Pharmacogn J. 2018; 10(3):429-434.
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com | www.journalonweb.com/pj | www.phcog.net

Original Article

Bactericidal Kinetics and Antibiofilm Efficacy of Pimarane-Type Diterpenes from *Viguiera arenaria* against Cariogenic Bacteria

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ABSTRACT

Background: Dental caries is an infectious disease related to biofilm formation. Among the microorganisms presents in the oral microbiota, *Streptococcus mutans* can form biofilm on the tooth surface in the presence of dietary carbohydrates. This study aimed to evaluate the bactericidal kinetics and antibiofilm efficacy of ent-pimara-8(14),15-dien-19-oic acid (compound 1) and ent-8(14),15-pimaradien-3β-ol (compound 2) obtained from *Viguiera arenaria* against oral pathogens; this study also aimed to determine the effect of these compounds on the morphology and architecture of the *S. mutans* biofilm by scanning electron microscopy (SEM). **Materials and Methods:** The bactericidal kinetics revealed different results depending on the tested bacteria. Compound 1 eliminated the viable bacteria within 24 hs of incubation. In the antibiofilm assay, compound 1 displayed promising results against *S. mitis* (ATCC 49456 and clinical isolate) and *L. casei* (clinical isolate), whereas compound 2 was not active at the evaluated concentrations. **Conclusion:** Compound 1 is an important metabolite in the search for new antibacterial agents against cariogenic bacteria both in the sessile and planktonic modes. The SEM image of *Streptococcus mutans* in the presence of compound 1 suggested that this metabolite acts by disrupting the bacterial membrane disrupting the bacterial membrane and/or cell wall and causing microrganism death.

Key words: Antibacterial activity, Bactericidal kinetics, Scanning electron microscopy, *Streptococcus mutans*, *Viguiera arenaria*.

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History

- Submission Date: 04-09-2017;
- Review completed: 25-09-2017;
- Accepted Date: 20-11-2017

DOI : 10.5530/pj.2018.3.70

Article Available online
http://www.phcogj.com/v10/i3

INTRODUCTION

Despite the widespread use of different sources of fluoride, dental caries continues to be the most prevalent and costly infectious disease worldwide.[1,2] According to Bowen and Koo,[3] virulent biofilms tightly adhered to oral surfaces are a primary cause of dental caries. Dental caries results from interactions of specific bacteria and their metabolic products with teeth. In particular, *Streptococcus mutans*, an acidogenic and acid-tolerant bacteria, plays a specific role in the development of biofilms in the presence of extracellular polysaccharide (EPS) from dietary sucrose.[4,5] *Streptococcus* and *Lactobacillus* species are also involved in the pathogenesis of the disease and in later formation of the dental biofilm.[6]

Mechanical removal of dental biofilm is the most efficient procedure to prevent caries. However, the use of chemicals to control dental biofilm formation is also necessary to reduce the emergence of biofilm.[7] Chlorhexidine (CHD) is the most often employed agent to prevent dental biofilm formation. However, CHD modifies the perception of food taste and leaves a burning sensation at the tip of the tongue.[8,9,1]

According to Cragg and Newman,[10] natural products are a promising source for the discovery of biologically active compounds. The use of natural products to prevent or treat oral diseases dates back to several thousand years, and they remain a largely unexplored source of effective antibiofilm molecules.[1] The term natural products is related to secondary metabolites produced by an organism and which often have the function to defend said organism against microorganisms, herbivores, insects, and competing plants.[11] Among the various classes of metabolites, diterpenes are recognized as a class displaying a wide spectrum of biological activities including their significant antibacterial activity.[12-17]

The species *V. arenaria* contains a class of pimarane type-diterpenes that have been proven to exhibit potential action against cariogenic bacteria, as reported in previous studies by our research group. Indeed,[12] have found Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values lower than 10 µg/mL for such diterpenes.

Based on a previous study of pimarane type-diterpenes against oral pathogens and on the high incidence of dental caries, the present study aims to analyze the bactericidal kinetics, verify the ability of cariogenic bacteria to form biofilms, evaluate

Cite this article: Ferreira JH, Garcia RM, Abrão F, Fernandez YA, Pires RH, Ambrósio SR, Veneziani RCS, Martins CHG. Bactericidal Kinetics and Antibiofilm Efficacy of Pimarane-Type Diterpenes from *Viguiera arenaria* against Cariogenic Bacteria. Pharmacog J. 2018;10(3):429-34.

the antibiofilm efficacy of pimarane type-diterpenes, and determine the effect of these compounds on the morphology and architecture of the *S. mutans* biofilm.

