

Antimicrobial Activity of Kaurane Diterpenes against Oral Pathogens

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Two kaurane diterpenes, *ent*-kaur-16(17)-en-19-oic acid (KA) and 15- β -isovaleryloxy-*ent*-kaur-16(17)-en-19-oic acid (KA-Ival), isolated from *Aspilia foliacea*, and the methyl ester derivative of KA (KA-Me) were evaluated against oral pathogens. KA was the most active compound, with MIC values of 10 $\mu\text{g mL}^{-1}$ against the following microorganisms: *Streptococcus sobrinus*, *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus sanguinis*, and *Lactobacillus casei*. However, KA did not show significant activity against *Streptococcus salivarius* and *Enterococcus faecalis*, with MIC values equal to 100 and 200 $\mu\text{g mL}^{-1}$, respectively. Our results show that KA has potential to be used as a prototype for the discovery of new effective anti-infection agents against microorganisms responsible for caries and periodontal diseases. Moreover, these results allow to conclude that minor structural differences among these diterpenes significantly influence their antimicrobial activity, bringing new perspectives to studies on the structure-activity relationship of this type of metabolites with respect to caries and periodontal diseases.

Key words: Kaurane Diterpenes, Oral Pathogens, Antimicrobial Activity, *Aspilia foliacea*

Introduction

Dental plaque is a biofilm consisting of microorganisms generally present on the tooth surface. This biofilm plays an important role in the development of dental caries and periodontal diseases (Koo *et al.*, 2000). Oral *Streptococci*, which are commonly isolated from human teeth, are the main microorganisms responsible for these infectious diseases (Hirasawa and Takada, 2002).

Extensive efforts have been made toward the search for antibacterial compounds that can be incorporated into dental products (Cai and Wu, 1996). Chlorhexidine, one of the most important antibacterial agents against oral pathogens (Decker *et al.*, 2005), has gained the approval of the American Dental Association Council on Dental Therapeutics (Cai and Wu, 1996). However, various adverse effects such as teeth staining and increased calculus formation are associated with the current use of products containing this compound (Cai and Wu, 1996). Also, chlorhexidine is much less effective at reducing the levels of

Lactobacillus, which are strongly related to caries progression in human mouths (Featherstone, 2006). These drawbacks justify the research on new effective antibacterial compounds that could be employed in caries prevention.

Natural products, especially those derived from higher plants, provide a rich source of novel and diverse antimicrobial agents (Cai and Wu, 1996; Koo *et al.*, 2000; Ríos and Recio, 2005). However, little is known about the potential of secondary plant metabolites against oral pathogens (Cai and Wu, 1996; Koo *et al.*, 2000). Many reports have extensively shown that kaurane-type diterpenes exhibit several important biological properties, including antiparasitic effects (Batista *et al.* 2007; Da Costa *et al.*, 1996), antispasmodic and relaxant actions on the smooth muscle (Ambrosio *et al.*, 2006; Tirapelli *et al.*, 2004), as well as analgesic and anti-inflammatory activities (Okuyama *et al.*, 1991; Paiva *et al.*, 2002). Members of this class of diterpenes have been reported to have cytotoxic effects, (Hanson, 2006; Ghisalberti, 1997), antiproliferative action on tumour cell cultures (Mongelli

et al., 2002), and significant antimicrobial activity against Gram-positive and Gram-negative bacteria and yeasts, including *Bacillus subtilis* (Ghisalberti, 1997; Slimestad *et al.*, 1995), *Staphylococcus aureus*, *Mycobacterium smegmatis* (Ghisalberti, 1997; Mitscher *et al.*, 1983), *Saccharomyces cerevisiae*, *Escherichia coli*, *Cladosporium herbarum*, *Candida albicans* (Ghisalberti, 1997), among others. Moreover, a previous screening using Brazilian plants against oral pathogens performed in our laboratories showed that plant extracts rich in kaurane diterpenes are very effective against these microorganisms. Our results pointed out that these metabolites are a promising source of new prototypes for the development of antibacterial agents against cariogenic microorganisms.

Considering the activity of plant extracts rich in kaurane diterpenes against oral pathogens, the aim of this study was to evaluate the activity of kaurane diterpenes isolated from *Aspilia foliacea*, as well as that of a semi-synthetic derivative, against some oral microorganisms, including *Streptococcus mutans*, which is considered one of the primary causative agents of dental caries.

Materials and Methods

Plant material

Aspilia foliacea (Spreng.) Baker was collected in Brazil by Walter Vichniewski at the GO-118 highway (km 11) in November 1995. The plant material was identified by João Moreira dos Santos (Museu Paraense Emílio Goeldi, PA, Brazil). A voucher specimen (Vichniewski # 367) was deposited in the herbarium of the Instituto de Biologia, Universidade de Campinas, SP, Brazil.

