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Essential Oil from *Psidium cattleianum* Sabine (Myrtaceae) Fresh Leaves: Chemical Characterization and *in vitro* Antibacterial Activity Against Endodontic Pathogens

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HIGHLIGHTS

- Viridiflorol, β -caryophyllene, 1,8-cineole and β -selinene were the major constituents found in PC-EO.
- Promising antibacterial activity of essential oil from *Psidium cattleianum* fresh leaves was evident, with MIC values below 100 $\mu\text{g}/\text{mL}$.
- Seven bacteria of endodontic interest were used.
- PC-EO exhibited high anti-*Aggregatibacter actinomycetemcomitans* activity (MIC = 6.25 $\mu\text{g}/\text{mL}$).

Abstract: Endodontic infections result from oral pathogenic bacteria which reach and infect dental pulp, as well as surrounding tissues, through cracks, unrepaired caries and failed caries restorations. This study aims to determine the chemical composition of essential oil from *Psidium cattleianum* leaves (PC-EO) and to assess its antibacterial activity against endodontic bacteria. Antibacterial activity of PC-EO was evaluated in terms of its minimum inhibitory concentration (MIC) values by the broth microdilution method on 96-well microplates. Bacteria *Porphyromonas gingivalis* (MIC = 20 $\mu\text{g}/\text{mL}$), *Prevotella nigrescens* (MIC = 62.5 $\mu\text{g}/\text{mL}$), *Fusobacterium nucleatum* (MIC = 12.5 $\mu\text{g}/\text{mL}$), *Actinomyces naeslundii* (MIC = 50 $\mu\text{g}/\text{mL}$), *Bacteroides fragilis* (MIC = 12.5 $\mu\text{g}/\text{mL}$), *Aggregatibacter actinomycetemcomitans* (MIC = 6.25 $\mu\text{g}/\text{mL}$) and

Peptostreptococcus anaerobius (MIC = 62.5 µg/mL) were evaluated and compared to chlorhexidine dihydrochloride (CDH), the positive control. PC-EO was obtained by hydrodistillation with the use of a Clevenger-type apparatus whereas its chemical composition was analyzed by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectrometry (GC-MS). Viridiflorol (17.9%), β-caryophyllene (11.8%), 1,8-cineole (10.8%) and β-selinene (8.6%) were the major constituents found in PC-EO, which exhibited high antibacterial activity against all endodontic pathogens under investigation. Therefore, PC-EO, a promising source of bioactive compounds, may provide therapeutic solutions for the field of endodontics.

Keywords: Endodontic infections; Chlorhexidine dihydrochloride; Pathogenic bacteria; *Aggregatibacter actinomycetemcomitans*; Alternative medicine.

INTRODUCTION

Endodontic therapy aims at total elimination or significant mitigation of pathogenic microorganisms with the use of chemical-mechanical preparations. Bacteria which cause primary and secondary intraradicular infections have been considered the main etiological agents of pulp and periapical pathologies, since they make infections persist due to endodontic treatment failure and/or coronary microinfiltration, which enables microbial re-contamination of radicular canals [1,2].

Epidemiological data have shown that from 30% to 50% of failures in conventional endodontic therapy are related to emergent, recurrent and persistent infections. Secondary infection is caused by microorganisms that are not part of the microbiota of primary infection but are carried to the system of radicular channels either during appointments or after the end of the endodontic treatment [3]. On the other hand, persistent infection is the one that persists even after disinfection procedures are carried out and after consequent changes in the microenvironment resulting from the endodontic treatment. Several studies have shown that both infections have mixed microbiota, but with less diversity and with predominance of Gram-positive bacteria [3]. Additional strategies are needed because microorganisms are found in regions which are inaccessible to conventional clinical procedures, intracanal medication, chemical substances and instrumentation, which leads to failure in endodontic therapy. The most cited treatments in the literature are the use of photodynamics therapy, use of chlorhexidine as final irrigant and ultrasonic activation of the irrigation solution. Despite all strategies to fight infection and avoid re-infection, some microorganisms, such as *Enterococcus faecalis*, can keep in latency state, with shortage of nutrients for long periods, and become pathogenic again when conditions in the microenvironment get favorable [3].

Medicinal plants have been used for treating several human diseases worldwide for thousands of years. Thus, natural products that derive from them have been promising alternatives in the development of new chemical products which have important pharmaceutical applications. In the area of health, professionals in Dentistry have effortfully searched for alternative treatments based on the use of bioactive compounds derived from plants [4]. Since essential oils extracted from several plant species exhibit high biological activity against different microorganisms which cause pathologies, they have drawn researchers' attention in the field of Dentistry [5].