MATERIALS AND METHODS

Compounds

Two pimarane type-diterpenes isolated from *Viguiera arenaria* Baker, *ent*-pimara-8(14),15-dien-19-oic acid (compound **1**) and *ent*-8(14), 15-pimaradien-3β-ol (compound **2**) were obtained by PORTO *et al.*[12]

Microorganisms

The tested strains were obtained from the American Type Culture Collection (ATCC). The following microorganisms were used in the present work: *Streptococcus mutans* (ATCC 25175 and clinical isolate), *Streptococcus sobrinus* (ATCC 33478 and clinical isolate), *Streptococcus sanguinis* (ATCC 10556 and clinical isolate), *Streptococcus mitis* (ATCC 49456 and clinical isolate), and *Lactobacillus casei* (ATCC 11578 and clinical isolate).

Antibiofilm activity

The Minimum Inhibitory Concentration of Biofilm ($MICB_{50}$) of compounds **1** and **2** against the bacteria evaluated in this study was determined on the basis of the minimum concentration of the antimicrobial agent that was able to inhibit 50% of biofilm formation or more.[18] For this purpose, a microtitration plate assay was used on the basis of the CLSI guidelines,[19] with some modifications. This method was similar to the MIC assay conducted for planktonic cells. Twofold serial dilutions of each sample were prepared in the wells of a 96-well polystyrene tissue culture plate (Costar) containing TSB broth (Difco) at a volume of 200 mL per well. The final concentration of compounds **1** and **2** ranged from 0.19 to 400 µg/mL. Chlorhexidine dichlorohydrate (CHD, Sigma) at concentrations between 0.115 and 59 µg/mL was the positive control; the bacterial strains in the absence of the antibacterial agent were the negative control. The cell suspension was added at concentrations of 10^9 CFU/mL. After 24 hs of incubation, biofilm inhibition was quantified by the following methodology. The plates were incubated in appropriate atmosphere, at a temperature of 37 °C (98.6 °F), for 24 hs. The concentration of the inoculum was adjusted according to McFarland standard 0.5. After incubation, the culture supernatants from each well were decanted, and planktonic cells were removed by washing with PBS, pH 7.2. The biofilm was fixed with methanol for 15 min and air dried at room temperature. It was then stained with 0.2% (w/v) crystal violet (Sigma) for 5 min and rinsed thoroughly with water until the control wells became colorless. To quantify biofilm formation, 200 µL of 33% acetic acid were added to each crystal violet-stained well. The plate was shaken at room temperature for 30 min, and the absorbance at 595 nm (A_{595}) was determined by using a microplate reader (ASYS, Eugendorf, Salzburg, Austria). The percentage of inhibition was calculated by using the equation [1 – (A_{595} of the test/A_{595} of non-treated control) x 100] as described by Wei *et al.*[18]

Bactericidal kinetics

The bactericidal kinetics of compound 1 was investigated in triplicate assays against *S. mutans* (ATCC 25175 and clinical isolate), *S. mitis* (ATCC 9811 and clinical isolate), *S. sanguinis* (ATCC 10557 and clinical isolate), *S. sobrinus* (ATCC 27609 and clinical isolate), and *L. casei* (ATCC 7469 and clinical isolate) as described by D'Arrigo *et al.*[20] Tubes containing compound 1 at final concentrations of one, two, and three times the MBC value were inoculated with the tested microorganism, to give a starting bacterial density of 5 x 10^5 CFU/mL. Next, the tubes were incubated at 37 °C. Samples were removed for determination of viable strains at 0, 30 min, 6 h, 12 h, 18 h, and 24 h after incubation, followed by dilution, when necessary, in sterile fresh medium. The diluted samples (50 mL) were spread onto tryptic soy agar plate supplemented with 5% sheep blood, incubated at 37 °C, and counted after 48 h. Time-kill curves were constructed by plotting $\log_{10}$CFU/mL versus time. The assays were performed in triplicate for each concentration and also for the positive (CHD) and negative controls (suspension of bacteria without added compounds **1**). CHD was used at its MBC.