Extraction and isolation

Underground parts of *Aspilia foliacea* were air-dried at 40 °C. The powdered material (650 g) was exhaustively extracted by maceration at room temperature using chloroform (5 L) to give the crude extract (6.1 g) after solvent evaporation under reduced pressure. This extract was re-suspended in methanol/water (9:1, v/v) and extracted with *n*-hexane, followed by extraction with dichloromethane, to give crude extracts (0.45 and 1.12 g, respectively) after solvent evaporation under reduced pressure.

The *n*-hexane extract (0.45 g) was chromatographed over silica gel 60 (0.063–0.200 mm) using classic chromatography with *n*-hexane and in-

creasing amounts of ethyl acetate as eluents. This procedure yielded 40 fractions (50 mL each), which were grouped into six new ones (Fr-1 to Fr-6) after TLC analysis. A solid mass appeared in Fr-2 (130 mg), and compound **1** [*ent*-kaur-16(17)-*en*-19-oic acid, KA, 70 mg] was obtained after washing the solid with methanol.

The dichloromethane extract (1.12 g) was chromatographed over silica gel 60H (Merck, art. no. 7736) using vacuum liquid chromatography (VLC) (Pelletier *et al.*, 1986) with increasing amounts of ethyl acetate in *n*-hexane. Solvent was removed under reduced pressure in a rotatory evaporator, and six fractions (Fr-1 to Fr-6, 150 mL each) were obtained. Fr-3 (150 mg) was fractionated by medium pressure chromatography ("flash" chromatography) (Still *et al.*, 1978) using silica gel 60 (Merck, art. no. 9385, 0.040–0.063 mm) and isocratic *n*-hexane/ethyl acetate (4:1) and 1% acetic acid as the mobile phase, furnishing compound **2** [15- β -isovaleryloxy-*ent*-kaur-16(17)-*en*-19-oic acid, KA-Ival, 8 mg].

Preparative thin layer chromatography [PTLC, silica gel PF₂₅₄, 1 mm thickness, Merck, art. no. 7730, *n*-hexane/ethyl acetate (3:2) and 1% acetic acid] of Fr-4 (60 mg) furnished a mixture (7 mg) containing the diterpenes 15- β -hydroxy-*ent*-kaur-16(17)-*en*-19-oic acid (**3**) and 17-hydroxy-*ent*-kaur-15(16)-*en*-19-oic acid (**4**, respectively).

KA (**1**) (about 50 mg) was treated with CH₂N₂ in Et₂O (Da Costa *et al.*, 1996), yielding the respective C-19 methyl ester derivative (KA-Me, **5**). This compound was purified by flash chromatography with *n*-hexane/ethyl acetate (9:1) as the mobile phase, and identified by means of spectrometric analysis.

The purity of the evaluated diterpenes **1**, **2** and **5** was estimated by thin layer chromatography using different solvent systems, as well as ¹H and ¹³C NMR analysis. We estimated that the purity of all the compounds evaluated against the microorganisms was in the range 95–98%.

Antimicrobial assays

The minimum inhibitory concentration (MIC) values of the diterpenes were determined in triplicate using the microdilution broth method (Andrews, 2001) in 96-well microplates. Standard strains from the American Type Culture Collection of the following microorganisms were used: *Enterococcus faecalis* (ATCC 4082), *Streptococcus*

salivarius (ATCC 25975), *Streptococcus sobrinus* (ATCC 33478), *Streptococcus mutans* (ATCC 25275), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556), and *Lactobacillus casei* (ATCC 11578). The samples were dissolved in DMSO (dimethyl sulfoxide) at 1 mg mL⁻¹, and were then diluted in tryptone soya broth; concentrations ranging from 200 to 5 µg mL⁻¹ were achieved. The final DMSO content was 4% (v/v), and this solution was used as negative control. The inoculum was adjusted for each organism, to yield a cell concentration of 5 · 10⁵ colony forming units (CFU) mL⁻¹. One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One non-inoculated well free of antimicrobial agent was also included to assure medium sterility. Chlorhexidine was used as positive control. The microplates (96-well) were incubated at 37 °C for 24 h. After that, resazurin (30 µL) in aqueous solution (0.02%) was added to the microplates to indicate the microorganism viability (Palomino *et al.*, 2002). The MIC was determined as the lowest concentration of the compound capable of inhibiting the microorganism growth. For the determination of either the bacteriostatic or bactericidal activities, the entire volume of each well of all the incubated microplates was subcultured on blood agar at 37 °C for 24 h. The absence of viable cell growth indicated a bactericidal effect. The minimum bactericidal concentration was defined as the lowest concentration that enabled no growth on blood agar.

Results and Discussion

The chemical structures of the diterpenes studied in this work are presented in Fig. 1. The spectral data of compounds **1** and **5** (Da Costa *et al.*, 1996), **3** and **4** (Yahara *et al.*, 1974), and **2** (Schteingart and Pomilio, 1981) are in agreement with those published in the literature.

