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Chemical composition and antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against oral pathogenic bacteria

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ABSTRACT

Tooth decay is a major public health problem which affects a large number of people in several countries. Even though more than 700 bacterial species have been detected in the oral cavity, *Streptococcus* and *Lactobacillus* stand out as the genera that cause tooth decay and other periodontal diseases. In this study, essential oils from *Citrus aurantifolia* leaves (CL-EO) and fruit peel (CP-EO) were obtained by hydrodistillation by a Clevenger-type apparatus whereas their chemical composition was analyzed by gas chromatography-flame ionization detector (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Limonene (77.5 %), linalool (20.1 %), citronellal (14.5 %) and citronellol (14.2 %) were the main constituents found in the essential oils from *C. aurantifolia* leaves and fruit peel. Antibacterial activity of essential oils was evaluated in terms of its minimum inhibitory concentration (MIC) values by the broth microdilution method in 96-well microplates. Both CL-EO and CP-EO displayed some activity against all oral pathogens under investigation; MIC values ranged from 20 to 200 µg/mL. CL-EO and CP-EO not only had promising activity against *Streptococcus mutans* (MIC = 20 µg/mL) and *Lactobacillus casei* (31.25 µg/mL), but also displayed antibacterial activity against all studied cariogenic bacteria. Efficacy of essential oils against *S. mutans* and *L. casei* is noteworthy and should be further investigated.

Key words: *Citrus aurantifolia,* limonene, cariogenic bacteria, essential oils, oral pathogens.

INTRODUCTION

The high prevalence of tooth decay worldwide has made the World Health Organization consider it a severe public health problem which affects people of all ages. Brushing and flossing are the most effective methods to remove microorganisms from

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the tooth surface and prevent tooth decay and gum diseases (Melo et al. 2017). Chlorhexidine has been widely applied to this purpose because it displays anticariogenic properties. However, its regular use in oral care products often leads to several side effects (Melo et al. 2017).

Citrus aurantifolia is a species that belongs to the family Rutaceae which has about 150 genera and 1600 species that are broadly distributed in tropical, subtropical and temperate zones around the world. In Brazil, the family Rutaceae is represented by about 29 genera and 182 species; some of them have economic importance (Campelo et al. 2013). *Citrus* is a genus that comprises about 70 species of subshrubs and shrubs which may be either grown or spontaneously found in Germany, Spain, Mexico, Venezuela, Cuba, Jamaica, Ecuador and in many regions in Brazil (Campelo et al. 2013).

Species of the genus *Citrus* have been highlighted because they are rich in essential oils which are very versatile and often used as flavorings in several goods, such as beverages, soaps, cosmetics and household products. Their essential oils have also been frequently used in medical treatments due to their antimicrobial, antifungal, antibacterial and antiparasitic properties (Estevam et al. 2016). Many recent studies that have attempted to determine the chemical composition of essential oils extracted from *C. aurantifolia*. Researchers have noted that the components of these oils display large intraspecific chemical variation (Tundis et al. 2012, Amorim et al. 2016).

The species *C. aurantifolia* has been known because it exhibits important biological activities, such as antimicrobial activity against several pathogens – *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas* spp, *Aspergillus niger* and *Candida albicans* – antiaflatoxigenic and anticancer activities (Aibinu et al. 2007, Abyaneh et al. 2009, Pathan et al. 2012, Narang and Jiraungkoorskul 2016). However, anticariogenic activity of essential oils from *C. aurantifolia* against oral pathogens has not been investigated yet.

As part of an ongoing project on the biological activity of essential oils (Xavier et al. 2016, Lemes et al. 2017), this study investigated the chemical constituents and the antibacterial activity of essential oils from *Citrus aurantifolia* leaves and fruit peel against a representative panel of cariogenic bacteria.

MATERIALS AND METHODS

PLANT MATERIAL

C. aurantifolia leaves and fruit peel were collected in the *Cerrado* region in Rio Verde, Goiás, Brazil, in July 2015. The plant was identified by the botanist Erika Amaral and a sample was deposited at the Herbarium Jataiense Professor Germano Guarim Neto at exsiccate number HJ 7444.

EXTRACTION OF ESSENTIAL OILS

Samples of *C. aurantifolia* fresh leaves and fruit peel were subjected to hydrodistillation for 3 hours by a Clevenger-type apparatus. In order to carry out the analysis, 300 g plant material was divided into three 100-g samples and 500 mL distilled water was added to each sample. After manual collection of the essential oil samples, traces of remaining water in the oils were removed with anhydrous sodium sulfate, which was followed by filtration. The extraction procedure was done in triplicate. The isolated oil was stored under refrigeration up to the analysis and test. Yields (w/w) were calculated from fresh leaf and fruit peel weight and expressed as the average of the triplicate analyses.