Psidium cattleianum Sabine, whose popular name is strawberry guava, (*araçá*, *araçá-do-campo* and *araçá-vermelho* in Brazilian Portuguese), is a member of the family Myrtaceae, which occurs naturally in Brazil, mainly in the Cerrado biome. The strawberry guava tree, a herbaceous species, may reach 2.5 m in height [6]. Its abundant fruits, which are small, globose and carry many seeds, have already been studied by sensory analysis, gas chromatography-olfactometry (GC-O) and quantitative analysis [7]. Even though strawberry guavas resemble guavas (*Psidium guajava*), the former are smaller, their taste is more acid and their odor is more intense, but both species are very rich in vitamin C [6].

Recent studies have shown that *Psidium cattleianum* has been employed as a medicinal plant because it has different biological activities, such as its peripheral analgesic potential, besides its antitumoral and antimicrobial activity against several microorganisms. In addition, its anticariogenic activity in rats has been described. In Brazil, it has been used as a popular home remedy for treating diarrhea, helping healing processes, regenerating tissue lesions, alleviating symptoms and soothing pain (as an analgesic). An ethnobotanic survey of species that belong to the genus *Psidium* in different parts of the country revealed that people have used leaves and barks directly, either by chewing them or by making tea (effusion). Topical use of the plant in either aqueous or alcoholic solution to relieve pain, such as toothache, upset stomach, sore throat and abdominal pain, was also reported [6]. Despite several applications of this species of Myrtaceae, antibacterial activity of essential oil from *P. cattleianum* leaves (PC-EO) against endodontic

pathogens has not been investigated yet, a fact that makes the study reported by this paper even more relevant.

A part of an ongoing project of biological activity of essential oils extracted from different plant species [8,9], this study investigated chemical constituents and antibacterial activity of PC-EO against a representative panel of endodontic pathogens.

MATERIAL AND METHODS

Plant Material

Psidium cattleianum leaves were collected in Limeira, (22°33'53"S and 47°24'06"W), São Paulo state (SP), Brazil, on September 15th, 2017, at 8 am. Voucher specimens of *P. cattleianum* (GERAES-20) were deposited in the herbarium that belongs to the Biology Department at the Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais, located in Machado, Minas Gerais, Brazil. Access activities were registered at SisGen (no. AEACDCA).

Extraction of essential oil

Samples of *P. cattleianum* leaves were subjected to hydrodistillation by a Clevenger-type apparatus for 2 hours. In order to carry out the analysis, 250 g plant material was divided into five 50-g samples and 500 mL distilled water was added to each sample. After manual collection of samples of essential oil, traces of remaining water in the oils were removed with anhydrous sodium sulfate, which was followed by filtration. The isolated oil was stored under refrigeration up to the analysis and test. Yield (w/w) was calculated from fresh leaf weight and expressed as the average of the triplicate analyses.

Identification of chemical composition of essential oil

Gas chromatography (GC) analyses were performed by a Shimadzu GC2010 Plus gas chromatograph equipped with an AOC-20s autosampler and fitted with a 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.; 0.25- μ m 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 μ L (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 μ m 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 μ L (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. The complete methodology of GC-MS and GC-FID is the one that has already been described by the authors of this study [8,9].

Identification of volatile components of *P. cattleianum* leaves (Table 1) 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 (C₈-C₂₀). 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 [10].

Bacterial strains and antimicrobial assays

Minimum Inhibitory Concentration (MIC), which is the lowest concentration of the test compound that is capable of inhibiting microorganism growth, of PC-EO was determined in triplicate. The broth microdilution method was employed on 96-well microplates. Standard strains were acquired from the American Type Culture Collection: *Porphyromonas gingivalis* (ATCC 33277), *Prevotella nigrescens* (ATCC 33563), *Fusobacterium nucleatum* (ATCC 25586), *Bacteroides fragilis* (ATCC 25285), *Actinomyces naeslundii* (ATCC 19039), *Aggregatibacter actinomycetemcomitans* (ATCC 43717) and *Peptostreptococcus anaerobius* (ATCC 27337). Culture media employed for the representative strains of endodontic infections were Schadler broth and Schadler agar (Difco), both supplemented with hemin (5.0 μ g/mL, Sigma, St. Louis, MO, USA),

