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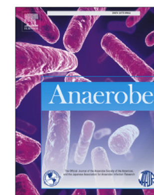

Anaerobes in human infections (dental/oral infections)

Mikania glomerata Sprengel extract and its major compound *ent*-kaurenoic acid display activity against bacteria present in endodontic infections

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ABSTRACT

The search for new, effective and safe antimicrobial compounds from plant sources has continued to play an important role in the maintenance of human health since ancient times. Such compounds can be used to help to eradicate microorganisms from the root canal system, preventing/healing periapical diseases. *Mikania glomerata* (Spreng.), commonly known as "guaco," is a native climbing plant from Brazil that displays a wide range of pharmacological properties. Many of its activities have been attributed to its phytochemical composition, which is mainly composed of diterpenes, such as *ent*-kaurenoic acid (KA). The present study evaluated the potential activity of an *ent*-kaurenoic-rich (KA) extract from *Mikania glomerata* (i.e. *Mikania glomerata* extract/MGE) and its major compound KA against bacteria that can cause endodontic infections. Time-kill assays were conducted and the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), anti-biofilm activity, and synergistic antimicrobial activity of MGE and KA were determined. The MGE exhibited MIC and MBC values, which ranged from 6.25 to 100 μg/mL and 12.5 to 200 μg/mL respectively. The MIC and MBC results obtained for the KA, ranged from 3.12 to 100 μg/mL and 3.12 to 200 μg/mL respectively. Time-kill and anti-biofilm activity assays conducted for KA at concentrations between 3.12 and 12.5 μg/mL exhibited bactericidal activity between 6 and 72 h of incubation and 50% inhibition of biofilm formation for *Porphyromonas gingivalis* (clinical isolate), *Propionibacterium acnes* (ATCC 6919), *Prevotella nigrescens* (ATCC 33563), *P. melaninogenica* (ATCC 25845), *Aggregatibacter actinomycetemcomitans* (ATCC 43717). For synergistic antimicrobial activity, KA combined with chlorhexidine dichlorohydrate (CHD) had an additive effect with increased efficacy against *P. gingivalis* (clinical isolate) compared to CHD alone. It was concluded that *M. glomerata* extract and its major compound *ent*-kaurenoic acid (KA) showed *in vitro* antibacterial activity, the latter being a potential biofilm inhibitory agent. They may play important roles in the search for novel sources of agents that can act against bacteria present in endodontic infections.

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1. Introduction

The main goal of endodontic treatment is to prevent or promote healing of apical periodontitis [1]. This is an inflammatory disease of the periradicular tissues induced by the presence of microorganisms within the root canals with necrotic pulp (primary endodontic infection) or within those with a failure of the endodontic treatment (secondary/persistent endodontic infection) [2].

The microbiota of the root canals of teeth with chronic apical periodontitis is composed mainly of bacteria organized in communities, forming a complex biofilm adhered to the intraradicular dentine [3,4]. Endodontic biofilms may even extend to other areas of the root canal system, including lateral canals, apical ramifications, isthmi, and recesses. In these areas, bacteria are more difficult

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to be reached and eliminated during treatment. Residual bacteria surviving in those areas may put the treatment outcome at risk, especially if they find a sustainable source of nutrient supply from the periradicular tissues [4].

There are over 750 bacterial species in the oral cavity, and a number of these species are the primary etiological agents of the root canal infection, including the strict anaerobic Gram-negative rods belonging to the genera *Fusobacterium, Prevotella, Porphyromonas;* the strict anaerobic Gram-positive rods including *Eubacterium* and *Filifactor,* the Gram-positive anaerobic cocci (GPAC) *Parvimonas* and the Gram-positive facultative cocci: *Streptococcus* and *Enterococcus* [2,5,6]

The endodontic treatment itself essentially consists of mechanical instrumentation aided by irrigation (known as chemo-mechanical preparation), with the purpose of removing microorganisms, debris, and potential substrate for microbial re-growth. An intracanal medication that remains between appointments may be necessary to reach maximal bacterial/endotoxin reduction in infected root canals [7–9].

Although several studies have demonstrated high levels of bacterial load reduction after chemo-mechanical preparation and the use of an intracanal medication, complete elimination of bacteria and their by-products has rarely been observed [8,9]. The traditional non-specific antimicrobial agents appear to be limited in controlling the infection. Research for an improved irrigants and intracanal medication includes the use of nanoparticles of chitosin, zinc oxide, polylactic co-glycolic acid (PLGA) nanoparticles encapsulated with photoactive drugs and natural plant extracts [10,11].

The rich biodiversity of the Brazilian plants and history of medicinal use of some of them favors the development of research involving herbal medicine, seeking ways to industrialize them [12]. The use of natural products could help to overcome bacterial resistance to old and new antimicrobials that are currently used in clinical therapy [13].

Mikania glomerata (Spreng.), commonly known as “guaco”, is a native climbing plant from Brazil that belongs to the Asteraceae family [14]. This plant displays a wide range of pharmacological properties, including anti-allergic [15], antimicrobial [16,17], analgesic [18], anti-inflammatory [18], anti-hemorrhagic [19], anxiolytic [20], antioxidant [20], and anti-diarrheal activities [21].

