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Antifungal and biofilm inhibitory effect of *Cymbopogon citratus* (lemongrass) essential oil on biofilm forming by *Candida tropicalis* isolates; an *in vitro* study

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ABSTRACT

Ethnopharmacological relevance: *Cymbopogon citratus* (lemongrass) essential oil has been widely used as a traditional medicine and is well known for antimicrobial properties. Therefore, it might be a potent anti-infective and biofilm inhibitive against *Candida tropicalis* infections. Until now, no ideal coating or cleaning method based on an essential oil has been described to prevent biofilm formation of *Candida* strains on silicone rubber maxillofacial prostheses, voice prostheses and medical devices susceptible to *C. tropicalis* infections.
Aim of the study: To investigate the antifungal and biofilm inhibitory effects of *Cymbopogon citratus* oil. Clinical isolates of *C. tropicalis* biofilms on different biomaterials were used to study the inhibitory effect.
Materials and methods: The efficacy of *Cymbopogon citratus, Cuminum cyminum, Citrus limon* and *Cinnamomum verum* essential oils were compared on biofilm formation of three *C. tropicalis* isolates on 24 well polystyrene plates. *C. citratus* oil coated silicone rubber surfaces were prepared using hypromellose ointment as a vehicle. The antifungal tests to determine minimum inhibitory and minimum fungicidal concentrations were assessed by a microbroth dilution method and biofilm formation was determined by a crystal violet binding assay.
Results: *C. tropicalis* strains formed more biofilm on hydrophobic materials than on hydrophilic glass. *C. citratus* oil showed a high antifungal effect against all *C. tropicalis* strains. For comparison, *C. limon* oil and *C. cyminum* oil showed minor to no killing effect against the *C. tropicalis* strains. *C. citratus* oil had the lowest minimal inhibitory concentration of all essential oils tested and inhibited biofilm formation of all *C. tropicalis* strains. *C. citratus* oil coating on silicone rubber resulted in a 45–76% reduction in biofilm formation of all *C. tropicalis* strains.
Conclusion: *Cymbopogon citratus* oil has good potential to be used as an antifungal and antibiofilm agent on silicone rubber prostheses and medical devices on which *C. tropicalis* biofilms pose a serious risk for skin infections and may cause a shorter lifespan of the prosthesis.

1. Introduction

Candida species are commensal yeasts to humans but also recognized as opportunistic pathogens. In case of damage to the skin or the mucosa or if the immune system is compromised, *Candida* species may elicit both local and systemic infections (Silva et al., 2009). Over the last decades the occurrence of *Candida* infections is growing (Sanguinetti et al., 2015; Silva et al., 2009). This is mainly due to an increasing use of medical implants and devices, the growing number of immune compromised patients (e.g. diabetes, renal failure, lung disease, AIDS and organ transplants) and patients treated with immunosuppressive therapy in cancer (Araújo et al., 2017; Ramage et al.,

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2006; Sardi et al., 2013; Silva et al., 2009). Besides these infections, *Candida* can also cause damage to medical implants and devices such as the deterioration of silicone rubber voice and maxillofacial prostheses (Ariani et al., 2013; Neu et al., 1993; Visser et al., 2008). For maxillofacial silicone rubber prostheses (Ariani et al., 2013; Visser et al., 2008), it has been reported that their lifespan is relatively short (on average 1.5 years) and for voice prostheses (Neu et al., 1993) even shorter (on average 3–6 months), mostly due to early deterioration of the silicone rubber caused by the ingrowth of *Candida* strains. Recently, dominance of non-albicans strains, such as *C. tropicalis*, on malfunctioning voice prosthesis has been described (Somogyi-Ganss et al., 2017). A major characteristic of *C. tropicalis* infections is the formation of surface attached microbial communities, which are known as biofilms (Ramage et al., 2006). Biofilm formation on the surface of medical devices and implants such as catheters, voice prostheses, artificial heart valves, maxillofacial prostheses, pacemakers, prosthetic joints and contact lenses, poses serious risks for functional, medical and esthetic problems (Chen et al., 2013; Swartjes et al., 2015). They result in a substantial increase of healthcare costs generated by prolonged hospital stays, revision of surgeries, frequent remakes of prostheses and clinical visits (Salwiczek et al., 2014; Soto, 2014). Therefore antibiofilm strategies, aiming to reduce the rate of infections and the degradation of prostheses are very important and generally address: (1) prevention of microbial adhesion, (2) killing of microorganisms on the biomaterial, (3) inhibition of quorum sensing and (4) the disruption of early biofilm formation (Bruellhoff et al., 2010). In recent years, there is a growing focus on the development of anti-adhesive and of biofilm-inhibiting biomaterial surfaces in order to prevent biomaterial associated infections (Ariani et al., 2015; Campoccia et al., 2013).

Various strategies to prevent biofilm formation on biomaterials have been described in literature. However, until now no cheap, effective and easily available solution that can be used by a patient for daily use, is available. The increasing resistance of microorganisms to antimicrobial drugs and the inadequacy to eradicate microorganisms in the biofilm mode of growth have boosted research on natural antimicrobial products (Saharkhiz et al., 2012). Essential oils, originating from a variety of aromatic plants, have been reported to display antimicrobial properties against several pathogenic microorganisms (Adukwu et al., 2012; Bazargani and Rohloff, 2016). Essential oils such as *Cymbopogon citratus*, *Cuminum cyminum, Citrus limon* and *Cinnamomum verum* have been traditionally used in different parts of the world to treat various microbial diseases (Chaturvedi et al., 2016; Khan and Ahmad, 2012; Rana et al., 2018; Mtambo et al., 1999; Prabuseenivasan et al., 2006; Rattanachaikunsopon and Phumkhachorn, 2010). *Cymbopogon citratus* is a tropical grass with thin, long leaves. It belongs to the Poaceae family. It is known as one of the most important medicinal and aromatic plants cultivated mostly for its essential oil in tropical and subtropical regions of Asia, South America and Africa. Various chemical compounds in *C. citratus* essential oil have been reported to possess antibacterial, antifungal and analgesic properties (Boukhatem et al., 2014). *C. citratus* oil is traditionally used to reduce inflammation associated with rheumatism, cold and flu, and bacterial and fungal infections of the throat, urinary and vaginal tract (Boukhatem et al., 2014; Khan and Ahmad, 2012).

