SUPPLEMENTARY MATERIAL

Antibacterial activity of commercially available plant-derived essential oils against oral pathogenic bacteria

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Antibacterial activity of commercially available plant-derived essential oils against oral pathogenic bacteria

This work investigated the antibacterial activity of fifteen commercially available plant-derived essential oils (EOs) against a panel of oral pathogens. The broth microdilution method afforded the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of the assayed EOs. The EO obtained from *Cinnamomum zeylanicum* (Lauraceae) (CZ-EO) displayed moderate activity against *Fusobacterium nucleatum* (MIC and MBC = 125 μg/mL), *Actinomyces naeslundii* (MIC and MBC = 125 μg/mL), *Prevotella nigrescens* (MIC and MBC = 125 μg/mL), and *Streptococcus mutans* (MIC = 200 μg/mL; MBC = 400 μg/mL). (Z)-isosafrole (85.3%) was the main chemical component of this oil. We did not detect cinnamaldehyde, previously described as the major constituent of CZ-EO, in specimens collected in other countries.

Keywords: *Cinnamomum zeylanicum*; *Streptococcus mutans*, cariogenic bacteria

Experimental

Essential oils and GC-MS analysis

The fifteen essential oils (EOs) tested in this study were obtained by hydrodistillation and donated by the company Body & Mind Beautiful Aromatherapy (Franca, SP, Brazil). The EOs and their corresponding lot numbers were as follows: *Boswellia carteri* Birdw. (Burseraceae) (**BC**, 118); *Cedrus atlantica* (Endl.) Manetti ex Carrière (Pinaceae) (**CA**, 101); *Citrus x bergamia* Risso & Poit. (Rutaceae) (**CB**, 114); *Citrus limonum* Risso (Rutaceae) (**CL**, 121); *Commiphora myrrha* (T. Nees) Engl. (Burseraceae) (**CM**, 140); *Citrus paradisi* Macfad. (Rutaceae) (**CP**, 145); *Cupressus sempervirens* L. (Cupressaceae) (**CSe**, 126); *Citrus sinensis* (L.) Osbeck (Rutaceae) (**CSi**, 100); *Cinnamomum zeylanicum* Blume (Lauraceae) (**CZ**, 099); *Eucalyptus globulus* Labill. (Myrtaceae) (**EG**, 125); *Melaleuca alternifolia* Chell (Myrtaceae)(**MA**, 167); *Pinus sylvestris* L. (Pinaceae) (**PS**, 139); *Salvia sclarea* L. (Lamiaceae) (**SS**, 197); *Thymus vulgaris* L. (Lamiaceae) (**TV**, 137); and *Zingiber officinale* Roscoe (Zingiberaceae) (**ZO**, 253).

Gas chromatography (GC-FID) and Gas chromatography–mass spectrometry (GC-MS) analysis

The essential oil of *Cinnamomum zeylanicum* (CZ-EO) was analyzed by gas chromatography (GC) on a Hewlett-Packard G1530A 6890 gas chromatograph fitted with FID and data-handling processor. An HP-5 (Hewlett-Packard, Palo Alto, CA, USA) fused-silica capillary column (30-m length × 0.25-mm i.d.; 0.33-μm film thickness) was employed. The operation conditions were as follows: the column temperature was programmed to rise from 60 to 240 °C at 3 °C/min and then held at 240 °C for 5 min; carrier gas = H_2, at a flow rate of 1.0 mL/min; injection mode; injection volume = 0.1 μL (split ratio of 1:10); injector and detector temperatures = 240 and 280 °C, respectively. The components concentrations were obtained by relative peak area normalization (%). The relative areas were the average of triplicate GC-FID analyses.

GC-MS analyses were carried out on a Shimadzu QP2010 Plus (Shimadzu Corporation, Kyoto, Japan) system equipped with an AOC-20i autosampler under the previously reported conditions (Magalhães et al., 2012). The chemical constituents of

CZ-EO were identified on the basis of their retention indices on a RTx-5MS relative to a homologous series of *n*-alkanes (C_8-C_{20}), using an Rtx-5MS (Restek Co., Bellefonte, PA, USA) fused silica capillary column (30-m length × 0.25-mm i.d. × 0.25-μm film thickness) under the same operating conditions as well as computer matching with the Wiley 7, NIST 08, and FFNSC 1.2 spectral libraries. The constituents were also determined by comparison of their mass spectra with those reported in the literature (Adams, 1995). Authentic standards available in our laboratory were also co-eluted with CZ-EO to confirm the identity of some EO components.

