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Antibacterial activity of *Pinus elliottii* against anaerobic bacteria present in primary endodontic infections

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ABSTRACT

Endodontic infections have a polymicrobial nature, but anaerobic bacteria prevail among the infectious microbes. Considering that it is easy to eliminate planktonic bacteria, biofilm-forming bacteria still challenge clinicians during the fight against endodontic diseases. The chemical constituents of the oleoresin of *Pinus elliottii*, a plant belonging to the family Pinaceae, stand out in the search for biologically active compounds based on natural products with potential application in the treatment of endodontic infections. Indeed, plant oleoresins are an abundant natural source of diterpenes that display significant and well-defined biological activities as well as potential antimicrobial action. In this context, this study aimed to (1) evaluate the in vitro antibacterial activity of the oleoresin, fractions, and subfractions of *P. elliottii* as well as the action of dehydroabietic acid against 11 anaerobic bacteria that cause endodontic infection in both their planktonic and biofilm forms and (2) assess the in vitro antibiofilm activity of dehydroabietic acid against the same group of bacteria. The broth microdilution technique helped to determine the minimum inhibitory concentration (MIC) of the oleoresin and fractions. This same technique aided determination of the MIC values of nine subfractions of Fraction 1, the most active fraction. The MIC, minimum bactericidal concentration, and antibiofilm activity of dehydroabietic acid against the tested anaerobic bacteria were also examined. The oleoresin and fractions, especially fraction PE1, afforded promising MIC values, which ranged from 0.4 to 50 μg/mL. Concerning the nine evaluated subfractions, PE1.3 and PE1.4 furnished the most noteworthy MIC values, between 6.2 and 100 μg/mL. Dehydroabietic acid displayed antibacterial activity, with MIC values lying from 6.2 to 50 μg/mL, as well as bactericidal effect for all the investigated bacteria, except for *Prevotella nigrescens*. Assessment of the antibiofilm activity revealed significant results – $MICB_{50}$ lay between 7.8 and 62.5 μg/mL, and dehydroabietic acid prevented all the evaluated bacteria from forming a biofilm. Hence, the chemical constituents of *P. elliottii* are promising biomolecules to develop novel therapeutic strategies to fight against endodontic infections.

1. Introduction

It is well established that primary endodontic infection has polymicrobial origin, and that Gram-negative bacteria are the main causative agent of this condition [13]. The main component of the cell wall of these bacteria is an endotoxin, the lipopolysaccharide (LPS) [17,19,24]. LPS released during bacterial disintegration, multiplication, and death [13] can egress into periradicular tissue and be a potent stimulus against different cells, which can lead to periapical inflammatory responses and bone destruction [15,29].

Eliminating microorganisms from infected radicular canals has been one of the major concerns in the field of endodontics. This has been demonstrated by intense research into the efficacy of mechanical instrumentation and the influence of irrigation via intracanal and systemic medication [7,36].

Considering that a number of methods can easily remove planktonic microorganisms, biofilm-forming antimicrobial-resistant bacteria continue to be the most significant challenge that

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researchers have to deal with [27]. Microorganisms that can develop as biofilms in competitive environments generally display larger resistance to antimicrobial agents, as a result of their low metabolic rate [33]. Depending on the biofilm structure that confers bacterial resistance to the pathogenic microorganism, the host cell immune response cannot control or neutralize the infectious process [1,22]. In this sense, anaerobic bacteria in infected radicular canals exert a fundamental role in enzyme and endotoxin production, which may culminate in reactions that potentialize the infection. The low oxyreduction potential that favors anaerobic bacterial growth in teeth, especially in the apical portion, and the interaction among bacteria are perhaps the most important determiners of bacterial survival in infected radicular canals.

The plant kingdom significantly contributes to supplying natural products that are potentially applicable in the treatment of diseases affecting the humankind. In this context, *Pinus elliottii* has attracted researchers' interest. This plant is a conifer belonging to the gymnosperm family Pinaceae. All the genera stemming from this family typically produce a resin. *P. elliottii* originates in southeastern USA. However, it is widely cultivated as a subtropical crop in Brazil, India, and China. It is usually employed to fabricate oleoresin and furniture [23].

