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Antimicrobial Activity of Terpenoids from *Copaifera langsdorffii* Desf. Against Cariogenic Bacteria

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In the present work, the anticariogenic activities of nine labdane type-diterpenes and four sesquiterpenes were investigated. Among these metabolites, (-)-copalic acid (CA) was the most active compound displaying MIC values very promising (ranging from 2.0 to 6.0 μg/mL) against the main microorganisms responsible for dental caries: *Streptococcus salivarius, S. sobrinus, S. mutans, S. mitis, S. sanguinis* and *Lactobacillus casei*. Time kill assays performed with CA against the primary causative agent (*S. mutans*) revealed that, in the first 12 h, this compound only inhibits the growth of the inoculum (bacteriostatic effect). However, its bactericidal effect is clearly noted thereafter (between 12 and 24 h). Also, CA did not show a synergistic effect when combined with the anticariogenic gold standard (chlorhexidine, CHD) in the checkerboard assays against *S. mutans*. In conclusion, the results points out CA as an important metabolite in the search for new effective anticariogenic agents. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: dental caries; *Copaifera langsdorffii* Desf.; labdane-type diterpenes; cariogenic bacteria; *Streptococcus mutans*; antimicrobial activity.

INTRODUCTION

Cariogenic bacteria are a group of microorganisms responsible for dental caries, one of the main oral diseases which affect humankind (More *et al*., 2008). This pathology is caused by a biofilm on the tooth surface (dental plaque) that is associated with the growth of *Streptococcus* and *Lactobacillus* species (Chung *et al*., 2006). *Streptococcus mutans* is considered one of the main cariogenic microorganisms, since it is responsible for the beginning of the caries process. Other aerobic bacteria such as *Enterococcus faecalis, Lactobacillus casei, Streptococcus mitis, S. sanguinis*, *S. sobrinus* and *S. salivarius* are also important in the later formation of the dental biofilm (Chung *et al*., 2006).

The mechanical removal of the dental plaque is the most efficient procedure to prevent caries; however, the use of chemicals as a complementary measure is also necessary and has demonstrated to be of great value with respect to decreasing the tooth surface biofilm (Furiga *et al*., 2008). Nowadays, chlorhexidine is considered a gold standard anticariogenic and has received the approval of the American Dental Association Council on Dental Therapeutics (Ambrósio *et al*., 2008). However, the regular use of oral care products containing this chemical are often associated with tooth and restoration staining, changes in the taste of food, and a burning sensation at the tip of the tongue (Greenberg *et al*., 2008; More *et al*., 2008; Porto *et al*., 2009b). In addition, chlorhexidine is much less effective in reducing the levels of *Lactobacillus*, which are strongly related to caries evolution (Ambrósio *et al*., 2008). These problems, therefore, denote that finding new effective anticariogenic compounds is essential.

Natural products have been a rich and promising source for the discovery of novel biologically active compounds (Newman, 2008). Several classes of secondary metabolites are synthesized by plants and, among them, diterpenes are recognized as a class with a wide spectrum of biological activities (Ambrósio *et al*., 2006), including their significant antibacterial activity (Kúzma *et al*., 2007; Almeida *et al*., 2008; Porto *et al*., 2009a).

Recently, our research group has demonstrated that some *ent*-kaurane and *ent*-pimarane type diterpenes are able to inhibit the growth of the main microorganisms responsible for dental caries with very promising minimal inhibitory concentration (MIC) values (ranging from 2 to 10 μg/mL). In these previous works, it was also pointed out that some of these metabolites may be potentially useful in the further development of natural anticaries agents (Ambrósio *et al*., 2008; Porto *et al*., 2009b). These results allowed us to conclude that this class of natural compounds is an important source for the discovery of new efficient bioactive compounds for dental application.

In agreement with our early findings and as part of our ongoing efforts to explore the antimicrobial potential of diterpenes against the pathogens responsible for dental caries, our research group has decided to investigate the activity of a different class of diterpenes.

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For this purpose, a commercially available and well documented source of natural labdane-type diterpenes (Veiga Júnior and Pinto, 2002), namely the oleoresin of *Copaifera langsdorffii*, was chosen. Moreover, an antimicrobial evaluation was also carried out of some semi-synthetic diterpene derivatives and the main sesquiterpenes present in this plant material.

MATERIALS AND METHODS

Plant material. Authentic oleoresin from *Copaifera langdsdorffii* was kindly provided by the Brazilian company Apis-Flora Comercial e Industrial.

