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Chemical composition and biological effect of essential oils from *Pachygone ovata* (Poir.) Miers ex Hook.f. & Thomsonon oral biofilm bacteria and on the cell viability of raw 264.7 macrophages

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Abstract

Essential oils (EOs) have the ability to reduce preformed dental biofilm, delay its development and, consequently, periodontal diseases. The chemical composition and biological effects of essential oils from the bark (EOB) and leaf (EOL) of *Pachygone ovata* (Poir.) Miers ex Hook.f. & Thomson were evaluated in this work. The GC-MS technique determined the chemical composition. The antimicrobial activity of the EOs was evaluated against selected gram-positive and gram-negative bacteria by the microdilution method, specifying the minimum inhibitory concentrations (MICs). Antibiofilm activity with F. nucleatum was determined on polystyrene microplates, and the MTT test evaluated the cytotoxic activity on macrophages. The GC-MS data showed that EOL and EOB have the same chemical constitution, but EOL has a higher proportion of phytoconstituents, mainly α-pinene (53.69%). *F. nucleatum* was the pathogen that presented the lowest MIC, 250 µg/ml for EOB and 62.5 µg/ml for EOL. In the antibiofilm activity, both oils inhibited biofilm formation between 1000-125 µg/ml, and in the concentration of 50 µg/ml, only the EOB did not present cytotoxicity on macrophages. So, the EOs from *P. ovate* showed anti-adherent and antibacterial activity against several bacteria constituting dental biofilms and can be used to treat periodontal diseases.

1. Introduction

Until recently, *Pachygone ovata* (Poir.) Miers ex Hook.f. & Thomson was a little-known member of the Menispermaceae family, but that has changed. *P. ovata* is a genus of plants that can be found in the southern Indian states of Tamil Nadu, Karnataka, and Andhra Pradesh (Dasgupta *et al*., 2004). The dried fruit of this plant was considered medicinally important by the ancient people because it was used as a fish poison and vermicide; the leaves were used to lower body temperature and improve fertility; and it has a variety of pharmacological actions, including analgesic, CNS stimulant, and treatment of hypothermia and leucorrhoea. Evidence for the pharmacological features of *P. ovata* is limitedly supported by scientific evidence (Marahel *et al*., 2014). Consequently, this investigation was carried out to determine the plant's phytochemical activity and antioxidant potential. It is one of the few plants that has not been adequately explored in terms of active phytoconstituents is a source of pride (Bushra, *et al.*, 2020). In the stems, leaves, and roots of *P. ovata*, researchers discovered a range of benzyl-isoquinoline-derived alkaloids that were shown to be present (Bhat *et al.*, 2004; Elhan Khan *et al.*, 2021). So, given the biological perspectives in the use of plants of the genus *P. ovate*, this research evaluated the antibacterial activity against aerobic and anaerobic pathogens of the oral cavity (Umesha *et al.*, 2013). The cytotoxic aspects in macrophage cultures and the chemical characteristics of essential oils obtained from bark and leaf of *P. ovate*.

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2. Materials and Methods

2.1 Plant material collection and authentication

The raw material of *P. ovata* was (Figure 1) collected from Tirupati, the Eastern Ghats of Andhra Pradesh, in October 2018 and authenticated by Professor T N Mary (Retd, Head Dept. of Botany Acharya Nagarjuna University). The herbarium voucher number is ANBH 121/2018. We dried the samples (leaf and stem parts) in the shade at room temperature for a full one month before using them.

2.2 Obtaining essential oils

Samples of the bark and leaves of *P. ovata* were collected, stored in thermal boxes to preserve their volatile constituents, and then submitted to the hydrodistillation process in a clevenger apparatus to obtain the essential oils. In this extractive method, the proportion

of 200 g of plant material to 800 ml of distilled water was used. The oils were collected by liquid:liquid separation by decanting. The yield of this extractive method was calculated as a function of time to improve the hydrodistillation process. After collection, the essential oil samples were stored in a freezer at –15ºC, to preserve their volatile constituents.

