European Journal of Medicinal Plants
4(7): 794-809, 2014

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Phytochemical Analysis of *Plectranthus sp.* Extracts and Application in Inhibition of Dental Bacteria, *Streptococcus sobrinus* and *Streptococcus mutans*

Neusa L. Figueiredo[1], Pedro Luis Falé[2,3], Paulo J. Amorim Madeira[2], M. Helena Florêncio[1,2], Lia Ascensão[3,4], Maria Luisa M. Serralheiro[1,2*] and Ana Rosa L. Lino[1,2]

[1]Departament of Chemistry and Biochemistry, Faculty of Science, University of Lisbon, Campo Grande 1749-016, Lisbon, Portugal.
[2]Centre of Chemistry and Biochemistry, Faculty of Science, University of Lisbon, Campo Grande 1749-016, Lisbon, Portugal.
[3]Centre of Plant Biotechnology (IBB), Faculty of Science, University of Lisbon, Campo Grande 1749-016, Lisbon, Portugal.
[4]Departamentof Plant Biology, Faculty of Science, University of Lisbon, Campo Grande 1749-016, Lisbon, Portugal.

Authors' contributions

This work was carried out in collaboration between all authors. Authors NLF and PLF performed the biological activity test, the statistical analysis and together with the authors PJAM and MHF identified the components of the plant extracts. Author LA collected and identified the plant species. Authors ARL and MLS designed the study and discussed the results. All the authors wrote the first draft of the manuscript and approved the final version.

Original Research Article

Received 24th October 2013
Accepted 20th December 2013
Published 25th March 2014

ABSTRACT

Aims*:* Evaluation of leaves methanol extracts from *Plectranthus barbatus* and *Plectranthus ecklonii* (Lamiaceae) against oral pathogens.
Place and Duration of the Study: *P. barbatus* and *P. ecklonii,* cultivated in Botanic Garden of the University of Lisbon, were collected during winter 2009.
Methodology: Methanol extracts were prepared and the compounds separated and

**Corresponding author: Email: mlserralheiro@fc.ul.pt;*

identified by HPLC-DAD and mass spectrometry. The anticariogenic activity was determined by measuring the inhibition activity towards the growth of the pathogens *Streptococcus mutans* and *S. sobrinus* together with the inhibitory activity against the enzyme glucosyltransferase (GTF) involved in the biosynthesis of glucans.
Results: Phytochemical analysis of the extracts revealed the presence of two abietane diterpenoids in *P. barbatus* and two quinone methides together with rosmarinic acid in *P. ecklonii*. The two *Plectranthus* extracts showed bacteriostatic activity with minimum inhibitory concentrations (MIC) of 0.3 mg/mL. The minimum bactericidal concentrations (MBC) obtained for both extracts were 0.6 mg/mL against *S. sobrinus* and 0.8 mg/mL against *S. mutans*. After exposing both strains during 2h to *P. ecklonii* extract, 80% of inhibition against viable cells on a 24h old biofilm was observed. When methanol extracts of *P. barbatus* and *P. ecklonii* were used to inhibit the growth of the two bacterial strains in biofilm, IC_{50} (inhibitory concentration) values were 1.9 mg/mL and 0.57 mg/mL against *S. sobrinus* biofilm and 0.7 mg/mL and 0.8 mg/mL against *S. mutans* biofilm, respectively. *P. barbatus* IC_{50} values for the biofilm formation were 0.63 mg/mL and 0.13 mg/mL against *S. sobrinus* and *S. mutans,* respectively. *P. ecklonii* IC_{50} values for the biofilm formation were 0.07 mg/mL and 0.12 mg/mL against *S. sobrinus* and *S. mutans* biofilm. GTF from *S. sobrinus* was inhibited in 30% when 0.3 mg/mL of *P. barbatus* extract was used.
Conclusions*:* These extracts are important in the control of biofilms and useful in the prevention of oral diseases.

Keywords: Plectranthus methanol extracts; diterpenoids; flavonoids; Streptococcus species and biofilm formation.

