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THE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS FROM DIFFERENT SPECIES OF LIME, LEMON AND ORANGE AGAINST ORAL PATHOGENS

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ABSTRACT

This study investigated in vitro the antimicrobial activity against oral pathogens of essential oils (EOs) from different species of lime, lemon, and orange. The EOs were chemically characterized by gas chromatography coupled to mass spectrophotometry (GC-MS) and tested for their antimicrobial activity (MIC and MBC/MFC) against *Candida spp.*, *Fusobacteriumnucleatum*, *Porphyromonasgingivalis*, *Prevotella intermedia*, *Streptococcus mutans*, *Streptococcus mitis* and *Streptococcus sanguinis*. Data were analyzed descriptively and by one-way analysis of Variance (ANOVA) considering a 5% significance level ($P < 0.05$). Limonene was the major compound tentatively identified in the samples. Among the EOs, *C. sinensis* showed lower MIC values against *C. krusei*, *C. albicans* and *C. tropicalis*. Nystatin showed MIC of 0.125 mg/mL against all *Candida spp.* strains and MFC values $\geq$ 1 mg/mL. *C. sinensis* EO and chlorhexidine showed MIC/MBC values against bacterial strains ranging from 1 to 8 mg/mL and 2 to 8 mg/mL, respectively. To conclude, *C. sinensis* EO was the most effective sample against *Candida spp.* Overall, the EOs showed weak antibacterial activity with bacteriostatic effects, except for *C. latifolia*, which showed strong antimicrobial activity against *P. intermedia*.*C. sinensis* EO showed bactericidal effects against *S. mitis*.

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INTRODUCTION

The oral microbiome is a highly dynamic complex ecosystem that shelters a wide variety of commensal microorganisms and opportunistic pathogens. Evidence has shown that the oral microbiome is formed by approximately 1,000 different species and that most of them coexist and communicate through biofilm communities (Wilson, 2004). Biofilm growth is associated with specific co-adhesion and coaggregation mechanisms and the production of an extracellular matrix (Newman, 2012). Several oral diseases are biofilm-dependent, including dental caries (Karadağlioğlu, 2019), candidiasis (Hartmann, 2017), gingivitis, and periodontitis (Rams; Sautter; Van Winkelhoff, 2020; Pérez-Sayáns, 2020; Lopes, 2020). Whilst the oral microbiome is usually in balance with the host, local and systemic shifts can trigger a dysbiotic state that favors pathogenic biofilm buildup and the onset of infectious diseases (Furletti, 2011; Larsen and Fiehn, 2017). *Candida spp.*are the best example of opportunistic microorganisms colonizing the saliva and oral biofilms that can transition into a pathogenic form in susceptible individuals (Kanagalingam, 2015).

Essential oils (EOs) are plant secondary metabolites capable of penetrating through the cell membrane and disrupting the biofilm structure and associated virulence mechanisms. EOs have been traditionally used in folk medicine to treat a wide spectrum of infectious diseases (Aires, 2018) and have been incorporated into the food, beauty, and pharmaceutical industry as ingredients of vegetal oils, mouthwashes, cosmetics, etc (Filogônio, 2011). Yet, little is known in the literature about the antimicrobial activity of lemon, lime, and orange EOs against oral pathogens. The plant species used in our study were selected based on their pharmacological activity (Viegas; Bolzani; Barreiro, 2006; Scur, 2016; Bragueto Escher, 2019), availability in the Brazilian market (Bizzo; Hovell; Rezende, 2009), and antibacterial potential evidenced in previous publications (Vitti; Brito, 2003; Benavente-Garcia; Castillo, 2008; XI, 2017).