Figure 1: Habit of *P. ovata*.

2.3 Gas chromatography coupled to a mass spectrometer (GC - MS)

For quantitative and qualitative chemical evaluation, the CG-MS-QP 2010® device was used, with the ion source-detector at 200°C and the interface at 280°C, in scan mode: 40-80 m/z. An RTX SMS® column, 30 m long, 0.25 mm in internal diameter and 0.25 µm in particle size, with an oven at 80°C, was used; the injection gas was He (helium), at a flow of 1 ml/min^{-1}. The sample was submitted to a pressure of 65 kpA, with a linear velocity of 36.8 cm.s^{-1}; with split injection mode, at a ratio of 1:50, injection volume of 0.3 µl and a run time of 63 min. The oven was programmed using an isothermal method. For the analysis of essential oils, 10 mg of the oils (OEC and EOL) were solubilized in 500 µl of ethyl acetate, before injecting them into the equipment. In addition, the injection of a homologous series of hydrocarbons (C8-C20) as a reference was used for the identification, for the calculation of the retention index (RI), according to the following formula:

$$I = 100 \times \left[n + (N - n) \frac{t_{r\,(unknown)}}{t_{r(N)} - t_{r\,(n)}} \right]$$

where

I = retention index; n = number of carbon atoms of the lower n-alkane series; N= number of carbon atoms in the larger n-alkane series; $t_{r(unknown)}$ = retention time of the unknown compound; $t_{r(n)}$ = carbon retention time of the smallest n-alkane series; $t_{r(N)}$ = carbon retention time of the larger n-alkane series.

The Retention Indexes (RI) of the compounds present in the essential oil samples were calculated and, together with the fragmentation patterns determined by the GC-MS, the compounds were identified by comparing them with the values presented in the literature using NIST 05 mass spectral library.

2.4 Evaluation of antimicrobial activity against oral pathogens

In the evaluation of antimicrobial activity, ATCC standard strains of *Streptococcus parasanguinis* (15912), *Streptococcus salivarius* (9759), *Streptococcus oralis* (35037) and *Streptococcus mutans* (55677) were used, using the microdilution method. The periodontal pathogens represented by *Prevotella intermedia* (*33277*), *Treponema denticola*(35405), *Fusobacterium nucleatum* (25586) and *Eubacterium nodatum* (33009), using the microdilution method for anaerobic bacteria described by clinical standards. All *Streptococcus* sp. were cultivated in BHI broth (Brain Heart Infusion), under microaerophilic conditions, in an anaerobic jar, at 37 ± 0.5ºC for 24 h. Anaerobic microorganisms were cultivated in TSB (Trypticase Soy Broth) containing 1 mg/ml of yeast extract, 5 µg/ml of hemin and 1 µg/ml of menadione, under anaerobic conditions, with an atmosphere of 90% N_2, 5% CO_2, and 5% H_2, for 48 h at 37 ± 0.5°C. The bacterial inoculum for *Streptococcus* sp. was used at a concentration of 1.0 ×10^6 CFU/ml, and for period on to pathogens at a concentration of 5×10^8 CFU/ml, standardized in a spectrophotometer at a wavelength of 625 nm. Microdilution was carried out in 96-well microplates (TPP®), using test solutions at concentrations of 4000 µg of the samples of essential oils from the bark (OEC) and from the leaf (EOL): 1 ml of solvent (100 µl of 96% alcohol: 900 µl of distilled water), with eight serial dilutions being carried out in the wells of the respective microplate columns. Solvents were used as a negative control and 0.12% chlorhexidine digluconate (Merck) as a positive control. The tests were performed in triplicate, and the microplates were incubated in anaerobic conditions for 48 h at 37 ± 0.5ºC. After this period, to determine the minimum inhibitory concentration (MIC), 20 µl of resazurin (Merck) at 0.01% was added, verifying the colorimetric reduction of the dye to blue in the wells where there was no presence of viable bacteria (Toole *et al.*, 2021).