Cinnamomum verum is an evergreen tree from tropical Asia and Africa and belongs to the Lauraceae family. The essential oil extracted from it has been reported to have antibacterial, antifungal, antiviral, antidiabetic and antioxidative properties. Additionally, *C. verum* oil has been used in the treatment of gonorrhea, typhoid fever and other microbial diseases (Rattanachaikunsopon and Phumkhachorn, 2010). *Citrus limon* is known as one of the important medicinal plants from tropical and subtropical Southeast Asia and it belongs to the Rutaceae family. Different parts of it (stem, root, leaves, and flower) have been reported to have antibacterial activities against clinically significant bacterial strains (Chaturvedi et al., 2016). Apart from these, *C. limon* is also known as anti-inflammatory, antiallergic, antiviral, anticarcinogenic and stimulant (Campêlo et al., 2011) and has been traditionally used to treat people suffering from throat and urinary tract infections (Chaturvedi et al., 2016). *C. cyminum* is known as a small, slender and an annual plant cultivated in Arabia, India, China and in the countries bordering the Mediterranean Sea. It belongs to the Apiaceae family and the essential oils of cumin seeds are reported to have important antibacterial activities (Hajlaoui et al., 2010; Rana et al., 2018). Additionally, it has been traditionally used to treat skin disorders, bronchitis, toothache, diarrhea, dyspepsia and epilepsy diseases (Hajlaoui et al., 2010; Rana et al., 2018). Considering all these antedecents, the aim of the present work was to investigate the antimicrobial and biofilm inhibitory effect of *Cymbopogon citratus*, *Cuminum cyminum*, *Citrus limon* and *Cinnamomum verum* oils on three *C. tropicalis* strains on different biomaterials. In addition, silicone rubber coated with a hydrophilic ointment containing *C. citratus* oil was investigated on inhibition of biofilm formation, in order to make the inhibitive effect of *C. citratus* oil against *C. tropicalis* more clinically relevant as silicone rubber is the main material of maxillofacial and voice prostheses.

2. Materials and methods

2.1. Essential oils

C. citratus oil (from *C. citratus* (Nees ex Steud.) J.F. Watson (Poaceae)) (lemongrass oil) was purchased from Yantra, Groningen, The Netherlands. Other essential oils, included for comparison, were *C. verum* oil (from *C. verum* J. Presl (Lauraceae)) (cinnamon oil), *C. cyminum* oil (from *C. cyminum* L. (Apiaceae)) (cumin oil) and *C. limon* oil (from *Citrus limon* (L.) Osbeck (Rutaceae)) (lemon oil), purchased from Lokmanhekim, Ankara, Turkey.

2.2. Materials used for prostheses and medical devices

Four different materials used for prostheses and medical devices were tested for sensitivity towards biofilm formation. Three of them are commonly used for prostheses and medical devices: polyethylene, polytetrafluoroethylene and silicone rubber. Glass was added because of its highly hydrophilic nature compared to the other three materials. Polyethylene and polytetrafluoroethylene were obtained commercially as sheets with a thickness of 0.2 mm and 0.5 mm, respectively. Silicone rubber (M511 Maxillofacial Silicone System, Technovent Ltd., South Wales, UK) for facial prostheses was used to prepare 60x60x1.5 mm sheets as described previously (Ariani et al., 2015). All polymers were cut into pieces of 10x10 mm. In addition, 10x10 mm glass slides with a thickness of 1 mm were used. The materials were sterilized with 70% ethanol and dried under sterile conditions in a laminar airflow hood.

2.3. Contact angle measurements

All materials were cleaned by rinsing with 2% Extran MA02 in water, followed by tap water, 70% ethanol and sterile demineralized water, and air-dried prior to contact angle measurements. Water contact angles were determined at 25 °C by placing a droplet of 1–2 μL ultra-pure water on the materials. The contact angles of the sessile droplets at the intersection of the droplets and smooth materials were determined by a home-made contour monitor (Millsap et al., 1999). Water contact angle measurements were performed in triplicate.

2.4. Microorganisms, growth conditions and harvesting

Three different *Candida* strains were used including *C. tropicalis* T26, *C. tropicalis* U71 and *C. tropicalis* V89. All strains were clinical isolates and previously characterized as highly biofilm forming (Sahal and Bilkay, 2018). Identification was done using the 18S Ribosomal RNA Gene Sequence Analysis (Ref Gen Biotechnology Co. Ltd., 2017; Ankara, Turkey).

All *Candida* strains were first grown on Brain Heart Infusion (BHI) agar (Lab M Ltd, Lancashire, UK) for 24 h at 37 °C. For pre-culture preparations, single colonies were inoculated into 10 mL BHI broth (Lab M Ltd, Lancashire, UK) and incubated overnight at 37 °C. This pre-culture was used to inoculate a second culture of 200 mL BHI broth, which was subsequently grown for 24 h at 37 °C. Cells were harvested by centrifugation at 3220*g* for 10 min at 5 °C (Eppendorf 5810R, with an Eppendorf Swing-bucket rotor A-4-62, Hamburg, Germany) and washed 3 times with 10 mM potassium phosphate buffer (pH 7). Cells were counted in a thoma counting chamber and diluted appropriately to obtain the cell concentrations required for the experiments.