Prior evidence indicated that lemon species have antimicrobial, anti-inflammatory, and antioxidant properties, and are associated with a decreased risk of cardiovascular disease and some types of cancer (Benavente-Garcia; Castillo, 2008; XI, 2017). Tahiti lime is a tropical fruit rich in vitamins, carotenoids, and essential oil (Mendonça, 2006), whereas Sicilian lemons are the third largest citrus species, after oranges and tangerines, and one of the most cultivated in Brazil (Miran, 2016; XI., 2017). The antimicrobial activity of *Citrus lemon* EO was attributed to the occurrence of terpenes, such as pinene, myrcene, and limonene, and the latter alone was reported to have strong antifungal effects. Oranges have well-known health-promoting effects due to their chemical composition rich in vitamins, minerals, phenolic compounds and terpenoids, limonene, linalool, β-myrcene, p-synephrine, an alkaloid, and flavonoids. Orange species were found to have antibacterial, antioxidant, pesticide, antidiabetic, antianxiety, and antiobesity mechanisms (Suntar, 2018). Orange EO derivatives are used in perfumes, soaps, cleaning materials, candies, beverages, and the pharmaceutical industry (Bizzo, 2009). Their antimicrobial activity is attributed to the presence of small terpenoids and phenolic compounds, which also exhibit antibacterial or antifungal activity in their pure form (Duarte, 2005). Persian lime (*Citrus latifolia*) showed strong antimicrobial activity, and its bark is popularly used in the treatment of sinusitis. D-limonene was the major compound detected in the bark EO, followed by β-myrcene and linalool (Vasudeva; Sharma, 2012; Lopes, 2014). Sweet orange (*Citrus sinensisMacfad*) is a natural antioxidant rich in vitamin C and secondary metabolites (Favela-Hernández, 2016). Bitter orange (*Citrus aurantiumRisso*), as it is popularly known, is widely used as an acidifier and flavoring for foods as well as in EO formulations (Suntar, 2018). Blood oranges (*Citrus sinensis L.*) are a great source of bioactive compounds, especially vitamin C, and have a high content of flavonoids. The predominant organic acid in their composition is citric acid (Cebadera-Miranda, 2019). Historically, natural products have been an effective source of novel molecules for drug discovery and development (Viegas; Bolzani; Barreiro, 2006; Scur, 2016). In this study, we investigated the antimicrobial activity of EOs obtained from lemon, lime, and orange species against oral pathogens. Collectively, our data provided insights into the biological potential of these EOs as adjuvants in the chemical control of oral biofilms. Our study hypothesis was that the EOs were effective against all strains.

MATERIALS AND METHODS

Ethical considerations: This study was previously approved by the Research Ethics Committee (#13.607-339), under protocol number 037/2019.

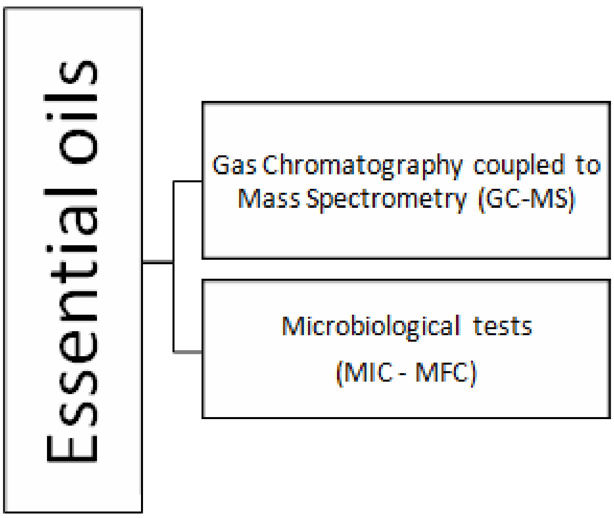

Figure 1. Methodology of the experimental phases of the research.

Essential oils: The EOs of Tahiti lime (*Citrus latifolia Tanaka*); Sicilian lemon (*Citrus lemon Tanaka*); Persian lime (*Citrus limettioides Swingle*); Sweet Orange (*Citrus sinensisMacfad*); Bitter Orange (*Citrus aurantiumRisso*), and Blood Orange (*Citrus sinensis L.*) were acquired from LASZLO®.