2.5 Antibiofilm activity against *Fusobacterium nucleatum*

In this analysis, it was necessary to use saliva, collected from the researcher, which followed the protocol by Marahel *et al.* (2016). In the biofilm assay, strains of *F. nucleatum* (ATCC 25585) were used, under the same cultivation conditions and inoculum concentration, described in item 2.5, following the protocol of He *et al.* (2016), with some modifications. First, 100 µl of saliva were pipetted into 96-well plates, kept at room temperature for 2 h. After this period, the salivary content was removed and the wells washed once with 100 µl of PBS buffer, then added 100 µl of TSB medium (containing 1 mg/ml of yeast extract, 5 µg/ml of hemin, 1µg/ml of menadione and 5% of glucose); 100 µl of the OEC and EOL sample solutions to be tested at various concentrations; and 100 µl of bacterial inoculum 5.0 × 10^8 CFU/ml. The tests were performed in triplicate and the plates were kept at 37 ± 0.5°C under anaerobic conditions. After this period, the contents of the wells were removed and they were washed twice with sterile saline solution to remove weakly adhered bacteria and kept at room temperature for drying. After drying, 200 µl of 1% crystal violet was added, kept for 40 min, then the wells were washed twice with sterile saline solution and kept at room temperature for 20 min for drying. Then, the contents of the wells were resuspended with 200 µl of ethyl alcohol at 95° for 40 min, after which 150 µl of the contents of the wells were transferred to another microplate, in which the optical density was measured using a spectrometer (Biotrak II Plate Reader) with a wavelength of 625 nm. The absorbance data of the wells with test solutions were compared to the wells with culture medium and without inoculum and to the wells with culture medium+inoculum; 0.12% chlorhexidine (Merck) was used as a positive control.

2.6 Macrophage cytotoxicity test

The experimental procedures of cell viability tests followed the methodology of He *et al.* (2016) proposed. Murine macrophages (RAW 264.7) in modified Dulbecco's Eagle Medium - DMEM (GIBCO®, United Kingdom) plus 10% fetal bovine serum (GIBCO®) and streptomycin (100 µg/ml) were used since these cells are related to the body's immune response in the development of periodontal diseases.

Cells were exposed to different concentrations of EOB and EOL in DMEM plus 1% fetal bovine serum (GIBCO®), assays were performed in sextuplicate and microplates were incubated for 48 h. After this period, the contents of the wells were discarded, and they were submitted to the MTT colorimetric test. Mitochondrial viability, and consequently, cell viability, was quantified by reducing MTT (a yellow-colored, water-soluble salt) to formazan (purple-colored, water-insoluble salt) by the activity of those enzymes. Thus, the reduction of MTT to formazan was considered directly proportional to mitochondrial activity and cell viability (Shailaja *et al.*, 2021).

2.7. Statistical analysis

For statistical analysis, the SPSS statistics software (The IBM® USA), version 16.0, Chicago, USA, was used, through which a database was created, and the averages of the results obtained in the tests of antibiofilm activity and cell viabilitywere analyzed through the ANOVA, with a significance level of 0.05.

3. Results

The major compounds of the essential oils of *P. ovata* were represented by: terpenes such as α-pinene (EOB:58.64%; EOL:53.69%), β-pinene (EOB:5.47%; EOL:5.57%) and D-limonene (EOB:4.61%; EOL:4.68%) and bicyclic sesquiterpenes such as γ-cadinene (EOB:6.98%; EOB:7.14%) and ε-cadinene (EOB:7.16%; EOL:1.78%). The thermal isomerization of β-pinene it possible to synthesize myrcene and D-limonene, which are used as intermediates to obtain terpene alcohols and chemical aromas. Although, it is not possible to elucidate the mechanisms for obtaining such compounds, myrcene was observed in the proportion of 1.32% in the EOB and 1.43% in the EOL.