1. INTRODUCTION

Plants with medicinal properties have been commonly used as remedies for many infectious diseases, such as digestive and intestinal disorders, malaria and tuberculosis [1]. *Plectranthus* species (Lamiaceae) contain many antioxidant compounds and exhibit several effects such as anti-inflammatory, antimicrobial and antifungal activities. These properties suggest that *Plectranthus* may be a promising medicinal genus for the discovery of medicinal compounds [2-4]. *P. barbatus* is one of the most studied species of *Plectranthus* due to its high diversity of uses. Although *Plectranthus* appears to be an old world genus absent from South America [5] *P. barbatus* and other related species are often found and used in Brazil. In Europe, namely in Portugal, several *Plectranthus* species are cultivated as ornamentals. In East Africa *P. barbatus* is used as a remedy for stomach-ache [3] and in Northeast of Brazil it is used in the treatment of digestive problems [4]. Decoctions of leaves from *P. barbatus* grown in Portugal showed high inhibitor activity against acetylcholinesterase and high antioxidant activity [6]. The main phytochemical compounds of the genus *Plectranthus* are terpenoids (mono-, sesqui- and diterpenoids) and phenolics [3] and several of these constituents are referred to inhibit the cariogenic bacteria growth [7].

In the last decades a remarkable potential has been observed for natural products and their bioactive compounds to interfere with survival and virulence factors of mutans streptococci [7-10]. Dental caries are known to be one of the most common oral diseases. Dental plaque is an oral biofilm formed on the tooth surface [11] that occurs by the action of acidogenic and aciduric bacteria [12], which interact with other microorganisms in dental biofilms [13]. Due to their action in dental biofilm regarding to their capability to synthesise glucans and their acid, *Streptococcus sobrinus* and *S. mutants* have been largely studied [14,15]. These bacteria

produce extracellular glucans from sucrose through the action of an enzyme – glucosyltransferase (GTF) and the glucans play an important role on the initial steps of colonization and accumulation of cariogenic microorganisms, contributing to biofilm formation [16,17].

Our previous work indicated that rosmarinic acid present in the *P. barbatus* and *P. ecklonii* aqueous extracts showed dental-bacteria inhibition activity and that the diterpenoids present in the *P. barbatus* extract were responsible for its higher activity [18], so it was decided to investigate an extract that could contain higher concentration of diterpenoids. Due to the lower water solubility of diterpenoids, an organic solvent like methanol was chosen. Methanol is an organic solvent able to extract compounds with higher bacteria growth inhibitory activity than aqueous extracts [19,20]. To the best of our knowledge no studies on methanol extracts of *Plectranthus* species against *S. sobrinus* and *S. mutans* were found in literature. Thus, in this study our aim was to evaluate methanol extracts of *P. barbatus* and *P. ecklonii* composition and its effects against to oral pathogens. To achieve this objective, leaves methanol extracts of *P. barbatus* and *P. ecklonii* were studied on what concerned viability of suspension cells, viability of biofilm cells, biofilm formation, synthesis of water insoluble glucans by extracellular GTF.

2. MATERIALS AND METHODS

2.1 Plant Material

Leaves of *P. barbatus* and *P. ecklonii,* cultivated in Botanic Garden of the University of Lisbon, were collected during winter 2009. The vouchers specimens from both species have been deposited in the Herbarium of the same Botanic Garden - *P. barbatus* Andr. (LISU: 214625) and *P. ecklonii* Benth (LISU: 146895).

2.2 Chemicals

Brain Heart Infusion (BHI-DIFCO Laboratories), yeast extract (DIFCO Laboratories), L-cysteine (Sigma) and sucrose (Panreac) were used for bacterial growth. Methanol and dimethyl sulphoxide (DMSO) used to prepare extracts were obtained from Panreac and Sigma, respectively. Methanol and TFA for HPLC analysis were HPLC grade and obtained from Merck.

2.3 Extract Preparation

The methanol extracts were prepared based on the literature [21]. The dried ground leaves of *P. ecklonii* and *P. barbatus* were extracted with methanol (5 mL/g), at room temperature for three hours using a magnetic mixer. After extraction, the mixture was filtered and the residue was re-extracted with fresh methanol (5 mL/g) overnight. The mixture of two methanol solutions was filtered through Whatman paper in vacuum condition and evaporated on a rotary evaporator. *P. barbatus* methanol extract (PBM) and *P. ecklonii* methanol extract (PEM) were reconstituted in DMSO to a concentration of 400 mg/mL for subsequent experimentation.