General. NMR spectra were recorded on a Bruker Avance DRX 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Samples were dissolved in $CDCl_3$, and TMS was used as internal reference. High performance liquid chromatography (HPLC) analyses were performed using a Shimadzu CBM-20A liquid chromatography controller, operating with the LC solution software, equipped with a Shimadzu UV-DAD detector SPD-M20A and a Shimadzu ODS column (4.6 × 250 mm, 5 μm, 100 Å). Gas chromatography (GC) analyses were carried out in Hewlett Packard GC equipment, model 6890N, equipped with a split/splitless injector inlet and a flame ionization detector (FID). The output was recorded using the workstation. An HP-5 capillary column (30 m of length × 0.32 mm of internal diameter × 0.25 mm of film thickness) was used. Hydrogen at a flow rate of 1.8 mL/min was employed as the carrier gas, and the GC oven temperature was programmed to rise from 100 to 140°C at 10°C/min, from 140 to 180°C at 2.8°C/min, maintained at 180°C for 1 min, followed by an increase from 180 to 280°C at 20°C/min, and kept at 280°C for 1 min more. The temperatures of the injector and detector ports were kept at 240°C and 290°C, respectively. The injector was operated in a split mode of 1/50. Gas chromatography/mass spectrometry (GC/MS) was performed using a Shimadzu – QP 2010 gas chromatography equipped with an automatic injector AOC – 20Si, a DB-5 column (30 m × 0.25 mm × 0.25 μm), and a mass spectrometer of the same company, which was operated in the EI mode (beam energy voltage 70 eV). Hydrogen at a flow rate of 1.8 mL/min was employed as the carrier gas. Both the injector and oven temperatures were programmed as described above. The main constituents were identified by comparing their retention indices (RI relative to C_9-C_{25} *n*-alkanes), which were obtained by GC-FID and GC/MS analyses, with those reported in the literature, as well as by comparison of the obtained mass spectra of the peaks with those either reported in the literature or available in the Wiley NBS data system library. In order to perform the antimicrobial evaluations against oral pathogens, the main sesquiterpenes identified in *C. langsdorffii* oleoresin were purchased from Sigma-Aldrich (*trans*-caryophyllene, Sigma, technical grade 98.5%; α-copaene, Aldrich, technical grade ≥ 90.0%; α-humulene, Aldrich, technical grade ≥ 98.0%).

Isolation of compounds. About 20.0 g of oleoresin was chromatographed over silica gel 60 H (Merck, art. 7736) using vacuum liquid chromatography (VLC) (Pelletier *et al.*, 1986) with increasing amounts of EtOAc in *n*-hexane as eluent. This procedure furnished six fractions (500 mL each) that were named F1 (4.9 g; *n*-hexane), F2 (3.1 g; 20% EtOAc), F3 (3.6 g; 40% EtOAc), F4 (2.2 g; 60% EtOAc), F5 (2.7 g; 80% EtOAc) and F6 (2.1 g; EtOAc) after solvent evaporation. Samples F1 and F2 were firstly analysed by GC and GC/MS, in order to identify their main volatile compounds. Fraction F3 (1.0 g) was fractionated over silica gel 60 (Merck, art. 7734) using classic chromatography (isocratic, *n*-hexane: EtOAc 7 : 3), to furnish compounds **1** (caryophyllene oxide; 117.0 mg) and **2** ((-)-copalic acid; 450.0 mg).

Both F4 and F5 were initially chromatographed by VLC over silica gel 60 H (Merck, art. 7736) as described above, to give additional fractions (F4.1–F4.5 and F5.1–F5.5). Compound **3** ((-)-acetoxycopalic acid; 230.0 mg) was obtained from F4.3 (880.0 mg) through medium pressure chromatography (flash chromatography) using silica gel 60 (Merck, art. 9385), isocratic *n*-hexane : EtOAc : $CHCl_3$ (5 : 2 : 3) as mobile phase, and a flow rate of 5 mL/min (Still *et al.*, 1978). Subfractions F5.2 (350.0 mg) and 5.4 (270.0 mg) were also chromatographed by flash chromatography as described above. These procedures led to the isolation of two compounds which, after NMR analysis, were identified as (–)-3-hydroxy- 14,15-dinorlabd-8(17)-en-13-one (**4,** 110. 0 mg), obtained from 5.2, and (-)-agathic acid (**5**, 150. 0 mg), originated from 5.4. Thin layer chromatography (TLC) analysis of F5.2.5 showed a main spot, which was later purified by preparative thin layer chromatography using silica gel PF_{254} (Merck art. 9385; 1 mm thickness) and isocratic *n*-hexane: EtOAc 1 : 1 as mobile phase. Compound **6** ((-)-hydroxycopalic acid, 130.0 mg) was obtained from this procedure and identified after NMR analysis.