Table 1: Essential oil constituents of the bark (EOB) and leaf (EOL) of *P. ovata* identified by GC-MS

Name of the compound	EOB			EOL		
	RT(min)	%	RI	RT(min)	%	RI
α-pineno	5.96	58.64	933.7	5.98	53.69	934.4
Canfeno	6.34	1.6	947.5	6.35	1.67	948
β-pineno	7.12	5.47	976.2	7.14	5.57	976.7
mirceno	7.49	1.32	989.6	7.5	1.34	990.1
D-limoneno	8.74	4.61	1026.8	8.76	4.68	1027.2
Cânfora	13.14	1.02	1142.3	13.15	0.98	1142.5
borneol	14.02	1.25	1163.5	14.03	1.19	1163.8
α-copaeno	22.8	2.19	1372.4	22.81	2.14	1372.7
(E)-cariofileno	24.59	3.2	1415.7	24.6	3.12	1415.8
γ-cadineno	27.09	6.98	1477.3	27.1	6.97	1479.5
ε-cadineno	28.76	7.16	1519.8	28.77	1.78	1520

R_T min: Retention time (in minutes); (%): Percentage of the total integrated area for the chromatogram; RI: Retention index

Table 2: Minimum inhibitory concentrations (MICs) of EOB and EOL against period on to pathogens and *Streptococcus* sp.

	MIC (µg/ml)			
Essencial oils	***S. mutans***	***S. oralis***	***S. parasanguinis***	***S. salivarius***
EOB	1000	500	500	>1000
EOL	500	500	500	>1000
	Treponema denticola	***P. intermedia***	***Eubacterium nodatum***	***F. nucleatum***
EOB	250	500	>1000	250
EOL	125	250	>1000	62,5

According to the GC-MS analyses, the EOB samples present a smaller amount of chemical constituents proportionally. In the evaluation of the activity of essential oils against the bacteria involved in the formation of dental biofilm (Table 2), there was a greater inhibitory action of the oils on the periodontopathogens, late colonizers, concerning *Streptococcus* sp., which are initial colonizers in the formation of dental plaque. This more effective action on gram-negative pathogens, such as *F. nucleatum* and *Treponema denticola*, may be related to the mechanism of action of essential oils, which are soluble in the bilipid layer of the cell membrane, and therefore, can destabilize and consequently, break or penetrate the lipid structure present in gram-negative bacteria,

which does not occur in gram-positive microorganisms, provided with a peptidoglycan barrier (Amalarasi *et al* ., 2019). Most essential oil ingredients have antibacterial properties (Yamina, 2019; Sethumathi *et al.*, 2021)) because they alter the structure of microbes' cell walls. For example, changing the gradient of hydrogen and potassium (H^+ and K^+ ions) alters the permeability of the cytoplasmic membrane. It disrupts essential cell processes such as electron transport and protein translocation. This results in the loss of chemiosmotic control in the affected cell and, consequently, cell death (Hancock *et al.*, 2021; Langeveld *et al.*, 2013).

F. nucleatum was the most sensitive microorganism; among the bacteria tested, it presented MIC of 250.0 µg/ml for EOB and 62.5 µg/ml for EOL. Data on antiadherent activity, in which a monospecies biofilm with this pathogen was developed, demonstrated that these oils are effective in planktonic and adherent cultures.