The oleoresins extracted from conifers belonging to the genus *Pinus* have countless applications. Some phytochemical studies have shown that these types of oleoresins consist mainly of tricyclic acid diterpenes of the pimarane and abietane class as well as bicyclic diterpenes, especially labdanes [23]. Among the various classes of plant terpenes, diterpenes stand out: they present several well-known biological activities such as antiparasite [35], anti-inflammatory [30], antifungal [8], and vascular smooth muscle relaxant [4,5,34] actions, just to mention a few.

Over the last decades, phytodrugs have assumed a prominent part as possible alternative therapy in dentistry. Indeed, they possess antibacterial activity against aerobic and anaerobic pathogens of the oral cavity [2,9,11,12,25,31,32]. Therefore, this study aimed to verify the antibacterial activity of the oleoresin, fractions, subfractions, and the major compound dehydroabietic acid isolated from *P. elliottii* against bacteria that cause endodontic infections.

2. Materials and methods

2.1. Plant material, bioguided-assay fractionation, and dehydroabietic acid isolation

Certified oleoresin of *P. elliottii* (PE; 100.0 mg) was kindly provided by ARESB (Associação dos Resinadores do Brasil). Bioguided-assay fractionation with PE was performed, because this oleoresin has been proven to be effective against a panel of bacteria that cause endodontic infections. This plant material was subjected to vacuum chromatography over silica gel 60H (500 g; Merck, art. 7736) using *n*-hexane and increasing amounts of ethyl acetate as an eluant (1500 mL each fraction). After solvent evaporation, this procedure afforded five fractions (PE1–PE5); these fractions were also assayed against endodontic microorganisms. Fraction PE1 (5.89 g) was the most active antibacterial extract. Hence, about 1.00 g of this fraction was partitioned by classic column chromatography over silica gel 60 (100 g; Merck, art. 7734) using *n*-hexane/ethyl acetate 8:2 as an eluant. Seventy fractions were collected and further combined into eight new fractions (PE1.1–PE1.8). PE1.5 (340.0 mg) was the most active against endodontic bacteria. ^{1}H and ^{13}C NMR analyses identified the diterpene dehydroabietic acid as the main constituent of PE1.5.

2.2. Bacteria used in the assays

The present study included eleven bacteria: eight were from the *American Type Culture Collection* (ATCC); three consisted of clinical isolates maintained at the culture collection of the Laboratory of Research in Applied Microbiology (LaPeMA/UNIFRAN), cryopreserved at −80 °C. More specifically, the following bacteria were employed: *Actinomyces naeslundii* (ATCC 19039), *Bacteroides fragilis* (ATCC 25285), *Prevotella nigrescens* (ATCC 33563), *Bacteroides thetaiotaomicron* (ATCC 20741), *Porphyromonas gingivalis* (ATCC 33277), *Fusobacterium nucleatum* (ATCC 25586), *Peptostreptococcus anaerobius* (ATCC 27337), *Propionibacterium acnes* (ATCC 6919), *Peptostreptococcus micros* (clinical isolate), *Prevotella buccae* (clinical isolate), and *Prevotella intermedia* (clinical isolate).

Table 1
In vitro antibacterial activity (MIC) of *P. elliottii* oleoresin and fractions against endodontic anaerobic bacteria.

Bacteria	Oleoresin (μg/mL)	Fractions (μg/mL)					Chlorhexidine (μg/mL)
		PE1	PE2	PE3	PE4	PE5	
B. fragilis ATCC 25285	12.5	6.2	25.0	50	–	–	7.4
A. naeslundii ATCC 19039	25	25	50	100	–	50	1.8
P. gingivalis ATCC 33277	0.4	0.4	0.8	3.1	6.2	25	0.9
P. nigrescens ATCC 33563	3.1	0.8	12.5	25	100	–	0.9
F. nucleatum ATCC 25586	12.5	12.5	50	100	–	–	1.8
P. acnes ATCC 6919	25	25	50	100	–	–	1.8
B. thetaiotaomicron ATCC 29741	100	50	100	–	–	–	29.0
P. anaerobius ATCC 27337	12.5	12.5	25	50	100	–	7.4
P. buccae Clinical isolate	6.2	1.6	6.2	6.2	12.5	25	1.8
P. micros Clinical isolate	50	6.2	12.5	25	50	–	1.8
P. intermedia Clinical isolate	12.5	12.5	100	–	–	–	1.8

–: Evaluated concentration considered inactive (MIC values > 100.0 μg/mL).