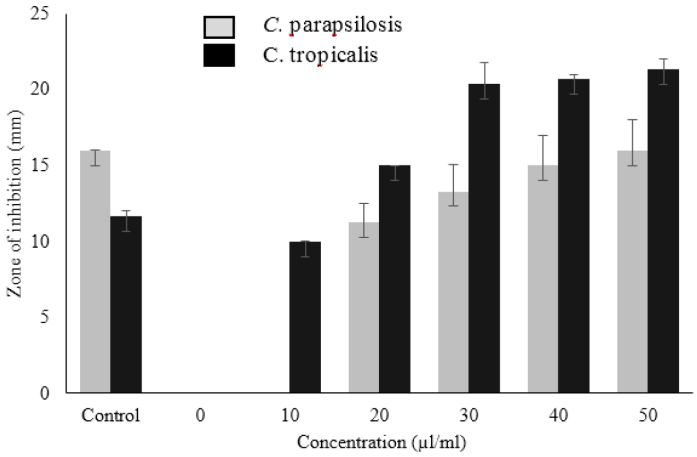

Figure 1: Antifungal activity of LGO against *C. parapsilosis* MTCC998 and *C. tropicalis* MTCC1000; Control-Amphotericin B (100 U).

Table 2: Minimum inhibitory concentration and minimum fungicidal concentration of lemongrass essential oil against *C. parapsilosis* and *C. tropicalis* (MIC and MFC in µL/ml median values, n=3).

Test organism	MIC	MFC	MIC	MFC
	LGO		Amphotericin B	
C. parapsilosis MTCC998	2.5	5.0	2	4
C. tropicalis MTCC1000	1.25	2.5	4	5

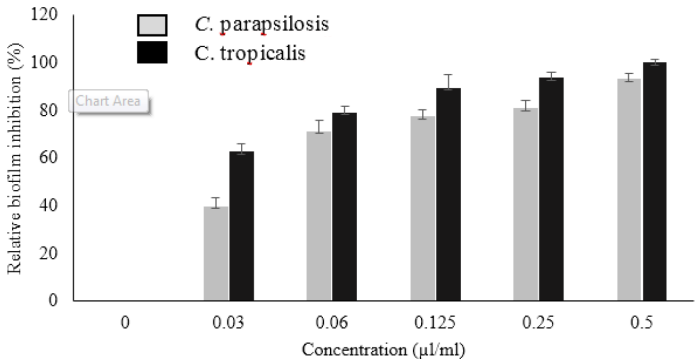

Figure 2: Antibiofilm activity of LGO against *C. parapsilosis* and *C. tropicalis* on polystyrene microtiter plate wells assayed by the spectrophotometric method using crystal violet. Biofilm inhibition was calculated relative to control wells without lemongrass oil. Error bars indicate standard deviations over triplicate experiments where *Candida* species are grown separately.

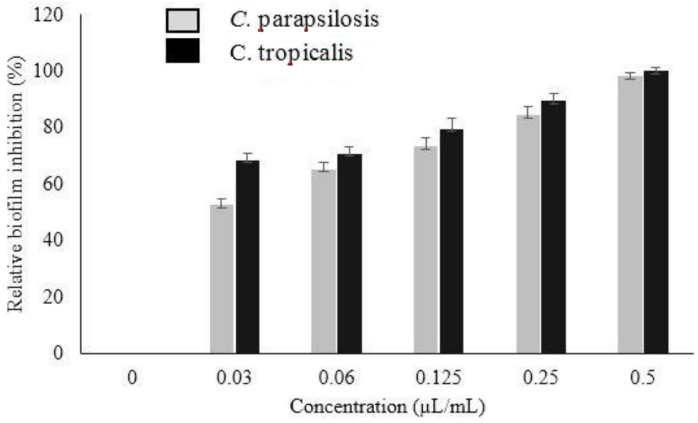

Figure 3: Anti-biofilm activity of LGO against *C. parapsilosis* and *C. tropicalis* on polyvinyl plastic coverslips assayed by the spectrophotometric method using crystal violet. Biofilm inhibition was calculated relative to control without lemongrass oil. Error bars indicate standard deviations over triplicate experiments in which *Candida* species were grown separately.

studied in polystyrene microtiter plate wells. LGO inhibited biofilm formation at an increased concentration (0.5 µl/ml) (Figure 2).

Concentration (0.03 µl/ml) of LGO reduced the biofilm formation by 39.72% in *C. parapsilosis* whereas, above 60% was inhibited in *C. tropicalis*. Further, an increase in the concentration of LGO reduced the adherence of cells in a polystyrene microtiter plate. The biofilm formation of *C. tropicalis* was completely inhibited at 0.5 µl/ml concentration whereas, only 93.2% inhibition was observed in *C. parapsilosis*. However, a higher concentration may inhibit biofilm formation completely in *C. parapsilosis*. Biofilm inhibition on polyvinyl plastic coverslips was also concentration-dependent (Figure 3). A higher concentration of LGO (0.5 µl/ml) inhibited biofilm formation completely in *C. tropicalis*, however, only 98.1% inhibition was observed in *C. parapsilosis*.