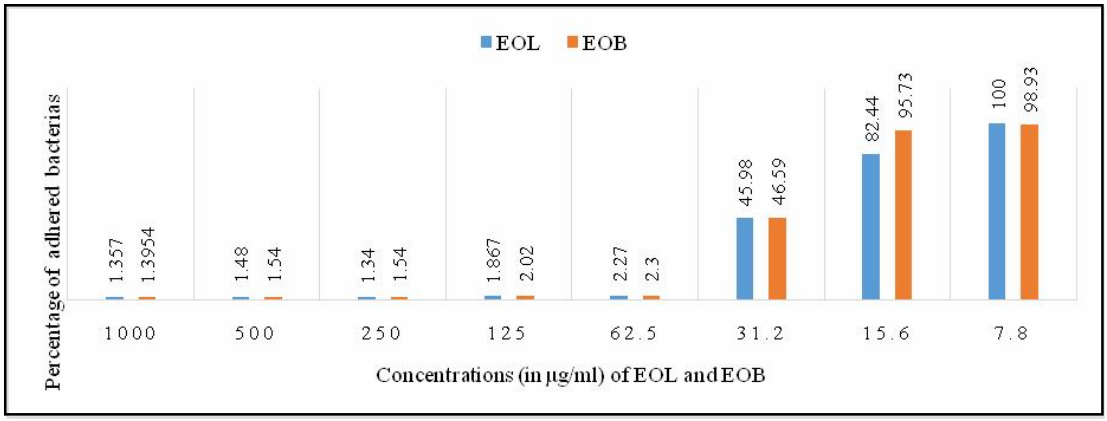

Figure 2: Percentage of *F. nucleatum*, adhered after 24 h of treatment, with different concentrations (in µg/ml) of EOB and EOL.

Table 3: Relationship of the mean cell viability for RAW 264.7 macrophages between the various concen-trations tested in the EOB and EOL samples

Tested concentration (µg/ml)	EOL concentration Mean ± SD	EOB concentration Mean ± SD	*p*-value
1000	16.26 ± 3.32	15.52 ± 2.33	0.766
500	16.89 ± 2.07	14.55 ± 1.97	0.23
100	14.85 ± 0.62	12.21 ± 1.30	0.34
50	24.00 ± 3.34	98.63 ± 9.02	0.000*
10	119.5 ± 1.54	176.46 ± 20.18	0.008*
5	101.02 ± 0.86	125 ± 3.14	0.000*
1	103.67 ± 10.35	117.21 ± 11.38	0.202

SD: Standard deviation; **p*-value: significant values obtained without ANOVA, with a significance level of 0.05.

Table 4: Relation of the averages of the percentages of *F. nucleatum* adhered, between the different concentrations tested of the samples of EOB and EOL

Tested concentration (µg/ml)	EOL Mean ± SD	EOB Mean ± SD	p-value
1000	1.48 ± 0.56	1.54 ± 0.94	0.12
500	1.34 ± 0.85	1.54 ± 1.21	0.85
250	1.86 ± 0.77	2.02 ± 0.42	0.055
125	2.27 ± 0.81	2.30 ± 0.64	0.066
62.5	45.98 ± 1.07	46.59 ± 0.96	0.238
31.2	82.44 ± 12.85	95.73 ± 2.45	0.055
15.6	101.42 ± 6.38	98.93 ± 8.76	0.213

SD: Standard deviation; **p*-value: significant values obtained without ANOVA, with a significance level of 0.05.

The biological behavior of the EOL is confirmed by the greater antibacterial activity, as given in Table 4, and the greater cytotoxic effect, as shown by the results of two cell viability tests. Salehi *et al.* (2019), studies suggested that either α-pinene or β-pinene, compounds present in the EOB and EOL samples, when isolated, show cytotoxicity on macrophages at a concentration of 250 µg/ml, reducing cell viability by 57.7%, or that explains the toxic effect of the samples in high concentrations in this study. When evaluated on other blood cells, such as lymphocytes, another analysis also found that α-pinene decreased cell viability, using LDH (lactate dehydrogenase) and MTT release tests, despite not causing DNA damage (Vo *et al*., 2020; Salehi *et al* ., 2019).

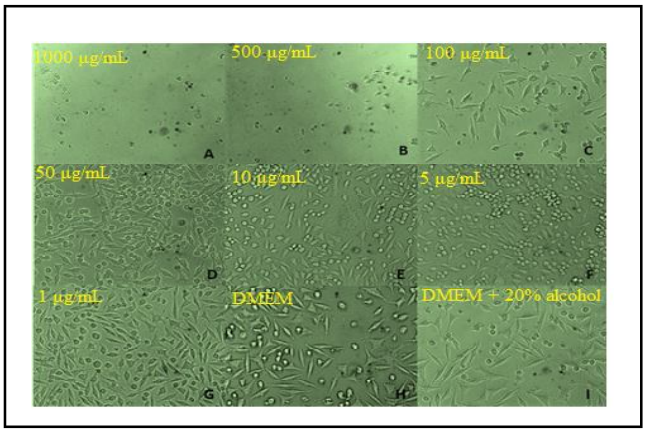

Figure 3: Photomicrographs of RAW 264.7 macrophage cultures taken under an inverted phase microscope after 48 h of treatment with different concentrations of *P. ovata* EOB (Nikon Eclipse TS 100), 100x.