2.5. Biofilm formation of candida strains on the different materials

Candida cell density of each strain was adjusted to 3×10^6 cells/mL in BHI broth. Of these suspensions 2 mL were added to each of the four materials in 24 well plates and they were incubated for 48 h at 37 °C. After 48 h, biofilm formation was determined using a crystal violet binding assay (O'Toole, 2011). Briefly, the materials with the biofilms were gently washed three times with 10 mM potassium phosphate buffer (pH 7) and stained with a 1% (w/v) solution of crystal violet (Merck) in sterile distilled water for 20 min at 25 °C. Subsequently, the materials were gently rinsed with 10 mM potassium phosphate buffer (pH 7) and crystal violet bound to the biofilm was dissolved in ethanol (96% v/v) for 20 min. Quantification of the biofilm was done by measuring the absorbance at 560 nm using a spectrophotometer (UV–Visible Spectrophotometer; Shimadzu UV-1700, Kyoto, Japan). Note, crystal violet binds not only to viable and dead cells but also to extracellular matrix of the biofilms (Kwasny and Opperman, 2010).

2.6. Minimum inhibitory concentrations (MICs) and minimum fungicidal concentrations (MFCs) of the essential oils

The minimum inhibitory concentrations (MICs) (the lowest concentration that inhibits microbial growth, the fungistatic activity) of lemongrass oil and the other three essential oils were determined using the broth microdilution method (Balouiri et al., 2016). Briefly, serial two-fold dilutions of each essential oil in BHI broth were prepared in 96-well microtiter plates covering the range from 0.49 µL/mL to 500 µL/mL. In 96-well microtiter plates, 10 µL of a *Candida* strain (adjusted to $1x10^7$ cells/mL in 10 mM potassium phosphate buffer, pH 7) were inoculated in 100 µL of BHI broth containing essential oil. The MICs were determined after incubation for 48 h at 37 °C without shaking. The lowest concentration of essential oil at which no growth was visually observed, was defined as the MIC. The wells that included essential oils without any *C. tropicalis* strain were used as negative controls and the wells that included *C. tropicalis* strains without essential oil were used as positive controls.

To determine the minimum fungicidal concentration (MFC) (the lowest concentration that kills all the microorganism), 10 µL were taken from each well after the 48 h incubation period, inoculated onto BHI agar and incubated for another 48 h at 37 °C (Taweechaisupapong et al., 2012). The lowest concentration of essential oils at which no growth was observed on BHI agar was defined as the MFC. MICs and MFCs were determined in triplicate with separately grown *Candida* cells and are reported as median values.

2.7. Application of essential oils on biofilm formation of C. tropicalis strains

Of each *C. tropicalis* strain, 50 µL ($1x10^7$ cells/mL in 10 mM potassium phosphate buffer, pH 7) were inoculated into a well of 24-well polystyrene plate and mixed with 500 µL of sub-MICs of essential oils in BHI broth. The plates were incubated for 48 h at 37 °C. Subsequently, biofilm formation of the *C. tropicalis* strains in each well was determined by a crystal violet binding assay as described before (O'Toole, 2011). Biofilm formation on the well without addition of any microorganism was used as a negative control. Biofilm formation in wells without any essential oil, were used as a positive control and set as 100%. Decrease (%) in biofilm formations relative to the positive control, was calculated using the following equation:

$$\% \text{ Decrease} = [(\text{Absorbance Control (560 nm)} - \text{Absorbance Treatment (560 nm)}) / \text{Absorbance Control (560 nm)}] \times 100\% \quad (1)$$

2.8. Biofilm formation of C. tropicalis strains on silicone rubber coated with C. citratus oil

Silicone rubber was coated with *C. citratus* oil in a vehicle containing *C. citratus* oil in the concentration of 0, 2, 4 or 8% (w/w). The vehicle was hypromellose ointment ((20 g hypromellose (hydroxypropylmethyl cellulose), 400 mPa s in 80 g white soft paraffin, according to the Dutch formulary *Formularium der Nederlandse Apothekers* (FNA) (Hypromellosezalf 20%, 2013) and obtained from Fagron, Capelle aan den IJssel, the Netherlands)). Hypromellose ointment is a hydrophilic ointment possessing good adhesive properties to wetted surfaces. The vehicle (sterile) was mixed with the *C. citratus* oil under aseptic conditions to obtain the desired concentrations using a mortar and a pestle. Sterilized silicone rubber pieces of 10x10 mm were coated with hypromellose ointment containing one of the concentrations of *C. citratus* oil.

To prepare a *C. citratus* oil coated silicone rubber surface, a layer of hypromellose ointment containing the oil was placed in a sterile Petri dish and a piece of sterile silicone rubber sheet was placed on top of that. Following this, gentle pressure was applied onto the silicone rubber sheet to let the ointment stick onto the material. Subsequently, the coated silicone rubber sheet was gently taken out of the Petri dish and placed inside sterile 6 well-plates for the biofilm formation experiments. The coating thickness was estimated by Optical Coherence Tomography (OCT) (Ganymade, Thorlabs INC., Munich, Germany) and was found to be approximately 0.3 mm.