Chemical identification by Gas Chromatography coupled to Mass Spectrometry (GC-MS): EO samples were submitted to GC-MS analysis as previously described by Markham (1996) and Proestos (2006). Briefly, 400 µL of each sample was placed into glass vials and 1 mL of a trimethylsilyl solution was added for silanization. The samples were analyzed in a gas chromatograph (HP-6890, Agilent Technologies) coupled to a mass spectrometer (HP-5975, Agilent Technologies), equipped with a DB-5MS capillary column (J&W Scientific, Palo Alto, CA; 30m x 0.25 mm x 02.5 µm). The detector operated at 70eV in scan mode (m/z 40-400). The temperature programming was 50ºC (0.3 min) to 285ºC (15 min), with an increment of 6ºC/min. The samples (0.5 µL) were injected by an auto-injector in splitless mode. The integration was carried out by the specific software of the equipment. Terpenes and sesquiterpenes were identified by comparison with authentic standards methylated and eluted under the same conditions.
The other chemical compounds were tentatively identified by comparison with the mass spectra data from the Nist-2011 library.

Microorganisms and growth conditions: *Candida albicans* CBS 562, *C. dubliniensis* CBS 7987, *C. krusei* CBS 573, *C. tropicalis* CBS 94 were obtained from the CentraalbureauVoorSchimmelcultures (CBS) collection and kept in a freezer at -70°C. The following reference strains were obtained from the American Type Culture Collection (ATCC): *Fusobacteriumnucleatum* ATCC 25586, *Porphyromonasgingivalis*ATCC 33277, *Prevotella intermedia* ATCC 25611, *Streptococcus mutans* ATCC 25175, *Streptococcus mitis* ATCC 49456, and *Streptococcus sanguinis* ATCC 10556. Yeast strains were grown in Saboraud Dextrose Agar or RPMI 1640 media (assays). P. intermedia was grown in Brain Heart Infusion (BHI) agar, and *F. nucleatum* and *P. gingivalis* were grown in blood agar. The medium used for the microbiological tests was BHI broth.

Inocula preparation: Inocula were prepared following the recommendations contained in the M27-A2 protocol for yeasts (CLSI, 2002) and the M7-A6 protocol for bacteria (CLSI, 2005). The inocula were serially diluted in RPMI 1640 or BHI broth to a final concentration of 5.0 x 103 cells/mL (yeasts) and 5.0 x 105 cells/mL (bacteria) in the microplate.

Minimum Inhibitory Concentration (MIC): The MIC of the selected EOs was determined as previously described. In a sterile 96-well microplate (8 rows A-H/ 1-12 columns), 100 µL of RPMI 1640 or BHI broth were added to the wells, with column #12 serving as microbial growth and media sterility controls. In column 1 - row A, 50 µL of the EO emulsion was added to check for sample sterility. An aliquot of 100 µL of the samples was added to line B, homogenized, and transferred to the well in the following line (C). This procedure was repeated until line H to obtain 1:2 dilutions. Then, 100 µL of the inocula (0.5 turbidity in the McFarland scale) were added to the wells to a final concentration of 1.5 x 103 cells/mL for yeasts and 1.5 x 106 cells /mL for bacteria. The plates were sealed with Parafilm® and incubated for 24-48 h at 37oC in a microaerophilic or anaerobic atmosphere depending on the requirement of the strain (CLSI, 2005). The MIC was considered as the lowest concentration of the EO that inhibited visible microbial growth. Nystatin (initial concentration of 25,000 U) and Chlorhexidine (initial concentration of 1%) were used as positive controls. To confirm the visual readings, 50 µL of a triphenyl tetrazolium chloride (TTC) solution (tartrazine dye) were added to the wells and the plates were reincubated for 3 h. The MIC was defined as the lowest concentration of the EO at which no red staining indicative of mitochondrial activity was observed. The plates were read in a microplate reader to confirm the presence or absence of microbial growth (CLSI, 2002; CLSI, 2005).

Minimum Fungicidal and Bactericidal Concentrations (MFC/MBC): Aliquots from the wells corresponding to the MIC and higher concentrations were subcultured on agar plates and incubated at 37°C for 24 h. The MFC/MBC were defined as the concentrations at which no microbial growth was observed on the solid media (CLSI, 2002; CLSI, 2005). The assays were performed in triplicate of independent experiments.

Data analysis: GC/MS data were analyzed descriptively, and MIC/MBC/MFC values were compared by one-way Analysis of Variance (ANOVA) followed by Tukey'sposthoc test. The results were analyzed in the Biostat 5.3 and Statistica 10.0 programs, considering statistical significance at $P < 0.05$.