Among the evaluated diterpenes, compound **1** (KA) displayed the highest antibacterial activity (Table I), with MIC values of 10 µg mL⁻¹ for the following microorganisms: *S. sobrinus*, *S. mutans*, *S. mitis*, *S. sanguinis*, and *L. casei*. For *S. salivarius* and *E. faecalis*, KA was not significantly active against these microorganisms, displaying MIC values of 100 and 200 µg mL⁻¹, respectively. The MIC values of the pure diterpenes **2** and **5** ranged from 50 to values higher than 200 µg mL⁻¹ (Table I).

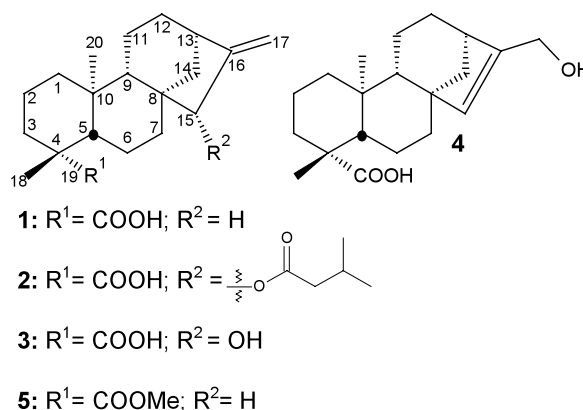


Fig. 1. Chemical structures of the kaurane diterpenes **1–5** from *Aspidia foliacea*.

Chlorhexidine was used as positive control, and its MIC values for each microorganism are described in Table I.

In recent years, the search for new anti-infection natural compounds has been the aim of many research groups (Ríos and Recio, 2005), since many bacterial pathogens have developed defense mechanisms and resistance against antimicrobial agents (Kuzma *et al.*, 2007). Therefore, it is reasonable to search for natural products that have anti-plaque properties and antimicrobial activity against oral pathogens (Koo *et al.*, 2000). In this work, we report on a comparison among structurally similar kaurane-type diterpenes with respect to their antibacterial activity against the main microorganisms responsible for dental caries and periodontal diseases for the first time.

Ríos and Recio (2005) analyzed the past, present and future of medicinal plants in the search for anti-infection agents, and they concluded that pure compounds displaying MIC values lower than or equal to 10 µg mL⁻¹ are very promising for the development of antibacterial drugs. In this sense, our results show that compound **1** has potential to be used as a prototype for the discovery of new effective anti-infection agents against microorganisms responsible for caries and periodontal diseases.

The results of the *in vitro* assays for **1** and **5** (Table I) clearly show that the presence of the carboxy group (C-19) causes an important contribution to the antibacterial activity against oral pathogens, and this activity decreases drastically in the case of the C-19 methyl ester. The structural analy-

Microorganism	Minimum inhibitory concentration [$\mu\text{g mL}^{-1}$ (μM)]			
	Chlorhexidine	1	2	5
<i>Enterococcus faecalis</i>	0.4 (0.69)	200.0 (661.26)	*	*
<i>Lactobacillus casei</i>	0.05 (0.09)	10.0 (33.06)	200.0 (496.81)	100.0 (315.98)
<i>Streptococcus mitis</i>	0.05 (0.09)	10.0 (33.06)	*	*
<i>Streptococcus mutans</i>	0.05 (0.09)	10.0 (33.06)	*	*
<i>Streptococcus sanguinis</i>	0.4 (0.69)	10.0 (33.06)	50.0 (124.20)	*
<i>Streptococcus sobrinus</i>	0.05 (0.09)	10.0 (33.06)	*	*
<i>Streptococcus salivarius</i>	0.1 (0.17)	100.0 (330.63)	*	*

Table I. *In vitro* antibacterial activity of the kaurane diterpenes **1**, **2**, **5** and chlorhexidine against oral pathogens.

* Inactive in the evaluated concentrations.

sis of compounds **1** and **2** allow to conclude that the introduction of an ester group (isovaleryloxy) at C-15 significantly decreases the investigated activity.

Mendoza *et al.* (1997) evaluated some kaurane diterpenes against Gram-positive bacteria. The results of their study showed that the introduction of a hydrophilic 3- β -OH group drastically reduces the antibacterial activity of these compounds. The marked differences in the MIC values of some structurally very similar kaurane diterpenes previously reported by Mendoza *et al.* (1997), as well as the MIC values found in our present study reveal the great importance of understanding the structure-activity relationships of these compounds, since minor structural alterations may improve their activities against several bacteria.

To date, the reports on the antimicrobial activity of natural products against oral pathogens are

scarce. However, comparing the MIC values against oral pathogens displayed by KA with those of previously described other secondary metabolites (Cai and Wu, 1996; Cunha *et al.*, 2007; Tsuchiya *et al.*, 1994), we can conclude that the kaurane-type diterpenes have potential use in the further development of natural anti-caries and anti-periodontal agents. Considering these results, it is important to emphasize that more biological, toxicological and structure-activity relationship studies must be undertaken, and the action mechanism of these diterpenes should be unveiled.

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