A sample of F6 was analysed by reversed phase HPLC using an analytical Shimadzu ODS column (4.6 × 250 mm, 5 μm, 100 Å; MeCN : H_2O 85 : 15; flow rate 1.0 mL/min; UV detection at 210 nm); compounds **5** and **6** were identified as the main constituents of this fraction.

Semi-synthetic derivatives. About 100.0 mg of compound **2** was treated with ethereal diazomethane and, after addition of a small amount of acetic acid to destroy the remaining diazomethane and elimination of the solvent, the methylated derivative **7** was obtained (Ambrósio *et al.*, 2008). Compounds **3**, **5** and **6** were submitted to the same treatment, to give compounds **8**, **9** and **10**, respectively.

Bacterial strains and antimicrobial testing. The minimal inhibitory concentration values (MIC; lowest concentration of the compound capable of inhibiting microorganism growth) and the minimal bactericidal concentration (MBC; lowest concentration of the compound at which 99.99% or more of the initial inoculum was killed) were determined in triplicate by using the microdilution broth method in 96-well microplates (Porto *et al.*, 2009b). The tested strains were obtained from the American Type Culture Collection (ATCC). The following microorganisms were used in the present work: *Streptococcus salivarius* (ATCC 25975), *Strepto-*

coccus sobrinus (ATCC 33478), *Streptococcus mutans* (ATCC 25275), *Streptococcus mitis* (ATCC 49456), *Streptococcus sanguinis* (ATCC 10556) and *Lactobacillus casei* (ATCC 11578).

Samples were dissolved in dimethyl sulfoxide (DMSO; Synth) at 1 mg/mL, followed by dilution in tryptic soy broth (Difco); concentrations ranging from 200.0 to 1.0 μg/mL were achieved. The final DMSO content was 5% (v/v), and this solution was used as a negative control. The inoculum was adjusted for each organism, to yield a cell concentration of 5×10^5 colony forming units (CFU) per mL, according to guidelines by the Clinical Laboratory Standards Institute. 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 employed, to ensure medium sterility. Chlorhexidine dihydrochloride (CHD) was used as positive control. The microplates (96-wells) were sealed with plastic film and incubated at 37°C for 24 h. After that, resazurin (30 μL) in aqueous solution (0.02%) was added to the microplates, to indicate microorganism viability (Porto *et al*., 2009b).

Before the addition of resazurin and in order to determine MBC, an aliquot of the inoculum was aseptically removed from each well presenting no apparent growth and then plated onto tryptic soy agar supplemented with 5% sheep blood. The plates were incubated as described previously. Determination of the MBC values were only performed for the most active compound (**2**, CA), against the strains whose MIC values were very promising (equal to or lower than 10 μg/mL) (Ríos and Récio, 2005).

Time-kill curves. Time-kill assays were performed in triplicate based on D'Arrigo *et al*. (2010) against *Streptococcus mutans*, which is considered one of the primary causative agents of dental caries (Chung *et al*., 2006). Compound **2** (CA) was chosen for time-kill curve assays because it displayed the highest antimicrobial activity.

Tubes containing CA at final concentrations of 7.0, 14.0 and 21.0 μg/mL (respectively one, two, and three-times the MBC of CA for *S. mutans*) were inoculated with the tested microorganism, resulting in a start bacterial density of 5×10^5 CFU/mL, and then incubated at 37°C. Samples were removed for the determination of viable strains at 0, 5, 15 and 30 min and 6, 12, 18 and 24 h after incubation, followed by dilution, when necessary, in sterile fresh medium. The diluted samples (50 μL) 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 the $\log_{10}$ CFU/mL versus time. The assays were performed in triplicate for each concentration and also for the positive (chlorhexidine dihydrochloride, CHD, 4 μg/mL) and negative controls (suspension of *S. mutans* without added CA). CHD was used at its MBC (4 μg/mL).