Bacterial strains and antimicrobial assays

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the EOs were calculated by using the broth microdilution method in 96-well microplates. The standard strains from the American Type Culture Collection (ATCC) employed in this study were as follows: Aerobic: *Enterococcus faecalis* (ATCC 4082), *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). Anaerobic: *Porphyromonas gingivalis* (ATCC 33277), *Prevotella nigrescens* (ATCC 33563), *Bacteroides fragilis* (25285), *Fusobacterium nucleatum* (25586), *Actinomyces naeslundii* (ATCC 19039), and *Peptostreptococcus anaerobius* (ATCC 27337).

The EOs samples were dissolved in dimethyl sulfoxide (DMSO; Synth, São Paulo, Brazil) at 2000 μg/mL, followed by dilution in tryptic soy broth (Difco, Detroit, MI, USA) for aerobic bacteria and Schaedler broth (Difco) supplemented with hemin (5.0 μg/mL) and vitamin K1 (10.0 μg/mL) for anaerobic bacteria. Concentrations ranging from 2000 to 0.976 μg/mL were achieved. The final DMSO concentration was 5% (v/v); this solution was used as negative control. According to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2009), the inoculum was adjusted for each organism to yield a cell concentration of 5×10^5 colony forming units (CFU)/mL, for aerobic bacteria, and 10^6 colony forming units (CFU)/mL for anaerobic bacteria (CLSI, 2007). One non-inoculated well free of antimicrobial agent was also included to ensure medium sterility. Chlorhexidine dihydrochloride (CHD) (C8527, Sigma-Aldrich, St. Louis, MO, USA) was dissolved

in tryptic soy broth (Difco) and used as positive control at concentrations ranging from 59.0 to 0.115 μg/mL. The microplates (96 wells) containing different concentrations of the EOs and the aerobic microorganisms were sealed with a plastic film and incubated aerobically at 37 °C for 24 h, whereas the microplates containing the anaerobic microorganisms were sealed with a plastic film and incubated for 48–72 h in an anaerobic chamber (Don Whitley Scientific, Bradford, UK), in 5%–10% H_2, 10% CO_2, 80%–85% N_2 atmosphere, at 37 °C. After that, 30 μL of 0.01% resazurin in aqueous solution was poured into each microplate reservoir, to indicate microorganism viability. The minimum inhibitory concentration (MIC) values were determined as the lowest concentration of the EO capable of inhibiting microorganism growth. Before the addition of resazurin and in order to determine MBC, an aliquot of the inocolum was aseptically removed from each well and then plated onto Blood agar for aerobic bacteria and Shaedler agar for anaerobic bacteria. The plates were incubated as described previously (Ríos and Recio, 2005). Three replicate assays were accomplished for each microorganism. The sterility controls of TSB and Schaedler broths, culture control (inoculum), chlorhexidine dihydrochloride, and the EOs were performed. The microplates (96 well) were sealed with parafilm and incubated at 37 ^{o}C, for 24 h.

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Table S1. *In vitro* antibacterial activity (MIC/MBC) of the selected essential oils against aerobic oral bacteria (in µg/mL).

EO	***S. mutans***	***S. mitis***	***S. sanguinis***	***S. salivarius***	***S. sobrinus***	***E. faecalis***	***L. casei***
BC	1000/–	200/200	–/–	1500/–	1500/–	–/–	100/100
CA	1000/–	1000/–	–/–	–/–	–/–	–/–	1500/–
CB	–/–	–/–	–/–	1500/–	1500/–	–/–	100/100
CL	–/–	–/–	–/–	–/–	–/–	–/–	–/–
CM	–/–	1000/–	–/–	–/–	–/–	–/–	350/400
CP	–/–	–/–	–/–	–/–	–/–	–/–	–/–
CSe	–/–	–/–	–/–	–/–	–/–	–/–	–/–
CSi	–/–	–/–	–/–	–/–	–/–	–/–	–/–
CZ	200/400	350/350	1000/–	1000/–	1000/–	–/–	300/400
EG	–/–	–/–	–/–	–/–	–/–	–/–	–/–
MA	1500/–	–/–	–/–	1500/–	1500/–	–/–	–/–
PS	–/–	1000/–	–/–	–/–	–/–	–/–	–/–
SS	1500/–	1000/–	–/–	–/–	–/–	–/–	1500/–
TV	500/500	500/500	500/500	500/1000	1000/–	1000/–	500/500
ZO	1000/1000	–/–	1500/–	1500/–	–/–	–/–	1000/1000
CHD	7.37	7.37	14.75	7.37	7.37	7.37	14.75