In vitro studies of cytotoxic effects on HaCatT keratinocytes cell line showed that low concentration retained cell viability

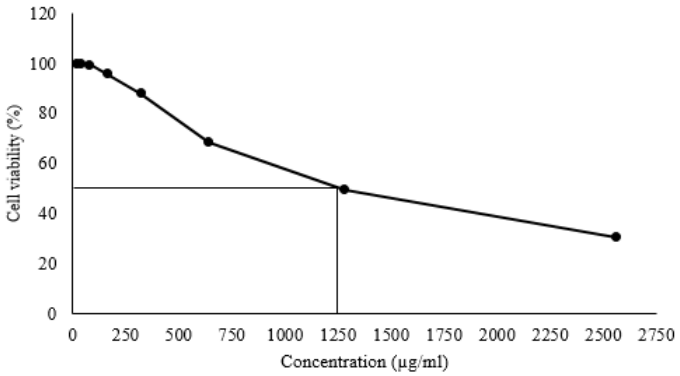

Figure 4: Cytotoxic effect of lemongrass oil on HaCatT keratinocytes cell lines calculated as the relative cell viability using MTT assay (IC_{50}=1250 µg/ml).

(Figure 4). The cell viability was maintained above 90% at 160 µg/ml of essential oil. Further, the IC_{50} value was found to be 1250 µg/ml of lemongrass oil. A higher above 1250 µg/ml concentration of lemongrass oil showed decreased cell viability.

DISCUSSION

Candida species are capable of forming biofilms on temporary medical devices and implants. Developing efficient methods to prevent biofilm formation on medical devices is under investigation. In phytochemical analysis, polyphenolic compounds such as 4-tert-butylcalix 4 arene (58.52%) were detected and that has been reported for antimicrobial activity.[20] The presence of a high number of polyphenols was detected in GCMS analysis in *C. flexuosus* oil. Polyphenols such as catechin, gallic acid, quercetin, isoquercetin, tannic acid, and rutin present in *C. citratus* were reported earlier.[21] These compounds exhibited antimicrobial activity against *C. albicans, C. tropicalis*, and *Aspergillus niger* and anti-biofilm activity.[22] Polyphenols denature the proteins, increase cell membrane permeability resulting in poor fungal growth and metabolism, and inhibit biofilm formation.[23] Additionally diethyl 3, 4-dihydro-1-naphthalenyl ester and 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione were also found in the *C. flexuosus* oil. Esters such as naphthyl acetate and terpenoids isolated from plant extracts were reported for antifungal activity.[24] The derivatives and branched peptides of boronic acid have been reported for antimicrobial activities on *Candida, Staphylococcus,* and *E. coli* and inhibiting biofilm formation in *C. albicans*.[25] Furthermore, 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione has been reported for biological activities especially, antiviral and antimicrobial activities.[26] Another compound present in the essential oil was hexadecanoic acid, which has been reported to inhibit the growth and biofilm formation in *C. tropicalis* by regulating virulence factors such as inhibiting various enzyme activity and cell surface hydrophobicity, and ergosterol biosynthesis.[27] These bioactive compounds from LGO showing anticandidal activity may be

useful in eradicating biofilm formation of *C. parapsilosis* and *C. tropicalis* in medical devices and implants.

The incidence of fungemia caused by *Candida* species is alarming. Fungal pathogens are becoming resistant to commonly used antifungal drugs. Most of the clinical isolates of *Candida* develop antifungal resistance through biofilm formation.[25] LGO has been reported earlier for antifungal activity against molds and yeasts.[12,22] Antimicrobial compounds from plant and natural sources may help in eradicating the resistance mechanisms of fungal pathogens. The inhibition of fungal growth and metabolism depends on the chemical components present in the oil and other cellular and genetic components of the cells. The chemical components of LGO were reported earlier for antifungal properties.[22] However, the results of the present study, MIC, and MFC of *Candida* species are much lower than antifungal agent amphotericin B tested as a control. Thus, lemongrass oil has less cytotoxicity to human cells and can inhibit biofilm formation in medical devices and implants.[12] *Candida* species are normally found on the skin and exist asymptomatically in healthy individuals. It becomes a major opportunistic pathogen in immunocompromised patients and leads to biofilm-related complications. Lemongrass oil showed inhibition of biofilm formation in *C. parapsilosis* and *C. tropicalis* at higher concentrations. It may be due to these Candida species' low adherence and viability in presence of essential oil.