2.4 Chemical Analysis

The extracts were analyzed by HPLC and the mainly compounds were identified by mass spectrometry. The HPLC analysis was carried out in Liquid Chromatograph Finnigan™ Surveyor ®Plus Modular LC System (MA, USA) equipped with a LiChroCART® 250-4 LiChrospher® 100 RP-18 (5µm) column, from Merck (Darmstadt, Germany), and Xcalibur software. The extracts were analyzed by HPLC injecting 25 µL of 0.5 mg/mL extract solution in DMSO, and using a gradient composed of solution A (0.05% trifluoroacetic acid), solution B (acetonitrile) and solution C (methanol) as following: 0 min, 70% A, 5% B, 25% C; 20-25 min 10% A, 10% B, 80% C; 30 min, 70% A, 5% B, 25% C. The detection was carried out between 200 and 600 nm with a diode array detector.The identification of the mainly compounds was performed in an Apex Ultra FTICR Mass Spectrometer from Bruker Daltonics (Billerica, MA, USA) equipped with an Apollo II Dual ESI/MALDI source and a 7 T actively shielded superconducting magnet. The samples were introduced, by means of an infusion pump from KD Scientific (Holliston, MA, USA), with a flow rate of 120 µL/h. The mass spectrometer was calibrated using a $2.8x10^{-6}$ mol/L solution of polyethylenglycol in methanol, and acidified with 0.1% (V/V) of acetic acid. The mass spectra were acquired in the positive ion broad band mode, with an acquisition size of 512 k, in the mass range of 50-500. The nebulizer gas flow rate was set to 2.5 L/min, the dry gas flow rate was set to 4.0 L/min at a temperature of 220ºC. The capillary voltage was set to 5000 V and the spray shield voltage was set to 4500 V. All mass spectra presented are the average of 32 mass spectra. The MS^2 experiments were performed using argon as collision gas.

2.5 Antibacterial Activity Test

2.5.1 Bacterial strain and growth condition

S. sobrinus, (CETC 4010) and *S. mutans* (CETC 479)were obtained from Colleccion Española de Cultivos Tipo and cultured in BHI and in BHI supplemented (BHI-S) with 0.5% yeast extract, 0.05% L-cysteine, and 1% sucrose in anaerobic conditions assured by degassing with 0.2 µm steril –filtered (Acro® 50 Vent Devices, Pall Gelman Corporation) oxygen free nitrogen for 15 minutes.

2.5.2 Determination of bacteriostatic and bactericide activity

The anti-streptococcal activity of extracts was evaluated through the determination of minimum inhibitory concentration (MIC), which was obtained by a modified microdilution broth method described by Cai and Wu [22], and minimum bactericidal concentration (MBC). Microorganisms, from overnight cultures, were adjusted to 0.5 (Absorbance Units) at 630 nm and diluted 1:100 in BHI broth (1. 5×10^6 CFU/mL). The crude of PBM and PEM extract, dissolved in DMSO (400 mg/mL), were serially diluted in BHI broth to achieve concentrations ranging from 0 to 1 mg/mL. In sterile 96-well microtitre plates 100 µL of diluted extract samples were added into wells containing 100 µL of bacterial suspension. DMSO solvent was tested and had no significantly inhibitory effect on the growth of *S. sobrinus* or *S. mutans*. To adjust the interference of color due to the extracts, a parallel series of mixtures, with uninoculated broth was prepared. Triplicate samples were taken for each test concentration. After incubation for 48 hours at 37°C under anaerobic conditions, *S. sobrinus* and *S. mutans* growth was estimated by absorbance at 630 nm. For the determination of the MBC, aliquots of 50 µL of all the wells after 48h of growth, diluted 10 fold in broth, were sub-cultured on BHI agar and incubated anaerobically for 48h. MIC corresponds to the extract

concentration inhibits 90% bacteria growth and MBC corresponds to the lowest concentration that did not permit any visible growth on the appropriate agar plate after the incubation period, i.e. the lowest concentration that kills the bacteria cells.