vitamin K1 (10 µg/mL, Sigma) and sheep blood (5%, Bio Boa Vista, Valinhos, SP, Brazil), as recommended by the Clinical Laboratory Standards Institute [10]. Samples were dissolved in dimethyl sulfoxide (DMSO) 1.0 mg/mL and diluted in the broth. Concentrations ranged from 6.25 to 62.5 µg/mL. Final DMSO content was 5 % (v/v), and this solution was used as negative control. Chlorhexidine dihydrochloride (CDH) was used as positive control and MIC varied from 1.844 to 14.75 µg/mL. The inoculum was adjusted to each organism, in order to yield cell concentration of 5×10^5 colony forming units (CFU) mL⁻¹ for aerobic and anaerobic facultative strains and 5×10^6 CFU mL⁻¹ for anaerobic strains, in agreement with a previous standardization carried out by the CLSI [11, 12]. Anaerobic strains were incubated in an anaerobic chamber (Don Whitley Scientific, Bradford, UK) for 72 h, in atmosphere with 5–10 % H₂, 10% CO₂ and 80–85 % N₂.

RESULTS AND DISCUSSION

Both GC-MS and GC-FID analyses identified thirty-one compounds in PC-EO, representing 95.4 % of its total compounds (Table 1). Table 1 shows these constituents with their retention indices, retention times and percentages.

Table 1. Chemical composition of essential oil from *Psidium cattleianum* fresh leaves

Compounds	RT (min)	RI _{calculated}	RI _{literature}	%RA
Hex-2(E)-enal	6.09	847	848	0.8
Myrcene	13.62	992	992	1.2
1,8-Cineole	16.35	1036	1036	10.8
Linalool	20.42	1104	1104	1.3
Butanoic acid, 3-methyl-, 3-methyl-3-butenyl ester	21.33	1117	1116	0.6
<i>cis-p</i> -Menth-2-en-1-ol	21.56	1121	1121	0.1
1-Terpineol	22.59	1136	1134	0.1
<i>p</i> -Menth-1-en-8-ol	24.12	1157	1162	0.2
1-Terpinen-4-ol	24.66	1165	1160	1.3
α-Terpineol	25.51	1177	1177	3.2
Carvone	28.13	1223	1223	0.1
α-Terpinyl acetate	33.09	1337	1336	2.6
Neryl acetate	33.69	1351	1351	0.2
α-Copaene	34.23	1364	1364	2.5
Neryl acetate	34.64	1373	1371	1.5
Longifolene	35.10	1384	1380	0.2
α-Gurjunene	35.68	1398	1398	1.4
β-Caryophyllene	36.47	1417	1418	11.8
Aromadendrene	37.10	1433	1433	5.0
α-Humulene	37.72	1449	1449	6.0
α-Muurolene	38.24	1462	1471	0.4
γ-Muurolene	38.59	1471	1471	2.8
β-Selinene	39.12	1484	1484	8.6
γ-Cadinene	40.09	1509	1509	2.6
Caryophyllene oxide	41.67	1551	1555	2.2
Nerolidol	42.34	1568	1568	2.5
Viridiflorol	43.37	1595	1595	17.9
Humulene epoxide II	44.12	1616	1609	4.1
α-Bisabolol	46.65	1686	1686	2.0
Farnesol	47.87	1721	1722	0.6
α-Cyperone	49.05	1755	1752	0.8

Monoterpene hydrocarbons	1.2
Oxygenated monoterpenes	19.9
Sesquiterpene hydrocarbons	42.8
Oxygenated sesquiterpenes	30.1
Others	1.4
Total	95.4

RT(min): Retention time in minutes; **RI_{calculated}**: Retention index relative to *n*-alkanes (C₈–C₂₀) on the Rtx-5MS column; **RI_{literature}**: Retention index found in the literature [10]. **%RA**: relative area.

PC-EO yielded (w/w on fresh weight basis) 0.3%, the same content found in essential oil from *Psidium laruotteanum* dry leaves, another species that belongs to the same genus and family [13]. Both GC-FID and GC-MS analyses revealed that sesquiterpene hydrocarbons (42.8 %) were the major constituents of PC-EO, followed by oxygenated sesquiterpenes (30.1%) and oxygenated monoterpenes (19.9 %). The major constituents of PC-EO were viridiflorol (17.9%, **1**), β -caryophyllene (11.8 %, **2**), 1,8-cineole (10.8%, **3**) and β -selinene (8.6%, **4**) (Figure 1, Table 1).