RESULTS

Chemical analysis: The major constituents of the EOs were tentatively identified by comparing their mass spectra with the NIST electronic library and retention indices described in the literature (Adams, 1998) under similar chromatographic conditions. Information concerning the chemical identification, retention time, and relative percentage of the compounds can be found in Table 1. Limonene was the predominant compound in the samples, followed by gamma-terpinene and beta-pinene to a lower extent.

Antimicrobial activity of essential oils against yeasts and periodontopathogens: The EOs of different species of lemon, lime, and orange were tested for their antimicrobial activity in vitro against clinically relevant yeast strains and periodontopathogens. Table 2 shows the MIC and MBC/MFC values of the EOs and positive controls (nystatin and chlorhexidine). *C. sinensisMacfad*, *C. aurantium*, and *C. sinensis L.* were effective against *C. krusei*, with MIC/MFC values of 0.06/4.0 mg/mL, 0.25/4.0 mg/mL, and 0.5/0.5 mg/mL, respectively. *C. sinensisMacfad* also inhibited *C. albicans* and *C. tropicalis* growth, with MIC of 0.25 mg/mL. As expected, nystatin inhibited all yeast strains, with MIC of 0.125 mg/mL and MFC ranging from 1.0 to 4.0 mg/mL.

The EOs were further tested against bacterial strains associated with the development of dental caries and periodontal diseases: *F. nucleatum*, *P. gingivalis*, *P. intermedia*, *S. mutans*, *S. mitis*, and *S. sanguinis*. *C. latifolia* EO (Tahiti lime) showed MIC values ranging from 0.125 mg/mL on *P. intermedia* to 2.0 mg/mL on *S. mitis*, 4.0 mg/mL on *S. mutans*, and 8.0 mg/mL on the other bacterial strains. *C. lemon Tanaka* EO showed MIC values ranging from 4.0 mg/mL against *S. mutans* to 8.0 mg/mL against the other bacterial strains. *C. limettioides Swingle* and *C. sinensisMacfad* showed MIC values of 1.0 mg/mL on *S. mutans* and 8.0 mg/mL on the other bacterial strains. *C. aurantiumRisso* showed MIC values of 1.0 mg/mL on *S. mutans* and *S. mitis* and 8.0 mg/mL on the other bacterial strains. *C. sinensis L.* showed MIC values of 1.0 mg/mL on *S. mutans*, 2.0 mg/mL on *S. mitis*, and 8.0 mg/mL on the other bacterial strains. This was the only EO that showed bactericidal activity, with MBC of 2.0 mg/mL on S. mitis. Chlorhexidine (positive control and gold standard) showed MBC values of 8.0 mg/mL and 2.0 mg/mL against *S. mutans* and *S. mitis*, respectively.

DISCUSSION

Over the last decades, natural antimicrobials have been tested for their ability to effectively assist in the treatment and/or prevention of oral diseases. Among them, EOs are reported to have antimicrobial, anti-inflammatory, antioxidant, and healing properties (Leach, 2008; Souza, 2016). In our study, we evaluated in vitro the antimicrobial activity of EOs against pathogens associated with the development of periodontal disease and caries. We found that the monoterpene limonene was the major bioactive compound present in the selected EOs, which is likely to be responsible for their biological activity (Vanitha, 2020). Beta-pinene and beta-myrcene were also tentatively identified in some samples, although to a lower extent. The benefits attributed to limonene include chemopreventive and chemotherapeutic properties against breast, skin, lung, and stomach cancer, while compounds such as beta-myrcene and beta-pinene are associated with the antimicrobial properties of lemon and orange EOs (Castaneda-Antonio, 2018). Citrus EOs have variations in their chemical composition depending on their origin, maturation stage, season, weather conditions, among other variables (Dosoky; Setzer, 2018). While limonene is the major compound found in most citrus EOs, minor constituents may also be acting synergistically to determine their antimicrobial properties (Ambrósio, 2019). Monoterpene or sesquiterpene hydrocarbons and their oxygenated derivatives are known to have antimicrobial activity (Hsouna, 2017), which is consistent with our GC/MS data showing their presence in the selected EOs. Our hypothesis that lemon, lime, and orange EOs were effective against the selected strains was partially accepted since some EOs had stronger antimicrobial activity than others. Literature reports suggest that MIC values up to 0.5%, 1.5%, and > 1.5% are indicative of strong, moderate, and weak antimicrobial activity, respectively (Aligiannis, 2001; Duarte, 2005). Of all the EOs tested in our study, *C. sinensisMacfad* demonstrated the best antimicrobial potential against all *Candida spp.* strains. Further research should investigate the association of *C. sinensisMacfad*EO and nystatin. The local treatment of fungal infections causes fewer adverse effects, and its effectiveness is directly related to the mucosal contact and exposure time, which is usually minimal when nystatin is used in suspension (Silva, 2017). *Citrus latifolia Tanaka* EO showed strong antimicrobial activity against *P. intermedia*. The other citrus EOs exhibited moderate to weak antimicrobial activity (De Freitas, 2020). *Citrus sinensis L.* showed bactericidal activity against *S. mitis* at a high concentration (2 mg/mL) (Furletti, 2014).