Candida cells were adjusted to a density of 3×10^6 cells/mL in BHI broth and 2 mL of these microbial suspensions were added to the coated (0, 2, 4 and 8% *C. citratus* oil) silicone rubber pieces for 7 days at 37 °C in 24-well plates under aerobic conditions. Then, the biofilm formation was determined by crystal violet staining (O'Toole, 2011) as described before. Biofilm formation on the silicone rubber surface without any coating (neither *C. citratus* oil-hypromellose ointment coating nor hypromellose ointment coating (vehicle alone)) was used as a positive control and set as 100%. Decrease (%) in biofilm formation relative to the positive control, was calculated using equation (1). Biofilm formation on the silicone rubber surface without any microorganism was used as a negative control.

2.9. GC-MS analysis of C. citratus oil

Among all four essential oils, *C. citratus* oil was used in further steps of this study. Therefore, the composition of it was determined by GC-MS analysis on a Shimadzu GCMS-QP2010 instrument (Shimadzu Benelux's-Hertogenbosch, the Netherlands). Column: InertCap 5MS/NP Proguard 2M, length 30 m, thickness 0.25 µm, inside diameter 0.25 mm (GL Sciences, Eindhoven, the Netherlands); injection temperature 250 °C; column oven temperature programme 50–280 °C with a ramp rate of 5 °C per minute and a hold time of 10 min at 280 °C; carrier gas He, flow 1 mL/min; mass spectrometry ion source electron ionization (EI), ion source temperature 160 °C, inlet pressure 100 kPa. Mass spectral data were compared with the NIST17 library (supplied by Shimadzu).

2.10. Statistical analysis

The Anderson-Darling test was applied to determine if biofilm

Table 1
Water contact angles of four different materials.

Material	Contact angle (degrees) (mean ± sd; n = 3)
Glass	22 ± 5
Polyethylene	71 ± 5
Polytetrafluoroethylene	96 ± 10
Silicone rubber	102 ± 5

formation and biofilm inhibition data were normally distributed. Following this, Levene's test was applied to understand homogeneity of variances. In case the results were found to be normally distributed, two factor experimental design was applied and pairwise comparisons were done by the Tukey test. In case the results were not found as normally distributed, non-parametric tests (Kruskal-Wallis and Mann-Whitney U tests) were used for evaluation of our results. Statistical analysis was performed using MINITAB 18 software.

3. Results

In Table 1, the equilibrium contact angles of ultra-pure water determined with the sessile drop on the three different biomaterials and on glass are listed. Glass is the most hydrophilic material, while polytetrafluoroethylene and silicone rubber are the most hydrophobic.

The biofilm formation of the *C. tropicalis* strains on different biomaterials were found to be normally distributed ($p = 0.508$) and homogeneity of variance assumption was met. ($p = 0.316$). Therefore, two factor experimental design was applied revealing that biofilm formation on different biomaterials statistically differ from each other ($p = 0.035$). In general, the *C. tropicalis* strains formed more biofilm on the hydrophobic materials (polyethylene, polytetrafluoroethylene and silicone rubber) than on hydrophilic glass (Fig. 1) and biofilm formation of all *C. tropicalis* strains was significantly more on polytetrafluoroethylene compared to glass (Tukey test; $p = 0.030$).

Minimum Inhibitory Concentrations (MICs) and Minimum Fungicidal Concentrations (MFCs) of the four essential oils against the *C. tropicalis* strains tested are listed in Table 2. The MICs and MFCs of *C. verum* and *C. citratus* oils were significantly lower than the MICs and MFCs of *C. limon* and *C. cyminum* oils for all *C. tropicalis* strains, meaning that they possess higher antifungal activity (Table 2).

C. cyminum oil was unable to inhibit growth of any *C. tropicalis* strain at the highest concentration tested (500 µL/mL), and *C. limon* oil displayed a very low antifungal activity.

The biofilm inhibitory effects of the four essential oils at sub-MIC values are given in Fig. 2.

In general, biofilm formation of all *C. tropicalis* strains were inhibited by almost all essential oils tested (see also Fig. 3).

According to the Anderson–Darling test, the biofilm inhibition

Table 2
The minimum inhibitory concentrations (MICs; µL/mL; median values, n = 3) and the minimum fungicidal concentrations (MFCs; µL/mL; median values, n = 3) of four essential oils against the *Candida* strains.

Essential oils (µL/mL)	*C. tropicalis* T26		*C. tropicalis* U71		*C. tropicalis* V89	
	MICs	MFCs	MICs	MFCs	MICs	MFCs
C. citratus	2.0	3.9	1.0	1.0	3.9	3.9
C. cyminum	> 500	> 500	> 500	> 500	> 500	> 500
C. limon	500	500	500	500	500	500
C. verum	7.8	7.8	15.6	15.6	3.9	3.9

results were found not to be normally distributed ($p = 0.001$). Therefore, the Kruskal-Wallis and Mann-Whitney U tests were applied to statistically evaluate the effect of sub-MICs of the essential oils against biofilm formation of the different *C. tropicalis* strains. Biofilm inhibitive effect of all essential oils against biofilm formation of different *C. tropicalis* strains were statistically significant ($p = 0.001$) and biofilm inhibition effect of sub-MICs of *C. verum* and *C. citratus* oils against all tested *C. tropicalis* strains were significantly different from *C. cyminum* and *C. limon* oils (Table 3).

Apart from these, all essential oils used in this study, inhibited biofilm formation of *C. tropicalis* U71 strain significantly more than the other *C. tropicalis* strains tested. Additionally, *C. verum* oil was significantly less inhibitive against biofilm formation of *C. tropicalis* T26 strain whereas *C. citratus* oil was significantly more inhibitive against biofilm formation of *C. tropicalis* U71 and *C. tropicalis* V89 strains (Table 3).