Scanning electron microscopy (SEM)

Streptococcus mutans was selected for SEM processing. To determine the effect of antibacterial drugs on the morphology and architecture of the *S. mutans* biofilm, the latter was prepared according to a previously published protocol.[21,22] Biofilm formation was tested in the absence of the tested compounds and in the presence of compound **1** to evaluate its possible mechanism of action. *S. mutans* biofilms were formed on sterile polyvinylchloride (PVC) disks within 12-well cell culture plates (Corning) by dispensing cell suspensions containing 10^9 cells/mL in BHI onto appropriate disks at 37 °C. The biofilms formed on these disks were fixed with 2% formaldehyde (v/v) and 3% glutaraldehyde (v/v) in 0.1 M potassium phosphate buffer (pH 7.2-7.4)] for 48 h. After three washes (30-100%), the biofilms were critical-point dried in CO_2 (MS 850, Electron Microscopy Sciences) and coated with gold in a Denton Vacuum Desk II coater. Following processing, the specimens were visualized by SEM (JSM 5410: JEOL, Tokyo, Japan). Experiments were repeated three times with at least three replicates for each time point.

RESULTS AND DISCUSSION

According to studies on *V. arenaria* published by our research group, the diterpenes isolated from this plant are a potential source of compounds for the development of new drugs to combat oral pathogens.[14,15,16] However, additional studies are important to evaluate the real ability of these compounds to act against oral bacteria in both the sessile and planktonic modes. Here, we have investigated the bactericidal kinetics of compound **1** and its antibiofilm activity against cariogenic bacteria.

Figure 1 shows the bactericidal kinetics of different concentrations of compound **1** against cariogenic bacteria in the planktonic mode, for different periods of incubation.

In a previous paper,[13] established the MBC concentrations evaluated in the present study. Here, we also assayed two and three times the MBC values for each bacteria. The times of death of the 10 cariogenic bacteria studied herein ranged from 30 min to 24 h.

Exposure of *S. sobrinus* (ATCC 27609) to compound **1** at concentrations of 5, 10, and 15 µg/mL eliminated the viable microorganisms within 24, 12, and 6 h, respectively. Increasing concentration of compound **1** decreased the time that was necessary to eliminate the microorganisms. A similar behavior emerged for *S. sobrinus* (clinical isolate), with the exception that at a concentration of 10 µg/mL compound **1** eliminated this bacterium within 24 h of incubation.

Exposure to compound **1** killed *S. sanguinis* (ATCC 10557) within 12 h (compound **1** at 6.75 µg/mL) and 24 h (compound **1** at 2.25 and 4.5 µg/mL). On the other hand, at the three tested concentrations (5, 10, and 15 µg/mL), compound **1** eliminated the clinical isolate within 24 h. At the three tested concentrations, compound **1** eliminated *S. mutans* (ATCC 25175 and clinical isolate) within 24 h of incubation. CFU/mL decreased after 12 h of incubation; total elimination only happened within 24 h.

At concentrations of 15, 10, and 5 µg/mL, compound **1** eliminated viable *S. mitis* (ATCC 9811) cells within 30 min, 6 h, and 24 h, respectively. At concentrations of 8, 16, and 24 µg/mL, compound **1** killed *S. mitis* (clinical isolate) within 24, 12, and 6 h of incubation, respectively.