The lipophilic components of EO constituents damage the integrity of the cell membrane and cause cell death. Gram-negative bacteria are less susceptible to the action of EOs since they have a different membrane structure compared to Gram-positive cells and the presence of lipopolysaccharides (Oyedemi, 2009; Rahman, Kang, 2009; Oliveira, 2016). Chlorhexidine is considered the gold standard antimicrobial for dental use (Karpiński; Szkaradkiewicz, 2015). In our study, chlorhexidine showed bacteriostatic activity against all tested strains and bactericidal activity against S. mutans and *S. mitis* (Hortense, 2017). These findings are in line with other studies showing that chlorhexidine effectively inhibited the growth of *P. gingivalis*, *F. nucleatum*, and different species of *Streptococcus* (Shetty, 2013; Bescos, 2020). However, the administration of chlorhexidine has been associated with harmful side effects. Hence, these citrus EOs should be further considered as potential alternative solutions for the use of effective antimicrobial formulations in dental care (Saffari, 2015).

In our study, nystatin showed antifungal activity against all strains at concentrations similar to those reported in the literature (Anna, 2010; Rangel, 2018). Nystatin has been routinely used in dental care to treat local fungal infections (Furletti, 2011). Hence, the effectiveness of citrus EOs could be enhanced through their combination with nystatin, which could help reduce the required concentration of each solution (Paiva, 2019). Chlorhexidine is known to produce color changes in the dental enamel and taste alterations (Saffari, 2015) whereas nystatin may cause nausea in some patients. In these cases, triazoles (e.g., fluconazole) can be prescribed, but they must be administered with caution due to nephrotoxic and hepatotoxic side effects (Sanitá, 2012). EO-containing formulations should be considered as adjuvants in the chemical control of biofilm-dependent oral diseases such as dental caries, gingivitis, and periodontitis (Sousa, 2014). Taken altogether, our study adds to the literature on the antibacterial and antifungal activity of EOs of different species of lemon, lime, and orange. To conclude, *C. sinensis* EO (sweet orange) was the most effective sample against *Candida spp.* Overall, the EOs showed weak antibacterial activity with bacteriostatic effects, except for *C. latifolia* (Tahiti lime), which showed strong antimicrobial activity against *P. intermedia*. *C. sinensis* EO showed bactericidal effects against *S. mitis*. Limonene was the major chemical compound in these citrus EOs and may be responsible for their antimicrobial effects.

Table 1. GC/MS identification of the major compounds present in the selected essential oils.

Essential oil	Rt (min)	Major compound	rel.%
Citrus aurantiumRisso	8.17	Limonene	94.96
	6.86	Beta-myrcene	2.49
Citrus sinensisMacfad	8.16	Limonene	68.87
Citrus sinensis L.	8.16	Limonene	94.49
	6.86	Beta-myrcene	2.39
Citrus limettioides Swingle	8.18	Limonene	86.23
Citrus lemon Tanaka	8.15	Limonene	54.34
	9.11	Gamma-terpinene	13.95
	6.52	Beta-pinene	13.34
Citrus latifolia Tanaka	8.16	Limonene	48.48
	9.12	Gamma-terpinene	14.95
	6.52	Beta-pinene	12.19

Rt = Retention time in minutes. rel. % = Relative percentage.