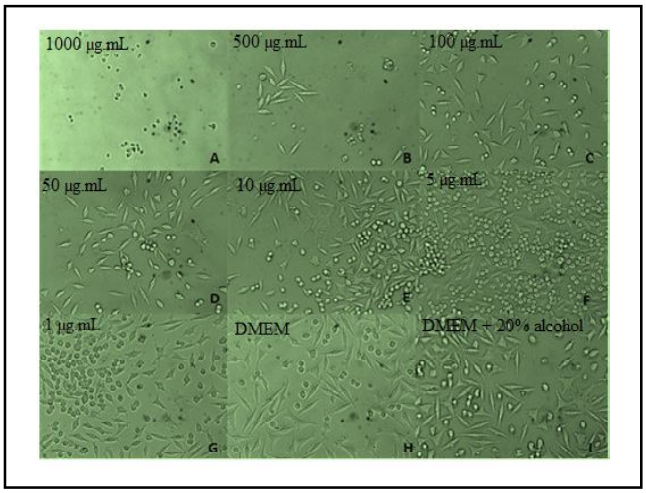

Figure 4: Photomicrographs of the RAW 264.7 macrophage cultures, after 48 h of treatment with different EOL concentrations of *P. ovata* viewed under an inverted phase microscope, 100x.

4. Discussion

According to Table 1, the oils showed similar antiadherent activities, considering that between 1000 and 125 µg/ml inhibited the adhesion of practically all *F. nucleatum* strains. While, at the concentration of 62.5 µg/ml, 45.98% (± 1.07) and 46.93% (± 0.96) of the bacteria remained adhered, respectively, in the tests with EOL and EOB. From the concentration of 31.2 µg/ml, practically all bacteria remained adhered, with no statistically significant differences between the means obtained for EOL and EOB. The results shown in Figure 2 indicate that *F. nucleatum* was able to adhere to polystyrene plates treated with saliva, forming monospecies biofilms, up to a concentration of 125 µg/ml, the essential oils were able to inhibit this grip. Although, there are limitations in this study, since we did not evaluate the action of these oils on the adherence of this pathogen to the dental structure, we can observe that there are perspectives in the use of the samples of essential oils evaluated in the treatment of periodontal disease. This aspect is important, since *F. nucleatum* is one of the gram-negative bacteria, which establish themselves in dental biofilms and play a fundamental role in the physical interactions between *Streptococcus* sp. and periodontopathogens, contributing to the establishment of conditions conducive to the aggregation of anaerobic, oxygen-intolerant bacteria (Toole *et al.*, 2021; He *et al*., 2016). In the analysis of the cytotoxicity tests with the essential oils of the plant, it can be observed through Table 3, the occurrence of variations in the toxicity of the oils. While at concentrations of 1000, 500 and 100 µg/ml, the samples showed cytotoxicity in the MTT test, reducing the percentage of viable macrophages among controls treated with a DMEM culture medium. Other concentrations did not induce the same biological behavior. It is noteworthy, through Table 3, that using EOL at 50 µg/ml, only 24% (± 3.34) remained viable, while the EOB, at the same concentration, did not exert toxic effects, remaining 98.63 % (± 9.02) of viable macrophages. This difference between samples being statistically significant (p<0.05). Thus, it can be mentioned that at a concentration of 50 µg/ml, the EOB was not cytotoxic, while this property was only achieved by the EOL at 10 µg/ml, according to data in Table 3.