IDENTIFICATION OF THE CHEMICAL COMPOSITION OF THE ESSENTIAL OILS

Gas Chromatography (GC) analyses were performed by a Shimadzu GC2010 Plus gas chromatograph equipped with an AOC-20s autosampler and fitted with fitted with Flame Ionization Detector (FID)

and a data-handling processor. An Rtx-5 (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) was employed. Operation conditions were as follows: column temperature programmed to rise from 60 to 240 °C at 3 °C/min and then hold at 240 °C for 5 min; carrier gas = He (99.999 %), at 1.0 mL/min; injection mode; injection volume, 0.1 µL (split ratio of 1:10); and injector and detector temperatures = 240 and 280 °C, respectively. Relative concentrations of components were obtained by peak area normalization (%). Relative areas were the average of triplicate GC-FID analyses.

GC-MS analyses were carried out by a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler. The column was an RTX-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary one (30 m x 0.25 mm i.d. x 0.25 µm film thickness). Electron ionization mode occurred at 70 eV. Helium (99.999 %) was employed as the carrier gas at a constant flow of 1.0 mL/min. The injection volume was 0.1 µL (split ratio of 1:10). Injector and ion-source temperatures were set at 240 and 280 °C, respectively. The oven temperature program was the same as the one used for GC. Mass spectra were taken at a scan interval of 0.5 s, in the mass range from 40 to 600 Da.

Identification of volatile components of *C. aurantifolia* leaves and fruit peel (Table I) was based on their retention indices on an RTX-5MS capillary column under the same operating conditions as the ones found in the case of GC, related to a homologous series of *n*-alkanes (C_8-C_{20}). Structures were computer-matched with the Wiley 7, NIST 08 and FFNSC 1.2 spectra libraries and their fragmentation patterns were compared with literature data (Adams 2007).

BACTERIAL STRAINS AND ANTIMICROBIAL ASSAYS

In vitro antimicrobial activities of CL-EO and CP-EO were determined by minimum inhibitory concentration (MIC) assays which were based on the broth microdilution method (CLSI 2009). *Streptococcus salivarius* (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556) and *Lactobacillus casei* (ATCC 11578) were the standard strains in the assays. Initially, bacteria were transferred to blood agar (Difco Labs, Detroit, MI, USA), and individual 24-h colonies were suspended in 10.0 mL tryptic soy broth (Difco). A spectrophotometer (Femto, São Paulo, SP, Brazil) at a wavelength (λ) of 625 nm was used for standardizing the suspensions of each microorganism so as to match the transmittance of 81, equivalent to 0.5 in the McFarland scale (1.5 x 10^8 CFU/mL). Dilution of the standardized suspension generated the final concentration of 5 x 10^5 CFU/mL. Essential oils (CL-EO and CP-EO) were dissolved in DMSO (Merck, Darmstadt, Germany) at 16.0 mg/mL. Concentrations ranging from 4000 to 3.9 µg/mL were achieved after dilution of essential oils in tryptic soy broth (Difco). After the dilutions, DMSO concentrations were between 4 % and 0.0039 % (v/v). Negative controls, three inoculated wells with DMSO at concentrations ranging from 4 % to 1 % and one non-inoculated well, free of any antimicrobial agent, were included. An inoculated well helped to test whether the broth was adequate for microorganisms to grow. The positive control was chlorhexidine dihydrochloride (CHD) (Sigma-Aldrich, St. Louis, MO, USA) at concentrations ranging from 5.9 to 0.115 µg/mL, diluted in tryptic soy broth (Difco). Ninety-six-well microplates were

sealed with parafilm and incubated at 37 °C for 24 h. After that, 30 mL of an aqueous solution with 0.02 % resazurin (Sigma-Aldrich, St. Louis, MO, USA) was added to each microplate well to indicate the viability of the microorganism (Palomino et al. 2002). The lowest concentration of the sample that inhibited microorganism growth (MIC value) was determined as the lowest concentrations of CL-EO and CP-EO that were able to prevent the resazurin solution from changing its color (Sarker et al. 2007). All assays were conducted in triplicate.

RESULTS AND DISCUSSION

Both analyses by GC-MS and GC-FID identified eighteen compounds in the essential oil from *C. aurantifolia* leaves and seventeen compounds in its essential oil from fruit peel, i.e., 99.5 % and 99.2 % of the total compounds, respectively (Table I). Table I shows these constituents with their retention indices, retention times and percentages.