There was no significant difference in biofilm inhibition when different concentrations of the essential oils were compared ($p = 0.980$) (Table 3).

In Fig. 4, the biofilm inhibitory effect of *C. citratus* oil-hypromellose ointment coated silicone rubber surface, against the three different *C. tropicalis* strain is shown.

The biofilm inhibition of different *C. tropicalis* strains on 0%, 2%, 4% and 8% *C. citratus* oil coated silicone rubber surfaces were found to be normally distributed ($p = 0.527$) and homogeneity of variance assumption was met ($p = 0.537$). Therefore, two factor experimental design was applied. Biofilm inhibitive effects of 0%, 2%, 4% and 8% *C. citratus* oil coated silicone rubber surfaces were statistically significant ($p = 0.001$). When the effect of these four different concentrations were compared with each other by the Tukey test, it was found that biofilm inhibition caused by 2%, 4% and 8% concentrations were significantly higher than biofilm inhibition caused by 0% (vehicle alone) (Table 4). Thus, *C. citratus* oil added to the vehicle had a significant inhibitory effect on biofilm formation, although not concentration dependent (Table 4). Vehicle alone showed biofilm inhibition up to 43%.

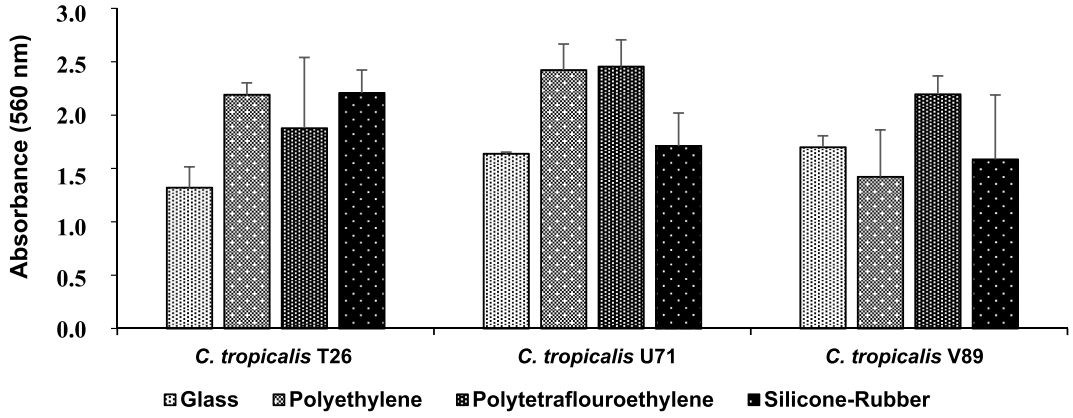

Fig. 1. Biofilm formation after 48 h of the three *C. tropicalis* strains on the four biomaterials as determined by a crystal violet binding assay.