Synergistic antimicrobial activity. Checkerboard assays were performed according to the protocol described previously by White *et al*. (1996). The objective was to investigate the *in vitro* antimicrobial efficacy of the combination of CHD with CA. The synergy tests were evaluated in triplicate, and concentrations of each compound (1/32 to 3 times of their MIC values) were combined in standard MIC format against 5×10^5 CFU/mL of *S. mutans*. To evaluate the synergism effect between CHD and CA, the fractional inhibitory concentration (FIC) index values were calculated on the basis of the equation previously established in the literature (White *et al*., 1996). FIC index values were analysed as follows: FIC index values ≤ 0.5, synergism; FIC index values > 4, antagonism. Indifference was defined as an FIC index of greater than 0.5 but of equal to or less than 4 (White *et al*., 1996).

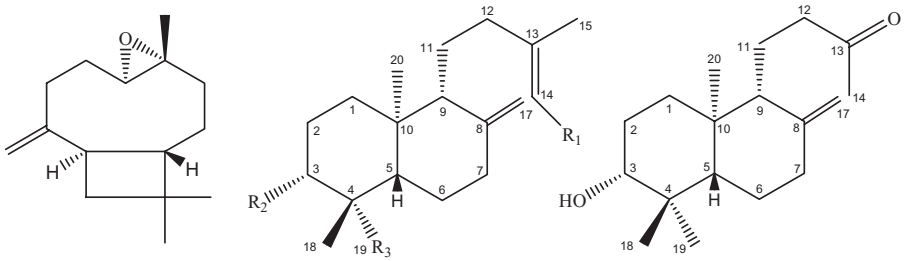
RESULTS AND DISCUSSION

Figure 1 shows the chemical structures of the metabolites that were evaluated in the present work. The spectral data of the compounds isolated from the oleoresin of *C. langsdorffii* are in agreement with those reported previously in the literature: **1** (Moreira *et al*., 2007), **2** (Ohsaki *et al*., 1994), **3** (Braun and Breitenbach, 1977), **4** (Romero *et al*., 2009), **5** (Zdero *et al*., 1991) and **6** (Romero *et al*., 2009). The structures of the semi-synthetic methyl derivatives (**7–10**) were confirmed on the basis of comparison of their nuclear magnetic resonance (^{1}H and ^{13}C NMR) data with their respective acid diterpenes. All isolated substances as well as those obtained by semi-synthetic means were evaluated by TLC using different solvent systems. According to these procedures and in addition to their ^{1}H and ^{13}C NMR data, the purity of such compounds was considered suitable for the antimicrobial assays. Analysis by GC and GC/MS of F1 and F2 allowed the identification of compounds **11** (*trans*-caryophyllene), **12** (α-copaene) and **13**(α-humulene) as the main metabolites of these fractions.

The MIC values for the investigated sesquiterpenes and diterpenes are shown in Table 1. Among all metabolites, compound **2** (CA) displayed the highest antibacterial activity, furnishing MIC values ranging from 2.0 to 6.0 μg/mL. On the other hand, compounds **3** and **4** demonstrated a moderate activity (MIC values between 40 and 80 μg/mL), while the other substances were not able to efficiently inhibit the growth of most pathogens (MIC values higher than 100 μg/mL).

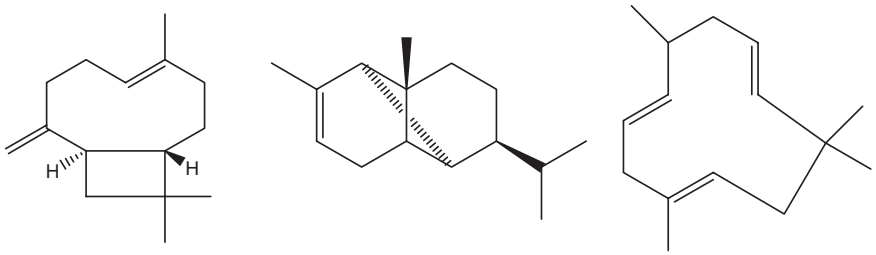
Time-kill curve assays (Fig. 2) were constructed against *S. mutans* (5×10^5 CFU/mL) only for CA, using the three different concentrations (7.0, 14.0 and 21.0 μg/mL). The resulting time-kill curves are presented in Fig. 2, which reveals that CA is able to kill *S. mutans* after 24 h of incubation. Analysis of these data also shows that CA drastically reduces the number of microorganisms after 12 h, in the three evaluated concentrations. No statistical differences were observed during the evaluated periods ($p < 0.05$). As for the synergistic antimicrobial test, the combinations of CHD with CA did not exhibit any synergic effects when the checkerboard methodology described by White *et al*. (1996) was employed. According to this author, the obtained FIC index = 2.02 means 'indifference'.