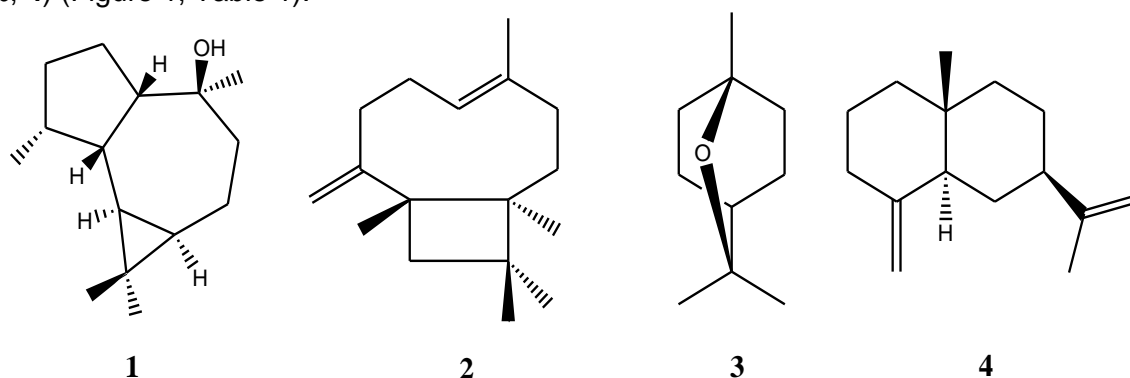


Figure 1. Chemical structures of major constituents of PC-EO: viridiflorol (**1**), β -caryophyllene (**2**), 1,8-cineole (**3**) and β -selinene (**4**).

Chemical composition of PC-EO collected in Limeira, SP, was similar to the ones from specimens grown in other Brazilian regions. The following major constituents were identified by Marques and coauthors [14] in essential oil from dry leaves of this species: α -thujene (25.2%), 1,8-cineole (16.4%) and β -caryophyllene (10.2%); however, only β -caryophyllene and 1,8-cineole were identified in PC-EO. Scur and coauthors [15] studied PC-EO collected in Paraná state, Brazil, and found the major constituents: terpenes α -copaene (22%), eucalyptol or 1,8-cineole (15%), δ -cadinene (9.63%) and α -selinene (6.5%). Regarding biological applications, Castro and coauthors [16] found that PC-EO exhibits promising antifungal and antioxidant activities, with no *in vitro* cytotoxicity. Scur and coauthors [15], however, reported that PC-EO was inactive against the following bacteria: *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Bacillus subtilis*, besides yeast *Candida albicans*.

The comparison between the chemical composition of PC-EO and the one of essential oil from the species collected in other countries showed different chemical profiles. Pino and coauthors [17] analyzed the phytochemical composition of *P. cattleianum* collected in Cuba and identified 18 compounds. Its major compounds were epi- α -muurolol (21.9%), α -cadinol (20%), epi- α -cadinol (16.7%) and caryophyllene (13.6%); it does not agree much with the study reported by this paper regarding the compounds, since only caryophyllene was the major compound in PC-EO. Two studies carried out by Chalannavar and coauthors [18,19] showed that PC-EO from KwaZulu-Natal, a province in South Africa, exhibited the following major constituents: caryophyllene oxide (29.56%), alloaromadendrene oxide (6.82%), 12-oxabicyclo [9.1.0] dodeca-3,7-diene (5.85%) and 1*H*-cycloprop[e]azulene (3.49%), caryophyllene oxide (12.43%), while other predominant constituents were bicyclo(4.4.0)dec-1-ene (6.61%), 2,3-butanediol diacetate (4.84%) and patchoulene (4.73%). PC-EO from Egypt had α -pinene (28.0%), β -myrcene (13.40 %) and β -caryophyllene (28.83 %) as its major constituents. Besides, it exhibited high antimicrobial activity against *Neisseria gonorrhoeae* [20].

A broad analysis enables the comparison between the chemical composition of PC-EO and the ones of other essential oils extracted from different *Psidium* species. β -caryophyllene (7.4%) is also the major

constituent of essential oil from *Psidium myrsinites* [21]. A study carried out by Medeiros and coauthors [13] showed that essential oil from *Psidium laruotteanum* leaves had high concentration of monoterpenes, such as *p*-cymene (19.4-34.8%), 1,8-cineole (6.9-19.2%) and α -pinene (9.2-11.4%), differently from PC-EO, which had high concentration of sesquiterpene hydrocarbons (42.8%). In addition, essential oil of *P. guineense* exhibited much variability in its chemical constituents. A recent study found the following: α -pinene, myrcene, limonene, β -caryophyllene, caryophyllene oxide, α -copaene, *ar*-curcumene, β -bisabolene, muurolo-4,10(14)-dien-1- β -ol, *epi*- β -bisabolol and β -bisabolol [22]. Only both constituents β -caryophyllene and 1,8-cineole were common to essential oils from most *Psidium* species previously mentioned.

Much variability in chemical composition of essential oils is expected, since it depends on several factors, such as local climate and environmental conditions, soil variation, season, geographical location, geology, stages of vegetative cycles, parts of plants and the method used for extracting them [23].