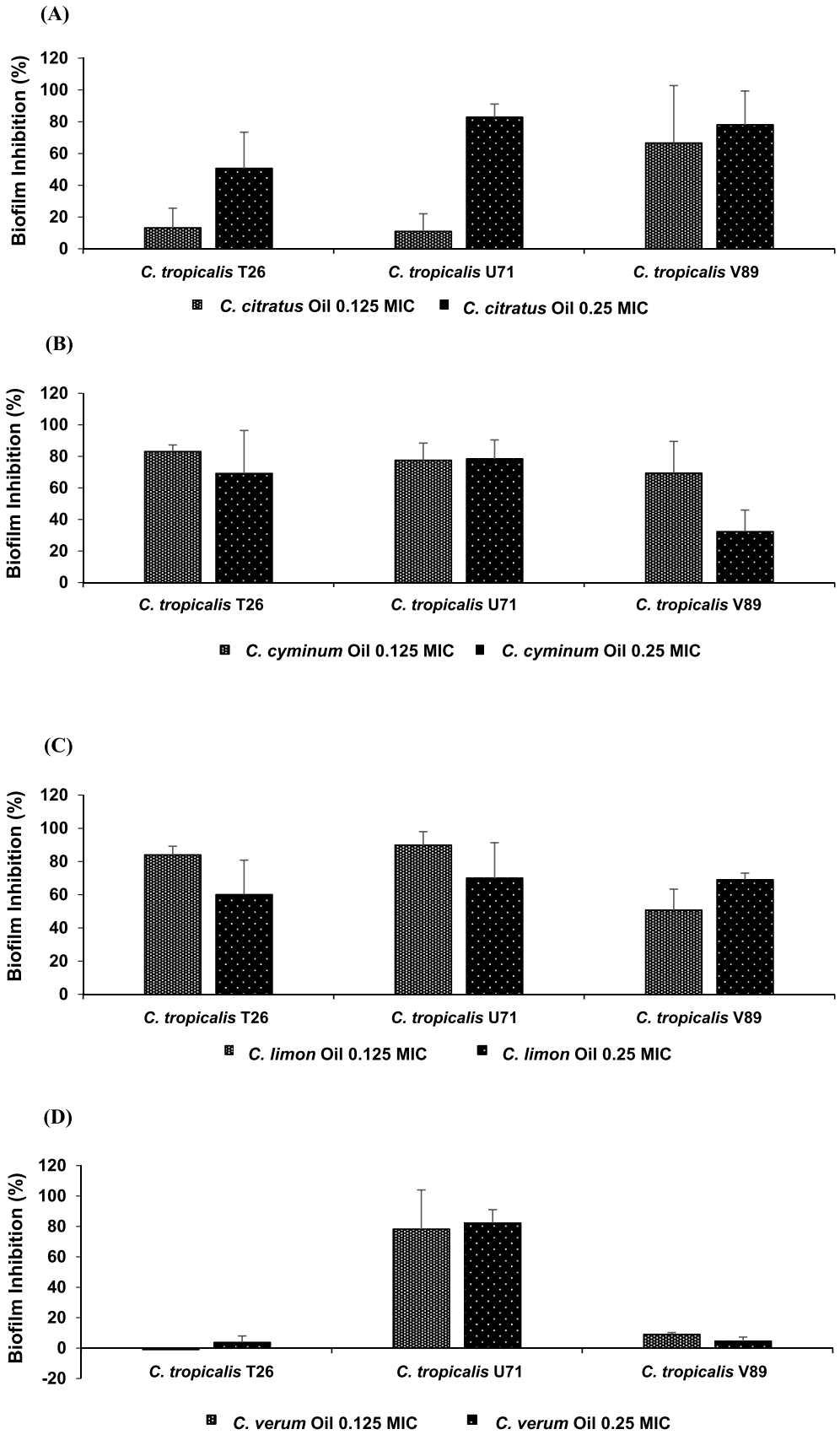

Fig. 2. Biofilm inhibition of (A) *C. citratus* oil (B) *C. cyminum* oil (C) *C. limon* oil and (D) *C. verum* oil and in 24-well polystyrene plates at sub-MIC values. Biofilm inhibition was calculated relative to the control (no essential oil).

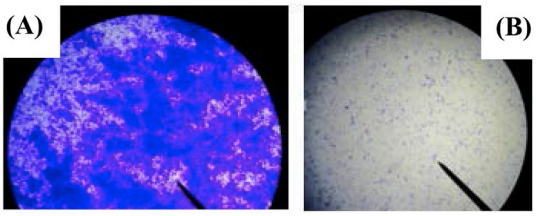

Fig. 3. Light microscopical images of *C. tropicalis* U71 biofilm (A) untreated (B) 0.25 MIC *C. citratus* oil treated biofilm. Images were taken with a bright field light microscope 4x magnification (LEICA DM500). The violet color indicates biofilm formation. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

According to GC-MS analysis, the following major constituents (> 0.5% of total peak area) were identified in the *C. citratus* oil ordered upon increasing retention time: Camphene (0.9%), 6-methyl-5-hepten-2-one (0.9%), beta-myrcene (0.4%), d-limonene (1.4%), 4-nonanone (0.3%), linalool (1.4%), exo-isocitral (0.7%), isoneral (0.7%), isogeranial (isocitral) (1.8%), alpha-terpineol (1.1%), neral (citral b, Z-citral) 34.8%, cis-geraniol (3.8%), geranial (citral a, E-citral) (46.3%), lavandulyl acetate (2.9%), caryophyllene (2.1%) and gamma-cadinene (0.7%).

Table 4
Tukey test results for biofilm inhibition by 0%, 2%, 4% and 8% *C. citratus* oil coated silicone rubber surfaces.

Concentration of *C. citratus* oil	P-value
0%–2%	0.005[a]
0%–4%	0.001[a]
0%–8%	0.001[a]
2%–4%	0.797
2%–8%	0.539
4%–8%	0.975

[a] Denotes the difference is significant at 0.05 alpha level.

Table 3
Descriptive statistics and analysis results for biofilm inhibition data.

	Mean	SE	Median	Grouping *	Test Results
Biofilm Inhibition Effect of Essential Oil					
C. citratus	43.31	8.44	37.54	A	$X^2 = 15.571$ $p = 0.001$[a]
C. cyminum	67.62	4.80	74.21	B	
C. limon	69.63	3.76	71.00	B	
C. verum	28.38	9.33	8.40	A	
Biofilm Inhibition of *C. tropicalis* Strain					
C. tropicalis T26	47.07	6.79	42.94	C	$X^2 = 11.520$ $p = 0.003$[a]
C. tropicalis U71	71.69	4.96	76.88	D	
C. tropicalis V89	46.77	5.73	48.74	C	
Biofilm Inhibition Effect of Essential Oil Concentration (Sub-MIC or µL/mL)					
0.125	54.04	5.62	62.22	E	$Z = -0.025$ $p = 0.980$[b]
0.25	56.63	4.62	62.93	E	
Biofilm Inhibition Effect of Essential Oil – Biofilm Inhibition of *C. tropicalis* Strain Interaction					
C. verum - *C. tropicalis* T26	1.93	1.79	1.24	F	$X^2 = 44.25$ $p = 0.001$[a]
C. verum - *C. tropicalis* U89	6.83	1.27	7.39	G	
C. citratus - *C. tropicalis* T26	29.29	9.62	31.78	G H	
C. citratus - *C. tropicalis* U71	39.70	18.00	23.80	G H I	
C. cyminum - *C. tropicalis* U89	50.85	8.99	47.14	H I	
C. limon - *C. tropicalis* U89	59.87	4.60	62.22	H I	
C. limon - *C. tropicalis* T26	69.05	7.14	78.54	H I	
C. citratus - *C. tropicalis* U89	72.30	12.60	77.60	I	
Cumin - *C. tropicalis* U71	78.00	3.74	74.21	I	
Cumin - *C. tropicalis* T26	76.13	7.77	81.35	I	
Cinnamon - *C. tropicalis* U71	80.68	6.46	81.75	I	
Lemon - *C. tropicalis* U71	78.83	6.34	88.60	I	

[a] Kruskal-Wallis
[b] Mann-Whitney U test results
* Means that do not share a letter are significantly different.