Our research group has concentrated efforts on the evaluation of the antimicrobial potential of diterpenes, aiming to discover new natural anticaries agents. It was



1 **4**

2: R_1 = COOH; R_2 = H; R_3 = H
3: R_1 = COOH; R_2 = $OCOCH_3$; R_3 = H
5: R_1 = COOH; R_2 = H; R_3 = COOH
6: R_1 = COOH; R_2 = OH; R_3 = H
7: R_1 = $COOCH_3$; R_2 = H; R_3 = H
8: R_1 = $COOCH_3$; R_2 = $OCOCH_3$; R_3 = H
9: R_1 = $COOCH_3$; R_2 = H; R_3 = $COOCH_3$
10: R_1 = $COOCH_3$; R_2 = OH; R_3 = H



11 **12** **13**

Figure 1. Chemical structures of the evaluated compounds.

Table 1. *In vitro* antibacterial activity (MIC) of the metabolites from *C. langsdorffii* against oral pathogens

	Minimum inhibitory concentration – μg/mL (minimum bactericidal concentration – μg/mL)													
Microorganism	**PC**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Streptococcus salivarius	0.9	*	2.0 (2.0)	12.0	80.0	*	*	120.0	180.0	120.0	200.0	*	*	*
Streptococcus mutans	0.9 (4.0)	*	3.0 (7.0)	40.0	60.0	*	*	100.0	150.0	100.0	120.0	200.0	*	*
Streptococcus mitis	3.6	200.0	5.0 (12.0)	60.0	80.0	*	*	80.0	120.0	80.0	150.0	150.0	*	*
Streptococcus sobrinus	0.9	*	3.0 (3.0)	40.0	40.0	*	*	150.0	100.0	120.0	180.0	180.0	*	*
Streptococcus sanguinis	3.6	*	6.0 (15.0)	60.0	40.0	*	*	60.0	80.0	100.0	120.0	200.0	*	*
Lactobacillus casei	0.9	150.0	4.0 (10.0)	50.0	60.0	*	*	80.0	150.0	80.0	150.0	150.0	*	*

* Inactive in the evaluated concentration (MIC values higher than 200 μg/mL); Positive control (PC), chlorhexidine dihydrochloride; Negative control (5% DMSO solution v/v) did not affect the growth of the microorganisms.

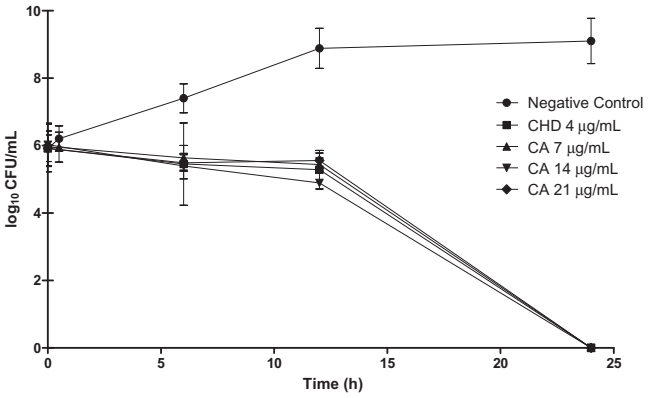


Figure 2. Time-kill curves for CA against *S. mutans*. Positive control: CHD 4 μg/mL.

demonstrated that kaurenoic acid and several pimarane-type diterpenes, isolated from Brazilian plants (Ambrósio *et al.*, 2008; Porto *et al.*, 2009b), could be used as prototypes for the discovery of new effective antiinfection agents against the microorganisms responsible for this pathology. Moreover, it was observed that minor structure modifications drastically affect the referred antimicrobial activity.

The present work investigated the effect of nine diterpenes and four sesquiterpenes from the oleoresin of *C. langsdorffii* against the main microorganisms responsible for human caries. From the evaluated metabolites, it is possible to observe that CA is the most effective compound (Table 1), since it furnishes very promising MIC values for all the investigated pathogens (MIC values lower than 10 μg/mL; Ríos and Récio, 2005). These values are much lower than some previously reported in the literature for compounds belonging to other classes of natural products, such as triterpenoids (Rivero-Cruz *et al.*, 2008), monoterpenes (Botelho *et al.*, 2007), phenolic compounds (Greenberg *et al.*, 2008) and lignans (Silva *et al.*, 2007).