Concerning the biological activity of PC-EO, *in vitro* antibacterial activity (MIC values, see Table 2) was evaluated against a representative panel of endodontic pathogens. Results were compared with CDH as positive control.

Table 2. Minimum inhibitory concentration (MIC = $\mu\text{g/mL}$) of PC-EO against endodontic bacteria under investigation

Bacteria	Minimum inhibitory concentration (MIC) - $\mu\text{g/mL}$	
	PC-EO	CDH*
<i>Porphyromonas gingivalis</i>	20	3.688
<i>Prevotella nigrescens</i>	62.5	1.844
<i>Fusobacterium nucleatum</i>	12.5	3.688
<i>Bacteroides fragilis</i>	12.5	14.75
<i>Actinomyces naeslundii</i>	50	3.688
<i>Peptostreptococcus anaerobius</i>	62.5	1.844
<i>A. actinomycetemcomitans</i>	6.25	1.844

PC-EO: Essential oil from *Psidium cattleianum* fresh leaves; **CDH*:** chlorhexidine dihydrochloride (positive control)

All MIC values of PC-EO were below 100 $\mu\text{g/mL}$. Results are remarkable, since the literature has reported that samples whose MIC values are below 100 $\mu\text{g/mL}$ are strong and may act as antibacterial agents [24-26]. Based on these criteria, PC-EO exhibited high activity against *P. gingivalis* (MIC = 20 $\mu\text{g/mL}$), *P. nigrescens* (MIC = 62.5 $\mu\text{g/mL}$), *F. nucleatum* (MIC = 12.5 $\mu\text{g/mL}$), *B. fragilis* (MIC = 12.5 $\mu\text{g/mL}$), *A. naeslundii* (MIC = 50 $\mu\text{g/mL}$), *A. actinomycetemcomitans* (MIC = 6.25 $\mu\text{g/mL}$) and *P. anaerobius* (MIC = 62.5 $\mu\text{g/mL}$) (Table 2). It should be highlighted that these pathogenic bacteria generate complicated endodontic infections which result from the easy access microorganisms have to the dental pulp, as well as surrounding tissues, via tooth cracks, unrepaired caries and failed caries restorations [27-28].

The mechanism of action of essential oils which leads to many important biological activities has not been clarified, but it is known that such activities may be associated with lipophilicity of chemical constituents found in essential oils, mainly monoterpenes and sesquiterpenes, which are often their main classes of compounds [29]. Lipophilia enables essential oils to propagate through cell membranes and cause the death of microorganisms, since they affect their metabolic paths and organelles. It is also worth emphasizing that essential oils are capable of inhibiting syntheses of DNA, RNA, proteins and polysaccharides in bacterial cells [30].

The sesquiterpene viridiflorol (17.9%) had its antibacterial activity proven when essential oil from *Allophylus edulis* leaves was investigated. This oil exhibited activity against *Mycobacterium tuberculosis*, besides antioxidant and anti-inflammatory potential, attributed to viridiflorol [31]. The second major constituent of PC-EO is β -caryophyllene (11.8%), which has already been identified at high contents in essential oils from species of Verbenaceae; these oils exhibited moderate antibacterial activity against certain types of bacteria, such as *Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli* [32]. The third major terpene of PC-EO is 1,8-cineole (10.8%), which had been identified in eucalyptus oil by a study that proved its antibacterial potential and suggested that it can improve the activity of some antiseptics [33]. Finally, β -selinene (8.6%), the fourth major constituent of PC-EO was also found to be a major one of essential oil from *Zanthoxylum caribaeum* leaves. Besides, Souza and coauthors described its antibacterial potential against serotypes of *Salmonella* [34]. The synergic effect among all constituents must also be taken into consideration, since it is responsible for potentializing different biological activities exhibited by essential oils [35]. Thus, this study, together with a recent one carried out by Buso-Ramos and coauthors, concludes that

P. cattleianum is really a promising species in the development of new antimicrobial agents of natural origin which exhibit strong activity against oral bacteria [36].

CONCLUSION

The PC-EO showed viridiflorol, β -caryophyllene, 1,8-cineole and β -selinene as the majority components and high *in vitro* antibacterial activity against *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Actinomyces naeslundii*, *Aggregatibacter actinomycetemcomitans* and *Peptostreptococcus anaerobius*. Future work to characterize this activity and its mechanism of action will be important and may lead to new therapeutic approaches for control of endodontic and other infections. In sum, further studies that aim at isolating, identifying and testing active chemical constituents of PC-EO are underway.

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