Phytochemical studies with *M. glomerata* have revealed the presence of numerous compounds in this species. Its major constituents are coumarin [22], coumaric acid, sesquiterpenes, and diterpenes [16,23]. Among these diterpenes, the most commonly found is *ent*-kaurenoic acid (*ent*-kaur-16-en-19-oic acid) (Fig. 1), which corresponds to approximately 48.94% of the extract from *Mikania glomerata* [17]. This diterpene has many pharmacological activities, such as anti-inflammatory [24], antibacterial [17,25], anti-leishmanial [26], and anti-convulsant [27] action, among others.

Finally, identification of new drug candidates to control endodontic infections is urgent, since complete elimination of bacteria and their by-products has rarely been achieved. Therefore, the present study aimed to investigate the *in vitro* antibacterial activity of an *ent*-kaurenoic-rich (KA) extract from *Mikania glomerata* Sprengel (MGE) and its major compound *ent*-kaurenoic acid (KA) against bacteria present in endodontic infections.

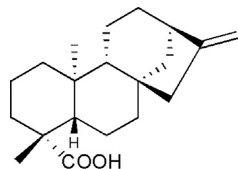

Fig. 1. Chemical structure of *ent*-kaurenoic acid (KA).

2. Materials and methods

2.1. Plant material and isolation of the major compound

Pure KA (purity above 99%) was provided by Dr. Rodrigo Cassio Sola Veneziani (University of Franca, UNIFRAN). The obtained MGE is detailed in Moreira et al. [17] and is briefly described as follows. The dried aerial parts of *M. glomerata* Sprengel were exhaustively extracted with dichloromethane, concentrated and suspended in methanol containing 10% water (methanol/H_2O 9:1). After filtration, the soluble fraction was partitioned with *n*-hexane and after solvent evaporation, the remaining fraction was successively chromatographed over silica gel 60 through using chromatography with *n*-hexane and then with 20% ethyl acetate (*n*-hexane/ethyl acetate 8:2). The *n*-hexane fraction was discarded and the *n*-hexane/ethyl acetate 8:2 was named MGE (Mikania glomerata extract) after solvent evaporation. All solvents used in the process were p a. grade.

2.2. Tested microorganisms

Bacteria were obtained from the American Type Culture Collection (ATCC) and from clinical samples collected from infected root canals. They were maintained in the culture collection of the Research Laboratory of Applied Microbiology (LaPeMA), University of Franca, State of Sao Paulo, Brazil, under cryopreservation (−80 °C) in Schadler broth supplemented with hemin (5 mg/mL, Sigma, St. Louis, MO, USA) and menadione (1 mg/mL, Sigma) [for strict anaerobic bacteria] and in Brain Heart Infusion (BHI; Difco Laboratories, Detroit, MI, USA) broth [for facultative bacteria], both containing glycerol at 20% (v/v) and 0.5 mL defibrinated sheep blood.

The microorganisms used were as follows: *Actinomyces naeslundii* (ATCC 19039), *Aggregatibacter actinomycetemcomitans* (ATCC 43717), *Enterococcus faecalis* (ATCC 4082), *Fusobacterium nucleatum* (ATCC 25586 and clinical isolate), *Porphyromonas gingivalis* (ATCC 33277 and clinical isolate), *Prevotella intermedia* (clinical isolate), *P. nigrescens* (ATCC 33563), *P. melaninogenica* (ATCC 25845) and *Propionibacterium acnes* (ATCC 6919).

In summary, facultative microorganisms such as *A. naeslundii, A. actinomycetemcomitans, E. faecalis,* were grown either on BHI broth or on BHI agar plates supplemented with 5% defibrinated sheep blood. The broth/agar plates were incubated at 37 °C in a CO_2 incubator (10% CO_2) for 24 h. The strict anaerobic bacteria (*F. nucleatum, P. gingivalis, P. intermedia, P. nigrescens, P. melaninogenica, P. acnes*) were grown either on Schadler broth (Difco) supplemented with hemin (5 mg/mL, Sigma) and menadione (1 mg/mL, Sigma) or on Schadler agar (Difco) supplemented with hemin, menadione and 5% defibrinated sheep blood for strict anaerobic bacteria. The broth/agar plates were incubated at 37 °C inside the anaerobic workstation (Don Whitley Scientific, Bradford, UK), in an atmosphere of 5–10% H_2, 10% CO_2, and 80–85% N_2 for 72 h.

2.3. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC was determined in triplicate in the exponential phase of bacterial growth using the broth microdilution method, conducted in 96-well microplates; methodology based on the Clinical & Laboratory Standards Institute (CLSI) [28,29], previously described by Leandro et al. [30].

Briefly, the MGE and KA samples (1 mg) were dissolved in 32 μL of dimethylsulfoxide (DMSO; Merck, Darmstadt, HE, Germany) and

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