Chemical analysis by gas chromatography coupled to mass spectrometry (GC-MS) showed that there are no qualitative differences between the oils from the bark and leaves of *P. ovata*. Results show that both oils have the same chromatographic spectrum, presenting compounds with similar peaks and retention times. However, there are quantitative differences. The essential oil obtained from the leaf has a higher content of metabolites, represented by the larger area presented under the peaks, compared to the oil extracted from the bark. The similarity was observed in the chemical constitution of the oils, verified by the pattern of ion fragmentation, obtained by mass spectroscopy and compared to the equipment library, NIST 05 Mass Spectral Library (Table 1). Therefore, this analysis was confirmed by comparing the retention index, obtained by injecting a series of standard carbons (C_8-C_{20}) that eluted under the same conditions as the oil samples, allowing the characteristics of the unknown compound to be compared, as fragmentation pattern, retention index and retention time, to the compounds referenced by NIST 05 library (Milman *et al*., 2016).

An investigation into 32 compounds, including β-phellandrene (20.4% in leaves), bicyclogermacrene (29.1% in flowers and 17.7% in leaves), and β-element (17.8% in flowers and 22.0% in leaves), was conducted using essential oils extracted from various parts of *P. ovata*, including leaves, flowers, roots, and wood bark collected in different regions of at various times of the day (Bhat *et al.*,2004). Although, this study did not identify the same major compounds, it is necessary to emphasize that some factors interfere in the

chemical composition inherent to plants. The climatic season, the period of collection, age, and physiological development cause circadian variations in the plant and interfere in the amount, in the relative proportion, and even in the nature of active constituents, such as essential oils. Mechanical factors to which plants are susceptible, such as injuries, or even stimuli caused by external agents, such as hail and the invasion of pathogens, can influence the expression of secondary metabolism, which explains the divergence of studies regarding the chemical composition of plants from the same species (Bhat *et al*., 2004; Amalarasi *et al.*, 2019).

In the evaluation of the activity of essential oils against the bacteria involved in the formation of dental biofilm (Table 2), there was a greater inhibitory action of the oils on the periodontopathogens, late colonizers, about *Streptococcus* sp., which are initial colonizers in the formation of dental plaque (Muthumperumal *et al*., 2009). This more effective action on gram-negative pathogens, such as *F. nucleatum* and *Treponema denticola*, may be related to the mechanism of action of essential oils, which are soluble in the bilipid layer of the cell membrane, and therefore, have the ability to destabilize and consequently, break or penetrate the lipid structure present in gram-negative bacteria, which does not occur in gram-positive microorganisms, provided with a peptidoglycan barrier (Hancock *et al.*, 2021). Through, the analysis of Table 2, it is verified that the essential oils obtained from the leaf presented a greater spectrum of action, both on facultative anaerobic bacteria such as *S. mutans*, (MIC of 500 µg/ml for EOL and 1000 µg/ml for EOB), and on restricted anaerobes such as *Treponema denticola* (250 µg/ml EOB and 125 µg/ml EOL) and *P. intermedia* (500 µg/ml EOB and 250 µg/ml EOL). This variation in the antibacterial activity may be related to the differences in the amounts of bioactive compounds present in the essential oils, the EOL samples have a more significant part of phytoconstituents, probably associated with their antibacterial activity. The action of the essential oils of *P. ovata* on *Treponema denticola* validates its antimicrobial spectrum in the treatment of periodontal disease. Among the various bacteria present in the subgingival biofilm, this pathogen is believed to be the main microorganism associated with chronic periodontitis. Evidence suggests that the reduction in the levels of this bacterium in periodontal pockets is associated with an improvement in the clinical signs of the disease, as this pathogen can invade junctional epithelial cells, in addition to inducing cell matrix degradation (Umesha *et al*., 2013).

Among the oral *Streptococci* tested, the most sensitive to EOB and EOL samples were *S. oralis* and *S. parasanguinis*, with a MIC of 500 µg/ml. These bacteria are involved in the production of H_2O_2, a substance that stimulates the release of bacterial DNA, facilitating the mechanisms of coaggregation in bacterial plaque; induces cell death by oxidative stress; and, consequently, can act as a cytokine, increasing the inflammatory response in periodontitis (Toole *et al*., 2021). Thus, these essential oils have broad perspectives to be used as chemical agents in treating periodontal disease.