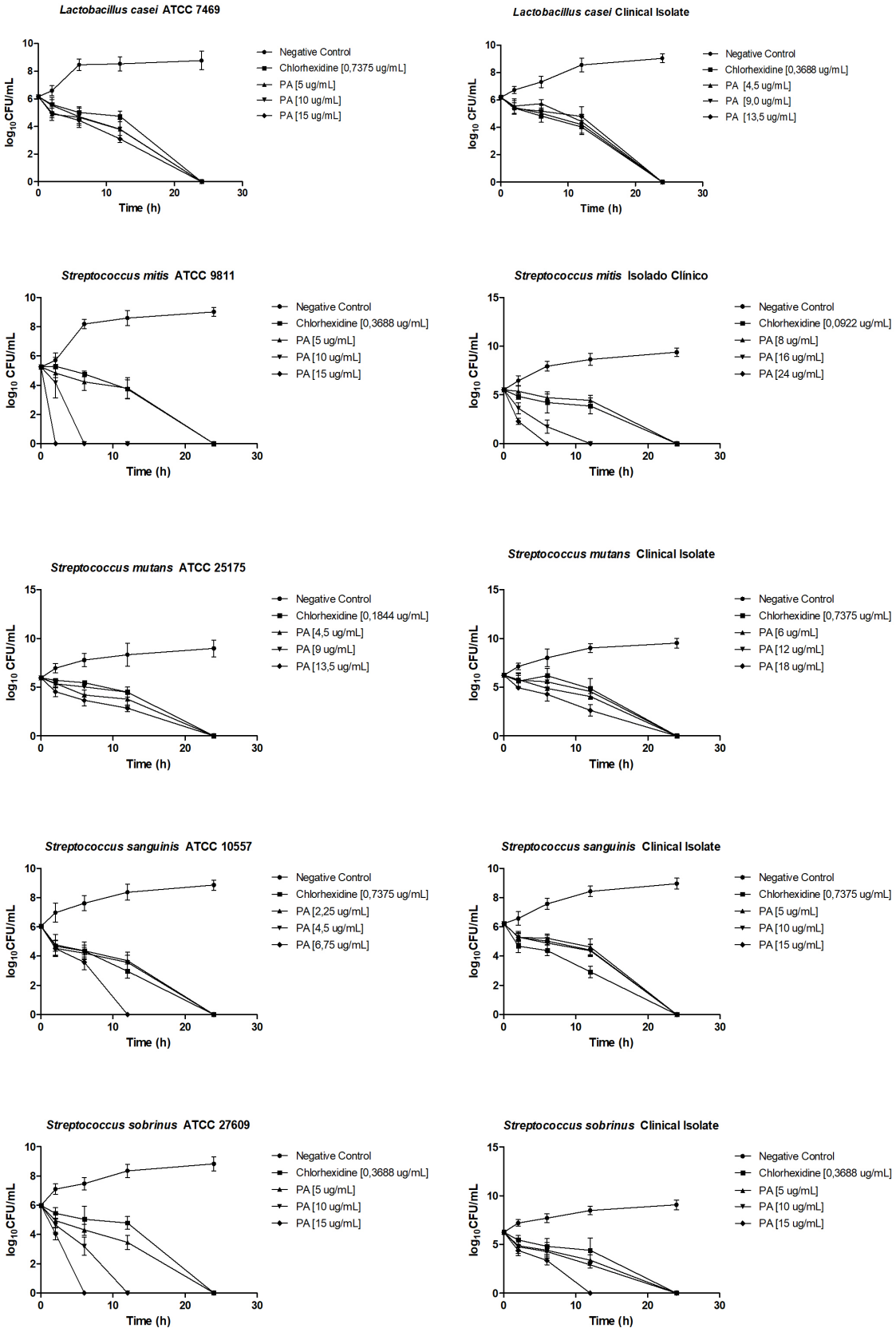

Figure 1: Bactericidal kinetics plots for pimara-ent-8(14),15-dien-19-oic acid (compound 1) against cariogenic bacteria.

Finally, at the three assessed concentrations, compound **1** eliminated *L. casei* (ATCC 7469 and clinical isolate) within 24 h of incubation.

Severiano *et al.*[23] evaluated the bactericidal kinetics of *S. mutans* (ATCC 25175) exposed to *ent*-8(14),15-pimaradien-19-ol and found that this compound only avoided growth of the inoculum within the first 12 h (bacteriostatic effect); however, its bactericidal effect became clear thereafter (between 12 and 24 h). These authors also combined *ent*-8(14), 15-pimaradien-19-ol with CHD. The bactericidal kinetics revealed that a significantly shorter time was necessary for the combination *ent*-8(14),15-pimaradien-19-ol + CHD to kill *S. mutans* as compared with the two chemicals alone. These results resembled the data obtained for this same pathogen in the present study—compound **1** required 24 h to eliminate this bacterium.

Souza *et al.*[14] examined the bactericidal kinetics of CHD against the primary causative agent of caries (*S. mutans*). Within the first 12 h of incubation, CHD only inhibited bacterial growth (bacteriostatic effect). However, its bactericidal effect became evident thereafter (between 12 and 24 h). These results were similar to the ones found here and confirmed the antibacterial activity of compound **1**.

We also evaluated the antibiofilm activity of compounds **1** and **2** against cariogenic bacteria and determined the $MICB_{50}$, listed in Table 1.