Essential oils from *C. aurantifolia* leaves and fruit peel showed high yield (w/w on fresh weight basis), i.e., 2.5 % and 3.0 %, respectively. Both analyses by GC-FID and GC-MS revealed that monoterpene hydrocarbons (84.6 %) were the main constituents of CP-EO and oxygenated monoterpenes (57.6 %) were the main constituents of CL-EO, whereas limonene (77.5 %, **1**), linalool (20.1 %, **2**), citronellal (14.5 %, **3**) and citronellol (14.2 %, **4**) were their major constituents (Figure 1, Table I).

The chemical composition of essential oils from *C. aurantifolia* leaves and fruit peel was similar to the ones from other species of *Citrus*, such as *C. limonia* and *C. reticulata* previously described in the literature since they all have the same major constituents (Martins et al. 2017). Limonene was found at large amounts in *C. aurantifolia* leaves and fruit peel, besides being the major constituent of essential oils from *C. sinensis*, *C. latifolia* and *C. limonia* (Eldahshan and Halim 2016, Estevam et al. 2016). Therefore, it should be highlighted that, since essential oils from *C. aurantifolia* found in southwestern Goiás are rich in limonene, linalool, citronellal and citronellol, they may be a new prospecting source of these bioactive compounds.

Previous reports on the essential oil obtained from others *C. aurantifolia* specimens have indicated that terpenes predominate in the oil, and that the chemical composition of the essential oil varies significantly depending on the origin of the plant. Samples from Italy contained limonene, β-myrcene, β-pinene, γ-terpinene, citral and β-bisabolene as the major compounds (Tundis et al. 2012, Spadaro et al. 2012). β-pinene and limonene were also the major components in an essential oil sample collected in South Korea (Hong et al. 2017). The chemical composition found in this study is similar to that previously reported by a study which quantified and identified the main chemical compounds of the volatile oil extracted from *C. aurantifolia* grown in Rio de Janeiro State (Amorim et al. 2016).

Plant derivatives, such as extracts, essential oils and pure compounds have already been evaluated regarding their antimicrobial effects against oral pathogenic agents. As a result, they have attracted the interest of research groups since they can be applied to the development of new solutions to mouth rinse used for oral hygiene (Estevam et al. 2016). However, reports of antimicrobial activity

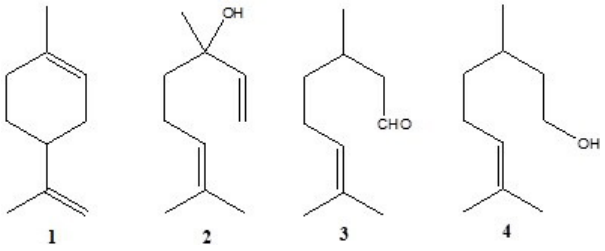

Figure 1 - Chemical structures of major constituents of essential oils from *C. aurantifolia*: limonene (**1**), linalool (**2**), citronellal (**3**) and citronellol (**4**).

TABLE I
Chemical composition of essential oils from *Citrus aurantifolia* leaves (CL-EO) and fruit peel (CP-EO).

RT (min)	*Compounds*	RI_{exp}	RI_{lit}	*RA %*	
				CL-EO	*CP-EO*
6.28	(*Z*)-Hex-3-en-1-ol	853	851	1.1	
9.09	α-Pinene	935	934		0.9
10.78	Sabinene	975	974		0.3
11.57	Myrcene	993	991	1.4	4.4
13.82	Limonene	1043	1040	**32.7**	**77.5**
14.32	*trans*-β-Ocimene	1054	1050	2.7	0.9
14.81	γ-Terpinene	1065	1062		0.6
17.00	Linalool	1106	1107	**20.1**	3.5
18.59	Limonene oxide	1133	1133	0.5	
19.97	Isopulegol	1141	1145	1.0	
19.31	Citronellal	1158	1153	**14.5**	3.2
21.13	α-Terpineol	1184	1185	0.6	0.7
21.34	Unknown	1198	1198		0.1
22.87	Citronellol	1230	1228	**14.2**	2.0
23.53	Neral	1242	1240	2.1	
24.12	Geraniol	1253	1255	1.0	
24.86	Geranial	1271	1270	2.6	
28.40	Citronellyl acetate	1354	1354	1.0	
31.30	*trans*-β-Caryophyllene	1420	1418	2.0	0.4
31.92	α-*trans*-Bergamotene	1435	1434	0.8	1.3
33.85	Germacrene D	1481	1480		1.5
34.48	Bicyclogermacrene	1499	1494		0.2
34.64	Unknown	1550	1549		0.2
34.89	β-Bisabolene	1510	1509	0.9	1.5
37.92	Caryophyllene oxide	1583	1581	0.3	
	Monoterpene hydrocarbons			36.8	84.6
	Oxygenated monoterpenes			57.6	9.4
	Sesquiterpene hydrocarbons			3.7	4.9
	Oxygenated sesquiterpenes			0.3	
	Others			1.1	
	Not identified				0.3
	Total			99.5	99.2

***RI_{exp}*:** Retention index relative to *n*-alkanes (C_8–C_{20}) on the Rtx-5MS column; ***RI_{lit}*:** Retention index found in the literature (Adams 2007). ***RA%*:** relative area.

of natural products against oral pathogens are still scarce (Estevam et al. 2016).