The phytoconstituents present in the EOB and EOL samples, represented in Table 2, are present in several studies with other plants that also have antibacterial action. Camphor and α-pinene have inhibited the growth of gram-positive and gram-negative bacteria; and D-limonene produced inhibition halos in agar diffusion tests with *Streptococcus* sp. In a study carried out with crude essential oils, consisting mostly of (E)-caryophyllene and germacrene-D, in microdilution tests on oral streptococci, it was found that when associated, these terpenes have a MIC of 20 µg/ml for *S. mutans* and 18 µg/ml for S. mitis. However, when isolated, the compounds showed a lower inhibitory effect, with a MIC of 400 µg/ml for both oral streptococci, indicating that several components of essential oils act through a synergistic mechanism (Langeveld *et al*., 2013).

These conclusions are supported by the chromatographic analysis, presented in Figure 2. The EOL has a greater amount of chemical constituents when compared to the EOB, and probably a greater amount of bioactives determines a more pronounced biological effect. Such biological behavior of the EOL is evidenced by the greater antibacterial activity, according to the data in Table 4, and the greater cytotoxic effect, as shown by the results of the cell viability tests. In addition, studies show that α-pinene and β-pinene, compounds present in EOB and EOL samples, when isolated show cytotoxicity on macrophages at a concentration of 250 µg/ml, reducing cell viability by 57.7%, which explains the toxic effect of samples at high concentrations in this study. When evaluated on other blood cells, such as lymphocytes, another analysis found that α-pinene decreased cell viability using LDH (lactate dehydrogenase) and MTT release tests, despite not causing DNA damage (Torrens *et al*., 2017). Considering that macrophages are essential cells for the body's immune response, this toxicological assay is of fundamental importance in screening natural products, with perspectives on periodontal disease. In periodontitis, there is an exacerbated inflammatory response, with the presence of bacterial antigens inducing defense cells, such as macrophages, to produce inflammatory mediators, such as interleukins, tumor necrosis factors, metalloproteinases and prostaglandins. Therefore, it is necessary to use, in therapy with natural products. These concentrations are not cytotoxic to defense cells, so as not to cause cellular damage that would increase the inflammatory response, resulting in greater damage to periodontal tissues (Vo *et al*., 2020).

An important observation that can be made through the results of the cytotoxicity tests refers to the increase in the number of viable cells, about the control groups treated with DMEM, when concentrations of 10 µg/ml of the samples were used. EOB and EOL, which can be seen in Figures 3-4. This biological behavior may be related to the presence of agents that delay oxidative degradation reactions, reducing the rate of cellular oxidation by complexing with metal ions or by inhibiting free radicals. Terpenes, such as pinenes and limonene, and sesquiterpenes, such as cadinenes, constituents of the tested samples, are examples of antioxidant agents found in essential oils from various vegetables (Torrens *et al.*,2017;Vo *et al*., 2020). These conclusions are supported by chromatographic analysis. EOL has a higher quantity of chemical constituents when compared to EOB, and probably a higher quantity of bioactives determines a more pronounced biological effect.

5. Conclusion

The essential oils of the bark (EOB) and the leaf (EOL) of *P. ovata* have the same chemical constitution. Still, no EOL has, proportionally, a higher quantity of volatile compost. The main composts identified are foram oils: α-pinene, delta-cadinene, beta-pinene and d-limonene.

The EOB and EOL samples were effective on the previously tested pathogens, with the exception of *Eubacterium nodatum* and *S. salivarius*, which did not show MICs for the concentrations tested; both EOB and EOL are effective in reducing the *in vitro* adhesion of *F. nucleatum*, indicating that they can be used as a possible antibiofilm phytotherapic formulation; the EOB samples are less cytotoxic than EOL, and at a concentration of 50 µg.ml they will allow a viability of 98.63% (± 9.02) for two macrophages.

Conflict of interest

The authors declare no conflicts of interest revelant to this article

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