BC: *Boswellia carteri* (Burseraceae); **CA:** *Cedrus atlantica* (Pinaceae); **CB:** *Citrus x bergamia* (Rutaceae); **CL:** *Citrus limonum* (Rutaceae); **CM:** *Commiphora myrrha* (Burseraceae); **CP:** *Citrus paradisi* (Rutaceae); **CSe:** *Cupressus sempervirens* (Cupressaceae); **CSi:** *Citrus sinensis* (Rutaceae); **CZ:** *Cinnamomum zeylanicum* (Lauraceae); **EG:** *Eucalyptus globulus* (Myrtaceae); **MA:** *Melaleuca alternifolia* (Myrtaceae); **PS:** *Pinus sylvestris* (Pinaceae); **SS:** *Salvia sclarea* (Lamiaceae); **TV:** *Thymus vulgaris* (Lamiaceae); **ZO:** *Zingiber officinale* (Zingiberaceae); **CHD**: chlorhexidine dihydrochloridrate.

Table S2. *In vitro* antibacterial activity (MIC/MBC) of the selected essential oils against anaerobic oral bacteria (in µg/mL).

EO[a]	***A. naeslundii***	***B. fragilis***	***F. nucleatum***	***P. anaerobius***	***P. gingivalis***	***P. nigrescens***
BC	–/–	–/–	–/–	1000/1000	250/250	250/250
CA	–/–	–/–	500/–	–/–	–/–	–/–
CB	–/–	–/–	–/–	–/–	–/–	500/–
CL	–/–	–/–	–/–	–/–	1000/1000	–/–
CM	250/250	–/–	250/250	1000/–	250/250	1000/–
CP	–/–	–/–	–/–	–/–	–/–	500/500
CSe	–/–	–/–	–/–	–/–	–/–	–/–
CSi	–/–	–/–	–/–	–/–	–/–	250/250
CZ	125/125	–/–	125/125	–/–	250/250	125/125
EG	–/–	–/–	1000/–	–/–	1000/1000	–/–
MA	–/–	–/–	–/–	–/–	–/–	250/250
PS	500/500	–/–	1000/–	–/–	–/–	1000/–
SS	–/–	–/–	2000/–	–/–	1000/1000	
TV	1000/1000	–/–	500/500	–/–	500/500	500/500
ZO	1500/1500	–/–	1000/–	500/500	500/500	125/125
CHD	7.37	7.37	7.37	7.37	7.37	7.37

[a] Abbreviations are the same as used in Table 1.

Table S3. Chemical composition of the essential oil from *Cinnamomum zeylanicum* (**CZ-EO**)..

Compound	**RI**	**% RA***	**Identification**
α-pinene	936	0.8	RL MS, Co
Camphene	951	0.4	RL MS
β-pinene	951	0.5	RL MS, Co
Limonene	931	0.2	RL MS, Co
Eucalyptol	1034	0.7	RL MS, Co
Linalool	1103	0.8	RL MS, Co
Camphor	1149	1.0	RL MS, Co
Borneol	1171	t	RL MS, Co
Terpinen-4-ol	1182	t	RL MS
α-Terpineol	1195	0.7	RL MS
(Z)-isosafrole	1315	85.3	RL MS
Cuparene	1512	0.3	RL MS
Spathulenol	1582	0.5	RL MS
Guaiol	1588	0.9	RL MS
NI	1623	1.9	RL MS
Torreyol	1647	1.0	RL MS
β-bisabolol	1652	0.5	RL MS, Co
Cadalene	1671	3.0	RL MS
NI	1678	0.5	RL MS
Alloaromadendrene oxide II	1697	0.6	RL MS

RI: retention indices relative to *n*-alkanes C_8-C_{20} on Rtx-5MS capillary column; **RA:** relative area (peak area relative to the total peak area in the GC-FID chromatogram); **RL:** comparison of the retention index with the literature; **MS:** comparison of the mass spectrum with the literature. Co: co-elution with authentic standards available in our laboratory; **tr**: trace (RA<0.1%). **NI**: not identified; * Average from three replicates.

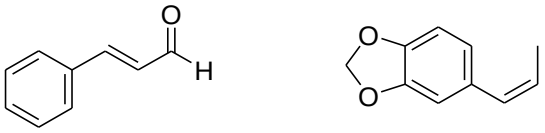

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Figure S1. Chemical structures of (*E*)-cynnamaldehyde (**1**) and (*Z*)-isosafrole (**2**).