Concerning the antibacterial activity of essential oils from *C. aurantifolia* leaves and fruit peel, the *in vitro* anticariogenic activity (MIC values, see Table II) of CL-EO and CP-EO were evaluated against a representative panel of cariogenic bacteria and results were compared with chlorhexidine dihydrochloride as a positive control. According to the literature, MIC values below 100 µg/mL, between 100 and 500 µg/mL and between 500 and 1000 µg/mL correspond to promising,

TABLE II
Anticariogenic activity of essential oils from *C. aurantifolia* (CL-EO and CP-EO) against oral bacteria under study.

Bacteria	Minimum inhibitory concentration (MIC) - µg/mL		
	CL-EO	CP-EO	CHD*
Streptococcus salivarius	200	200	0.922
S. mutans	20	20	0.922
S. mitis	200	100	3.688
S. sanguinis	200	100	0.922
S. sobrinus	100	200	0.922
Lactobacillus casei	31.25	31.25	1.844

CHD*: chlorhexidine dihydrochloride (positive control).

moderate and weak activities, respectively, whereas MIC values above 1000 µg/mL denote inactivity (Gibbons 2004, Rios and Recio 2005, Saleem et al. 2010). Based on these criteria, CL-EO and CP-EO displayed moderate activity against *Streptococcus mitis*, *S. sanguinis*, *S. sobrinus* and *S. salivarius*. On the other hand, CL-EO and CP-EO had promising activity against *S. mutans* and *Lactobacillus casei* (Table II).

Several mechanisms have been proposed to explain the antimicrobial activity of essential oils (Oliveira et al. 2016). Microbial growth inhibition by the activity of essential oils is due to direct damage caused to the integrity of cell membranes by lipophilic components of the essential oil, a fact that affects the maintenance of the cell pH and the balance of inorganic ions directly (Oliveira et al. 2016). Inhibitory effects of essential oils have also been described as consistent with the activity of monoterpenic and sesquiterpenic constituents in cell membranes while damage caused to the membrane is said to yield different effects on diverse microorganisms (Vieira et al. 2017). Correlation between the lipophilicity of oil constituents and the antimicrobial activity of essential oils has motivated researchers to investigate the antibacterial activity of some essential oils against cariogenic bacteria (Melo et al. 2017, Carneiro et al. 2017). In this study, CL-EO and CP-EO were found to display promising activity against *S. mutans* (MIC = 20 µg/mL) and *L. casei* (MIC = 31.25 µg/mL) (Table II). It is remarkable because *S. mutans* is one of the primary causes of tooth decay and its inhibition by natural compounds is unusual (Saleem et al. 2010).

Promising anticariogenic activity shown by essential oils from *C. aurantifolia* may be justified by their major constituents, i.e., limonene, linalool, citronellal and citronellol, whose antibacterial potential has already been reported by the literature (Valeriano et al. 2012, Millezi et al. 2014). In addition, Bezerra et al. (2013) stated that bacterial strains of *S. mutans*, *S. oralis* and *S. salivarius* were susceptible to the activity of phytoconstituents linalool, citronellol and limonene. In sum, antibacterial activity of CL-EO and CP-EO may also be correlated to other minor components that might either underline or even increase the activity of the major chemical constituents of these essential oils in a synergistic mechanism (Vieira et al. 2017). However, although the antimicrobial activity of CL-EO and CP-EO may be related to the lipophilicity of the monoterpenes thereof, the exact mechanism through which CL-EO and CP-EO exert their antimicrobial activity is not clear and should be further investigated.

Findings of this study highlight that essential oils from *Citrus aurantifolia* leaves and fruit peel display interesting and promising antibacterial activity against some important cariogenic bacteria, such as *S. mutans*. As a result, CL-EO and CP-EO might be used as promising components of new oral care products because *S. mutans* is one of the main causes of oral disorders, such as tooth decay. Further studies that identify active chemical constituents of CL-EO and CP-EO and determine their antimicrobial mechanisms are underway.

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