Time-kill curve assays (Fig. 2) denote, for all the evaluated concentrations, that CA is able to completely kill *S. mutans* after only 24 h of incubation. Figure 2 also

reveals that in the first 12 h, CA only inhibits the growth of the inoculum. This leads to the interpretation that CA displays a bacteriostatic effect during this period, but its bactericidal effect is clearly noted thereafter (between 12 and 24 h). No statistical differences were demonstrated within the periods of time investigated for each concentration, allowing us to conclude that no dose-dependent responses were verified for CA in the assay conditions. Analysis of Fig. 2 also shows that CA at 23.1 μM (7.0 μg/mL) exhibits a time-kill curve profile that is very similar to those demonstrated by CHD at 6.9 μM (4.0 μg/mL) ($p < 0.05$). In addition, the inoculum concentration is equivalent for both curves. These results confirm that CHD, the anticariogenic gold standard, is about three and half times more potent than CA. Nevertheless, several adverse effects are associated with the regular use of CHD. This reinforces the great importance of CA as a prototype for the development of novel anticaries agents (More *et al*., 2008).

Antimicrobial compounds have demonstrated different action mechanisms (Greenberg *et al*., 2008). Several authors have emphasized that diterpenes are an important class of plant metabolites for the search of new antibacterial agents; however, the mechanism(s) responsible for this property have not yet been very well elucidated (Urzúa *et al*., 2008). Urzúa *et al*. (2008) have suggested that these metabolites promote bacterial lysis and disruption of the cell membrane. According to these authors, the structural features that promote the efficient antibacterial activity include a lipophilic structure, capable of insertion into the cell membrane, and one strategically positioned hydrogen-bond-donor group (HBD; hydrophilic group), which interacts with the phosphorylated groups on the membrane. In these studies, it was also emphasized that a second HBD introduced in the lipophilic region led to reduction in or suppression of the activity. A careful observation of the results depicted in Table 1 reveals that compound **2,** which contains only one HBD at C-16, displays much lower MIC values than those achieved with diterpenes **3–10**, which contain two HBD in their structures. Based on these considerations, our results give support to the mechanism of action suggested by Urzúa *et al*. (2008). However, comparison of the MIC values displayed by CA (Table 1) and other acid diterpenes previously reported in the literature, such as *ent*-kaurenoic acid (KA) and *ent*-pimaradienoic acid (Ambrósio *et al*., 2008; Porto *et al*., 2009b) reveals that the antibacterial activity displayed by this class of natural products is also ruled by other structural features. These statements justify the need for information about the anticariogenic potential of a large number of diterpenes, so that further quantitative structure-active relationship (QSAR) studies can be accomplished.

Urzúa *et al.* (2008) have also demonstrated that the efficiency of hydrogen bond interactions between the phosphorylated groups on the membrane and the HBD in the diterpene are very important for the antibacterial activity. According to these authors, KA is much more active than its respective methyl ester (KA-Me). The docking of these compounds into a model using phosphatidylcholine bilayer revealed that KA-Me is more deeply embedded into the hydrophobic region of the membrane compared with KA, presumably diminishing the capacity of the molecule to disrupt or damage the cell membrane. This observation is also in agreement with our results when the MIC values displayed by the *ent*-labdanes diterpenes **2** and **7** are compared (Table 1).

According to several authors (Vuorela *et al*., 2004; Newman, 2008), drugs derived from medicinal plants can serve not only as new drugs themselves but also as prototypes suitable for optimization through several approaches, including medicinal and semi-synthetic strategies. In this sense, modifications of the carboxylic acid of CA into other hydrophilic moieties, such as an amino, aldehyde, salt, among others, should be investigated for improvement of its anticariogenic activity.

In conclusion, the results have shown that CA is an important metabolite in the search for new effective antibacterial agents against the pathogens responsible for dental caries. This is an important report, because very few natural products are known to inhibit the growth of oral pathogens (Saleem *et al*., 2010). Moreover, the antimicrobial results displayed by the *ent*-labdanes diterpenes described in the present work can be used to promote docking and QSAR studies, in association with data from other diterpenes reported previously. This should certainly aid total elucidation of the structural features involved in the anticariogenic effect displayed by these natural compounds.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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