In the biofilm, the bacteria displayed high resistance to antibiotics, disinfectants, and host immune system clearance. The importance of biofilm is well recognized in medical, environmental, and industrial contexts.[24] According to Ramage,[25] biofilms tend to be 10 to 1000 times more resistant to antimicrobial agents as compared with the planktonic mode.

In the present study, compound **1** gave the best $MICB_{50}$ results against *S. mitis* ATCC 49465 and clinical isolate—12.5 and 50 µg/mL, respectively. This compound was also effective against *L. casei* (clinical isolate) and *S. sanguinis* (ATCC 10556 and clinical Isolate), with $MICB_{50}$ of 50, 100, and 200 µg/mL, respectively. At the tested concentrations, compound **2** was not effective against the evaluated bacteria: $MICB_{50}$ results were higher than 400 µg/mL.

Finally, we also evaluated the effect of antibacterial metabolites on the morphology and architecture of *S. mutans* biofilm. **Figure 2** presents the SEM image.

As described by Carvalho *et al.*,[16] the mechanisms behind the antibacterial activity of this class of compounds have not yet been elucidated. Urzúa *et al.*[26] and Wilkens *et al.*[27] have suggested that these metabolites promote lysis when they insert into the lipophilic cell membrane, consequently disrupting it. Carvalho *et al.*[16] have provided support for the mechanism of action suggested by Urzúa *et al.*[26] and Wilkens *et al.*[27]

Both *ent*-pimara-8(14),15-dien-19-oic acid (compound **1**) as well *ent*-8(14),15-pimaradien-3β-ol (compound **2**) contain a HBD at C-3 or C-19 in their structure, different from another isolated molecules by Porto *et al.* (12) which has no HBD or has two HBD in their structure. According to Úrzua *et al.*[26] the presence of two HBDs decrease the lipophilicity of the hydrophobic moiety, hindering its interaction with the bacterial membrane and the intramolecular HBD group interactions compete with intermolecular hydrogen bonds between each HBD and the cell membrane. The results appointed in this work corroborate.[26,12]

According to the SEM images, compound **1** acted by disrupting the bacterial cell membrane and/or cell wall and killing the microorganism; i.e., the pimarane type-diterpene attacked the membrane of the bacteria in the sessile mode, which confirmed the suggestion.[16,28] have assayed the *Copaifera duckei* oleoresin, which is rich in diterpenes, against bacteria of clinical and food interest and have determined its possible mechanism of action. These authors found that this oleoresin acted on the bacterial cell wall by removing proteins and the S-layer, thereby interfering in the cell-division process.

Lee *et al.*,[29] examined the effect of garlic extract on the formation of *Streptococcus mutans* biofilms on orthodontic wire. Despite its antibacterial function, garlic extract increased *S. mutans* biofilm formation on orthodontic wire as desmonstrated by SEM via activaction of glucosyltransferase expression.

Jeong *et al.*,[30] investigated the anticariogenic properties of *ent*-kaur-16-en-19-oic acid (KA) isolated from *Aralia continentalis*. SEM confirmed the inhibitory effect of KA on biofilm formation. Treatment with 3 and 4 µg/mL KA inhibited and completely inhibited biofilm formation, respectively. The authors suggested that KA exerts its bactericidal effect by disrupting the *S. mutans* cell membrane.