Table 2. The antimicrobial activity (MIC and MBC/MFC) of EOs obtained from different species of lemon, lime, and oranges against oral pathogens

Strain	*Citrus latifolia*Tanaka		*Citrus lemon*Tanaka		*Citrus limettioides* Swingle		*Citrus sinensis*Macfad		*Citrus aurantium*Risso		*Citrus sinensis*L.		Chlorhexidine (positive control)		Nystatin (positive control)		P-value
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC	MIC	MFC	MIC/MBC/MFC
Fusobacteriumnucleatum ATCC 25586	8	*	8	*	8	*	8	*	8	*	8	*	1	*	-	-	<0.05
Porphyromonasgingivalis ATCC 33277	8	*	8	*	8	*	8	*	8	*	8	*	1	*	-	-	<0.05
Prevotella intermedia ATCC 25611	0.125	*	8	*	8	*	8	*	8	*	8	*	1	*	-	-	<0.05
Streptococcusmutans ATCC 25175	4	*	4	*	1	*	1	*	1	*	1	*	1	1	-	-	<0.05
Streptococcusmitis ATCC 9811	2	*	8	*	2	*	8	*	1	*	2	2	0.25	0.25	-	-	<0.05
Streptococcus sanguinis ATCC 10556	8	*	8	*	8	*	8	*	8	*	8	*	1	*	-	-	<0.05
Candida albicans CBS 562	4	4	8	8	8	8	0.25	2	1	1	1	1	-	-	0.125	2.0	<0.05
Candida dubliniensis CBS 7987	*	*	*	*	*	*	1	1	*	*	8	*	-	-	0.125	4.0	<0.05
Candida krusei CBS 573	2	4	4	4	4	8	0.06	4	0.25	4	0.5	0.5	-	-	0.125	2.0	<0.05
Candida tropicalis CBS 94	8	*	*	*	*	*	0.25	0,5	4	8	4	4	-	-	0.125	1.0	<0.05

MIC, MBC, and MFC values in mg/mL; (*) No inhibition at the tested concentrations; (-) Not applicable.

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Annex 1 – Approval Letter from the Research Ethics Committee

Comissão de Ética em Animal -CEUA- 2019-2020
Fone: (19)3543-1440
Parecer Nº081/2019

IDENTIFICAÇÃO DO PROJETO

Título: AVALIAÇÃO IN VITRO DA ATIVIDADE ANTIMICROBIANA DO ÓLEO ESSENCIAL DE Calendula officinalis SOBRE AS BACTÉRIAS ENVOLVIDAS NA DOENÇA PERIODONTAL

Título Inglês
IN VITRO EVALUATION OF ANTIMICROBIAI ACTIVITY OF ESSENTIAL OIL OF CALENDULA OFFICINALIS ON BACTERIAs INVOLVED IN PERIODONTAL DISEASE

Pesquisador Responsável: Fabio Venancio

Parecer: O Projeto Simplificado encontra-se em consonância com o estabelecido na legislação.
Decisão homologada na reunião do dia 12/11/2019

Doutor Rodrigo Augusto Dalia
Coordenador(a) do Comissão de Ética em Animal
-CEUA- 2019-2020

Comissão de Ética em Animal -CEUA- 2019-2020

Fundação Herminio Ometto

Título: ATIVIDADE ANTIMICROBIANA DE ÓLEOS ESSENCIAIS DE ESPÉCIES DE LARANJA E DE LIMÃO NA AVALIAÇÃO DO POTENCIAL ANTIBIOFILME DE STREPTOCOCCUS

Orientador Responsável: Vivian Fernandes Furletti de Goes

Aluno(s)

Lais Venancio Rorato

Curso: Odontologia (Bacharelado)

Nº de Inscrição no CEP: 065/2019

Data Apreciação do CEP: 11/09/2019

O Comitê de Ètica e Mérito Científico informa que o projeto acima especificado foi registrado em seus arquivos.

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