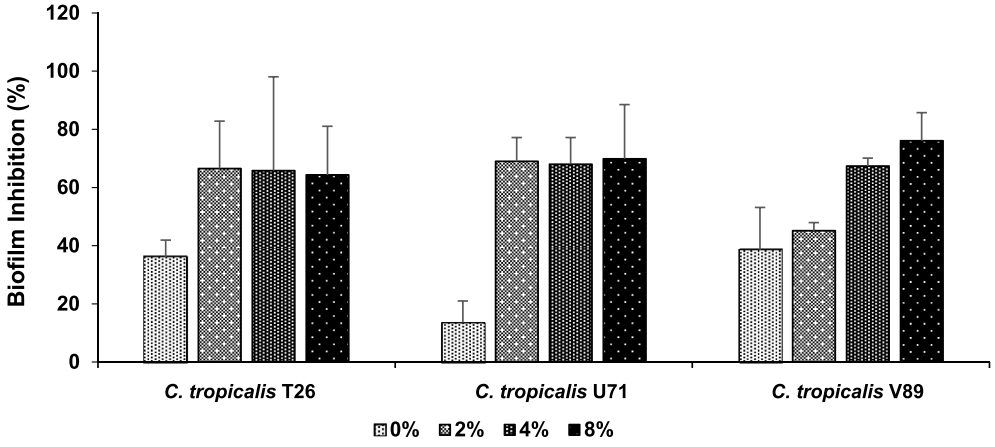

Fig. 4. Inhibitory effect on biofilm formation of three *C. tropicalis* strains, of a vehicle with *C. citratus* oil, coated on silicone rubber surfaces. Biofilm inhibition was calculated relative to untreated silicone rubber. 0% is the vehicle without *C. citratus* oil. Error bars indicate standard deviations over triplicate experiments with separately grown *C. tropicalis* cells.

4. Discussion

Candida species form a significant burden of infection especially to patients. Next to nosocomial blood stream infections, biofilm formation may occur on medical devices and prostheses (Ariani et al., 2013; Rajendran et al., 2016; Ramage and Williams, 2013; Silva-Dias et al., 2015). Since biofilm contaminated devices and prostheses are rarely cured with antimicrobial therapy, their removal is regarded as the only valid option to eliminate biofilm associated biomaterial infections (Bink et al., 2011). To prevent and inhibit biofilm formation on medical devices and prostheses it is necessary to develop and select appropriate biomaterials. In this framework, we investigated biofilm formation of three clinically isolated *C. tropicalis* strains on four different biomaterials as well as the effect of lemongrass oil and three other essential oils on growth and biofilm formation of these *C. tropicalis* isolates. In order to make it more clinically relevant, *C. citratus* oil was added to hypromellose ointment, which was used as a coating on silicone rubber, to study the effect on *C. tropicalis* biofilm formation.

Adhesion of a microorganism to a surface is the first step in biofilm formation (Pavithra and Doble, 2008). This is mediated not only by nutrient environment, pH and temperature, but also by physicochemical properties of the surface (Lorite et al., 2013). Hydrophobicity has been shown to be one of the most important determinants of microbial adhesion and/or biofilm formation (Pavithra and Doble, 2008).

Until now conventional antimicrobial therapies are inadequate to prevent or to treat biofilm related infections (Chen et al., 2013). Microorganisms in a biofilm environment undergo physiological and metabolic alterations adapting them to a slow growing or starved state and making them more resistant to antimicrobial drugs (Chen et al., 2013). Furthermore, clinical *Candida* isolates that demonstrated high biofilm formation capacity displayed a higher rate of antifungal resistance in one of our previous studies (Sahal and Bilkay, 2018). Therefore, alternative approaches are urgently needed to prevent biofilm associated biomaterial infections. Alteration of the surface of medical devices and prostheses and coating the surface with antimicrobial substances have been indicated as main focuses (Chen et al., 2013). Natural products such as plant extracts and essential oils have attracted attention in this respect (Bazargani and Rohloff, 2016). Additionally, considering the deleterious effects (causing changes in colour, hardness, and tear strength) of conventional solutions on various prostheses, plant extract solutions including of *Cymbopogon nardus* have been investigated for their capacity to eliminate mature biofilm on the surface of a particular material (Guiotti et al., 2016).

In our study, four materials with different hydrophobicities were tested for susceptibility to biofilm formation of three *C. tropicalis* strains. All isolates belonging to the *C. tropicalis* species displayed more biofilm on hydrophobic surfaces than on a hydrophilic surface. Apart from this, Four essential oils were tested. Most of them were inhibitory and fungicidal, and all of them displayed biofilm inhibitive effect (Table 2 and Fig. 2). All these findings validated the traditional use of *C. citratus*, *C. cyminum*, *C. limon* and *C. verum* oils for the treatment of various microbial diseases.

All *C. tropicalis* strains tested in this study were previously determined as resistant to fluconazole (MIC ≥ 64 mg/L) in an earlier study of us (Sahal and Bilkay, 2018). However, according to the results of this study, the MIC and MFC results of *C. citratus* oil were ≥15–60 times lower than MIC of fluconazole (MIC ≥ 64 mg/L) against the same strains showing that; *C. citratus* displays a substantial stronger antifungal effect against high biofilm forming *C. tropicalis* strains. Since the MIC and MFC of *C. citratus* oil was 100–500 times lower (see Table 2) than of *C. cyminum* and *C. limon* oil, and has been reported to have low cytotoxicity (Oliveira et al., 2017), we decided to add *C. citratus* oil to a vehicle, that can be applied on biomaterials. When *C. citratus* oil was added to a vehicle, it was still appeared to be highly effective in inhibiting biofilm formation on silicone rubber.