Biofilm acts as a reservoir for pathogenic yeast to escape from the host immune mechanisms and also helps in developing resistance to antifungal drugs.[28] It was observed that the sub MIC level of LGO inhibited the biofilm formation in both the species of *Candida*. In this study, the components such as polyphenols, hexadecanoic acid might have inhibited the biofilm formation in both *Candida* species. In addition to the antimicrobial activity, the anti-biofilm properties of LGO used as a coating are applicable for making anti-infective materials used for medical applications. *C. parapsilosis* is frequently found in the skin of healthcare workers and causes catheter-related infections in immunocompromised patients. It can form a biofilm with a negligible number of yeast cells and with a minimum extracellular matrix.[29] Conventional therapeutic methods are insufficient to prevent biofilm formation and treat related infections. Recently, coating the surfaces with some antimicrobial additives or altering the materials of medical devices and prostheses are the major focus to control biofilm formation. Several bioactive components present in *C. citratus* LGO applied on biomaterials using vehicles have been reported for anti-biofilm activity on *C. tropicalis*.[12] The essential oils were reported to change the hydrophobicity in *Candida* species,[30] resulting in an antibiofilm effect. Further investigations on phytochemicals, these novel approaches may lead to an alternative biomedical approach to control biofilm formation. The aldehydes and esters present in LGO might have interfered in cellular activities, membrane synthesis, respiration, and germination that are required for growth and multiplication.[31] *C. parapsilosis* is considered as one of the major biofilm-forming yeast after *C. albicans* among the *Candida* sp. Several nosocomial outbreaks have been reported worldwide even in NICU centers due to failure in infection prevention, improper control programs, environmental disinfection, and hand hygiene.[1] *C. flexuosus* oil and its components showed a low cytotoxic effect on epithelial cells compared to other species.[32] Further disinfecting medical devices and improving hand hygiene of healthcare workers to eliminate the *C. parapsilosis* by novel antifungal agents derived from plant origin such as LGO may help in reducing nosocomial infection in immunocompromised patients.

The cell viability studies also showed that lemongrass (*C. flexuosus*) oil had less toxicity with IC_{50} value of 1250 µg/ml, less than the reported *C. citratus* oil. The citral component in *C. citratus* LGO showed moderate cytotoxicity (IC_{50} = 39.48 µg/ml) and the viability of human dermal fibroblast cells was decreased up to 5% viability at 0.25% v/v.[33] Toxicity studies of *C. citratus* on W138 cells showed moderate toxicity (IC_{50}=39.77 µg/ml).[34] The cytotoxic studies were carried out in HaCaT keratinocytes because skin offers a stable environment for different types of microorganisms such as bacteria and fungi resulting in the formation of biofilms. Further, skin infections are directly linked with biofilm formation due to the extracellular matrix protection for the microbial community against host defense and antimicrobial agents.[35] The constituents present in LGO were less toxic to human cells. Therefore, based on the present study, the *C. flexuosus* lemongrass oil showed less cytotoxic effects on the HaCatT keratinocytes cell line. Further investigations are needed on other types of cell lines before using as an anti-biofilm agent.

CONCLUSION

Phytochemicals present in *C. flexuosus* essential oil showed antifungal activity against *C. parapsilosis* and *C. tropicalis* also interfered in biofilm formation in both polystyrene and polyvinyl plastic surfaces. The inhibition of biofilm formation may be due to the reduction in adherence ability and inhibiting quorum sensing molecules used for cell to cell communication. Deleterious effects of *C. flexuosus* essential oil on medical devices and prostheses, such as changing physicochemical properties need to be studied in detail. Modifying the surface of the materials using such phytochemicals in biomaterials may reduce the risk of biofilm formation and associated infections. Further studies on bioactive components and phytochemicals present in *C. flexuosus* LGO helps in eliminating mature biofilms from the surfaces by inhibiting biofilm formation and thereby controlling nosocomial infections.

ACKNOWLEDGEMENT

The author would like to thank the Deanship of Scientific Research at Shaqra University for supporting this work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

BSTFA: N,O-Bis(trimethylsilyl) trifluoroacetamide; **DMSO:** Dimethyl sulfoxide; **ELISA:** Enzyme-linked immunoassay; **GC-MS:** Gas chromatograph-mass spectrometry; **HaCaT:** Human keratinocytes cell lines; **IC_{50}:** Half-maximal inhibitory concentration; **LGO:** lemongrass oil; **MIC:** Minimum inhibitory concentration; **MTCC:** Microbial type culture collection; **MTT:** 3-(4, 5-dimethylthiazol-2,5-diphenyltetrazolium bromide; **TMCS:** trimethylchlorosilane; **CFU:** Colony forming units; **RPMI:** Roswell Park Memorial Institute.

SUMMARY

Antifungal and antibiofilm activity of *Cymbopogon flexuosus* (Lemongrass oil) against *C. parapsilosis* and *C. tropicalis* were studied. The major components present in lemongrass oil were determined by GC-MS analysis. The present study shows that *Cymbopogon flexuosus* oil has antifungal activity and has the ability to prevent biofim formation on polystyrene and polyvinyl plastic surfaces. Further, an *in-vitro* investigation shows that a low concentration of lemongrass oil has no cytotoxic effect on HaCat cells.

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Cite this article: Al-Ghanayem AA. Antifungal Activity of *Cymbopogon flexuosus* Essential Oil and its Effect on Biofilm Formed by Candida *parapsilosis* and *Candida tropicalis* on Polystyrene and Polyvinyl Plastic Surfaces. Ind. J. Pharm. Edu. Res. 2023;57(1):113-9.