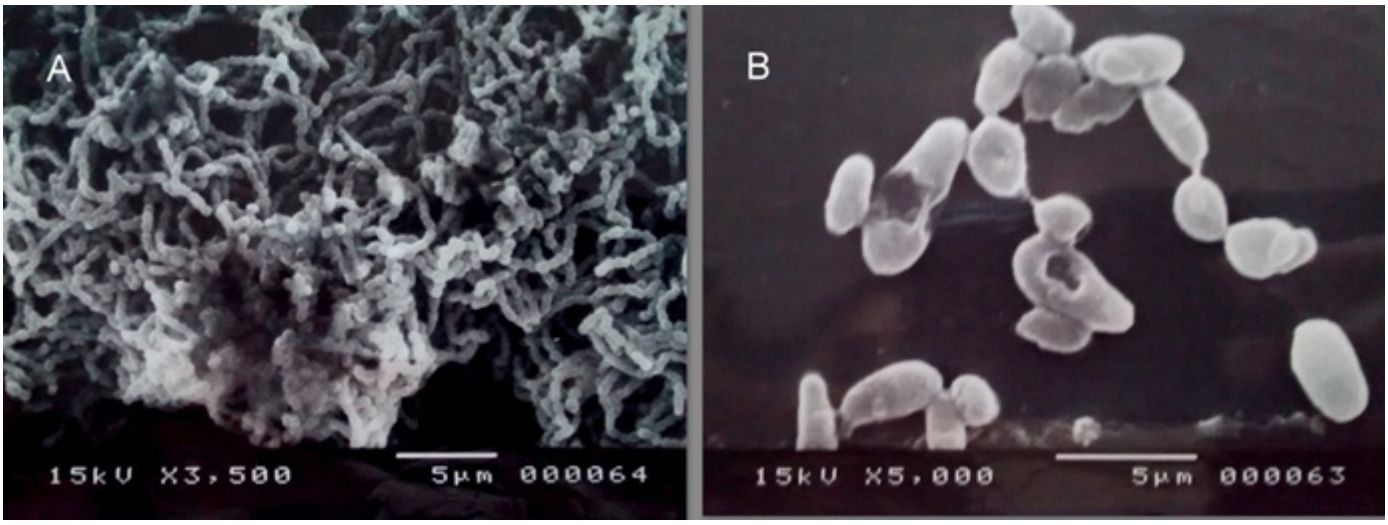

Figure 2: Scanning electron microscopy (SEM) images of *Streptococcus mutans* biofilms. Biofilms emerged after 24-hour incubation in 12-well plates. Preparation and observation under SEM were carried out as described in the text. The images showed thick *S. mutans* biofilms on the surface; these biofilms consisted of groups of cells separated by water channels. A: note a mature *S. mutans* biofilm consisting of a dense network; B: *S. mutans* after 24 hours of incubation with the tested metabolite (compound **1**).

Table 1: $MICB_{50}$ of diterpene type-pimarane against cariogenic bacteria.

Microorganisms	Compound 1	Compound 2
S. mutans (ATCC 25175)	>400	>400
S. mutans (Clinical Isolate)	>400	>400
S. mitis (ATCC 49456)	12.5	>400
S. mitis (Clinical Isolate)	50	>400
S. sanguinis (ATCC 10556)	100	>400
S. sanguinis (Clinical Isolate)	200	>400
S. sobrinus (ATCC 33478)	>400	>400
S. sobrinus (Clinical Isolate)	>400	>400
L. casei (ATCC 11578)	>400	>400
L. casei (Clinical Isolate)	25	>400

$MICB_{50}$ - The Minimum Inhibitory Concentration Biofilm is the lowest tested concentration of the plant compound that was able to inhibit ≥ 50% of the bacterial biofilm.

CONCLUSION

In summary, our results have shown that *ent*-pimara-8(14),15-dien-19-oic acid is an important metabolite in the search for new antibacterial agents against cariogenic bacteria in both the sessile and planktonic modes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this paper.

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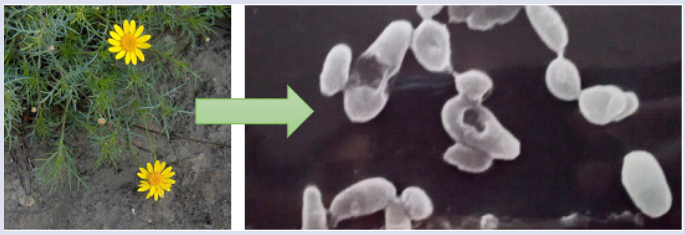
GRAPHICAL ABSTRACT



SUMMARY

- *Pimarane-type diterpene* demonstrated being a promising metabolite that can be used in treatment of dental biofilms.
- The authors suggested that pimara-ent-8(14),15-dien-19-oic acid exert its bactericidal effect by disrupting the *S. mutans* cell membrane and/or cell wall.
- Secondary metabolites *Viguiera arenaria* can be an important hole in the development of new anticaries agent.

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Cite this article: Ferreira JH, Garcia RM, Abrão F, Fernandez YA, Pires RH, Ambrósio SR, Veneziani RCS, Martins CHG. Bactericidal Kinetics and Antibiofilm Efficacy of Pimarane-Type Diterpenes from *Viguiera arenaria* against Cariogenic Bacteria. Pharmacog J. 2018;10(3):429-34.