C. citratus oil with citronellal and geraniol as major compounds (Sticher, 2015) has known broad spectrum antibacterial and antifungal properties (Aiemsaard et al., 2011). It induces shrinkage of the cell wall, disruption of the cell membrane resulting in lysis of the cells in yeast biofilms (Tyagi and Malik, 2010). In a recent study, comparable to our findings, 0.5 MIC (ranging from 50 to 180 µg/mL, depending on the *C. albicans* strain) of *C. citratus* oil showed an inhibitory effect on biofilm formation of *C. albicans* strains (Khan and Ahmad, 2012). Different from the other studies carried out with *C. citratus* oil, biofilm inhibitive effect of *C. citratus* oil against *C. tropicalis* strains has been reported in this study.

C. cyminum essential oil is obtained from *C. cyminum* seeds with cymene, gamma-terpinene, cumin aldehyde, beta-pinene, carvone and limonene as main components (Sticher, 2015). Different from our results, *C. cyminum* oil has been reported to be an important antimicrobial agent not only against many different human pathogenic bacteria and *Candida* species but also against various food pathogenic microorganisms (Kamble, 2015; Naeini et al., 2014). The reason of the poor antimicrobial effect of *C. cyminum* oil in our study may be related to the high biofilm forming abilities of the *Candida* strains used. However, in spite of its poor antimicrobial activity, all tested concentrations of *C. cyminum* oil inhibited biofilm formation of all *C. tropicalis* strains used in our study.

Different from *C. cyminum*, *C. limon* and *C. citratus* oils, *C. verum* oil does not contain monoterpenes as principal constituents. Instead, it mainly contains phenylpropanes with cinnamaldehyde as the major compound (Sticher, 2015). Our finding that antimicrobial properties of *C. verum* oil against *Candida* species were higher than those of *C. cyminum* and *C. limon* oil is in line with other studies (Almeida et al., 2016; Velluti et al., 2003).

These results emphasize that, in addition to antimicrobial effects, antibiofilm properties of natural products are relevant for the development of new anti-infective substances in alternative biomedical approaches. Since several essential oils are known to change hydrophobicity of *Candida* yeasts (Rajkowska et al., 2015), antibiofilm effects of the tested essential oils might be related to the changing effect of these oils on hydrophobic properties of clinical *C. tropicalis* isolates. Modification of surface material is regarded as an emerging approach to prevent biomaterial associated infections (Sadekuzzaman et al., 2015). Therefore, in the last part of our study, silicone rubber was coated with *C. citratus* oil in hydrophilic ointment basis.

Indwelling devices such as artificial heart valves, pacemakers and prosthetic joints, are unsuitable to be coated on a regular basis. For short term used biomaterials, essential oil containing hypromellose ointment coatings may be suitable since essential oils are volatile. Accordingly, prostheses (e.g. maxillofacial prostheses), which can be removed by the patient, can be coated on a daily basis with the product (*C. citratus* oil-hypromellose ointment as tested in the present study). Maxillofacial prostheses are usually made from silicone rubber (Ariani et al., 2013) and the *Candida* ingrowth is a major problem. Beside the deterioration and discoloration of the prosthesis itself, also skin and mucosa irritation underneath these prostheses due to a warm and humid environment is regularly seen (Abu-Serriah et al., 2000; Ariani et al., 2013; Holgers and Ljungh, 1999). These maxillofacial prostheses are cleaned by the patient daily and can also be treated daily with an antifungal product, for example the vehicle with *C. citratus* oil, which can increase the life-time of the maxillofacial prosthesis. Additionally, application of *C. citratus* oil containing hypromellose ointment on a silicone rubber surface (the surface that we have found as one of the most suitable surfaces for biofilm formation of *C. tropicalis* strains) was a novel approach in this study. According to our findings, the inhibitive effect of *C. citratus* oil against *C. tropicalis* may be clinically relevant as well, as silicone rubber is the main material of maxillofacial and voice prostheses. Therefore, different from other studies carried out with *C. citratus* oil so far, our study may contribute to increase the life-time of some silicone rubber prostheses on which *C. tropicalis* biofilms pose a serious risk for infections and which can be cleaned by the patient on a

daily basis such as maxillofacial prosthesis.

This approach may ultimately increase the quality of life for the patient and decrease healthcare costs. Beside maxillofacial prostheses, *C. citratus* oil can probably also be used for other medical devices and implants such as vaginal implants, next to the already existing, drug releasing intravaginal rings (Gunawardana et al., 2011).

5. Conclusion

In conclusion, lemongrass oil shows significant inhibition of *C. tropicalis* growth and biofilm formation, which in turn has a clinical potential for maintaining silicone rubbers used for silicone prostheses or medical devices. The essential oil is on the FDA GRAS-list, meaning generally recognized as safe.

Authors' contribution

Gulcan Sahal, participated in research design, conducting the experiments, data analyses and drafting the manuscript. Herman J. Woerdenbag, Wouter L.J. Hinrichs, Henny C. van der Mei and Isil Seyis Bilkay participated in research design, interpretation of data and revising drafts of the manuscript. Henny C. van der Mei and Isil Seyis Bilkay supervised the whole study. Herman J. Woerdenbag and Wouter L.J. Hinrichs also contributed through design and preparation of *C. citratus* oil containing vehicles. Anita Visser participated in supplying silicone-rubbers and revising drafts of the manuscript. GC-MS analysis part of the study were applied by Pieter G. Tepper and Wim J. Quax. All authors read and approved the final manuscript.

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Conflicts of interest

The authors declare that they have no conflict of interest.

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