2.6 Effects on Biofilm

2.6.1 Biofilm culture condition

S. mutans and *S. sobrinus* biofilm were formed on glass bottles at 37ºC for 24 and 48 hours anaerobically in BHI-S. To produce these biofilms 1 mL from a bacterial suspension in the exponential phase of growth (optical density of 0.5 AU) was inoculated with 9 mL of BHI-S. After incubation for 24 and 48h at 37ºC, the culture broth, together with the planktonic cells, was removed and the biofilm was washed with 0.1M sterile phosphate buffer to remove poorly-adherent bacteria.

2.6.2 Effect on biofilm formation

To assess the effect of PBM and PEM extracts on biofilm formation by *S. sobrinus* and *S. mutans,* bacteria were incubated in glass bottles at 37ºC for 18h anaerobically in BHI-S. To accomplish this, 1mL from a bacterial suspension in the exponential phase of growth (optical density of 0.5 UA) were inoculated in small glass bottles with 9 mL of BHI-S containing concentrations of PBM and PEM ranging from 0-1 mg/mL for *S. sobrinus* and for *S. mutans*. After incubation at 37ºC for 48h planktonic cells were removed, sessile cells were re-suspended in 10 mL of water, sonicated (3x5 seconds burst with 15 seconds intervals) and cell were counted using an hemocytometer. Controls without extract but with DMSO (in the same final concentration) and without inoculation were prepared for all the assays. IC_{50} was defined as the lowest agent concentration that showed 50% of inhibition on the biofilm formation. Each assay was performed in triplicate.

2.6.3 Effects in removal old biofilm

The effect of PBM and PEM extracts in removal of old biofilm cells was studied to determine the influence of concentration of these extracts in removal of an old biofilm cells. For this assay, a 48h old biofilm formed, as previous described, was used. To the adherent biofilm, new culture broth containing over MIC concentrations of PBM and PEM extracts were added. After incubation at 37ºC for 48 h, sessile cells were re-suspended in 10 ml of water, sonicated (3×5 seconds burst with 15 seconds intervals) cells were counted using a hemocytometer and variations in pH were carried out. Positive controls without extract and with DMSO (same proportion) were prepared for all tests. Each assay was performed in triplicate. IC_{50} was defined as the lowest extract concentration that showed potential to remove 50% adherent biofilm.

2.6.4 Effects on biofilm cells viability

To evaluate the effects of PBM and PEM extracts on the biofilm cells viability, the influence of treatment time was evaluated. For this assay, a 24 hours biofilm was treated with 3×MBC concentration of PBM and PEM extracts in 0.01M potassium phosphate buffer (KPB) for 0, 1, 2 and 4 hours. After the incubation time, the bacterial cells in the biofilm were dispersed using a sonicator, diluted (10^5) and spread on BHI agar. The plates were incubated at 37ºC for 48 hours anaerobically, at which time the number of colonies was determined. The

vehicle control was 3×MBC% of DMSO (v/v, final concentration) in the absence of extract. Killing curves were constructed by the CFU per milliliter (% of control) versus exposure time.

2.7 Effects on GTF Activity

2.7.1 Preparation of extracellular GTF

A crude GTF preparation was extracted according to the method described by [23] with some modifications. Actively growing *S. sobrinus* and *S. mutants* were incubated in 1000 mL of BHI. After incubation for 48 h at 37ºC under anaerobic conditions, the bacteria were removed by refrigerated centrifugation. The pH of culture supernatant was adjusted to 6.8 by addition of 2M NaOH. The supernatant was treated with ammonium sulfate at 50% saturation and then centrifuged. The precipitate was dialyzed against PBS, pH 6.8, containing 1mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The dialyzed preparation was used as crude extracellular glucosyltransferase and stored at -20ºC.

2.7.2 Water-insoluble glucan formation by GTF

Quantification of glucan synthesis was carried out according to a previous method [24] with some modifications [21]. The reaction mixtures contained sterile 0.1M sucrose as a substrate and 50 µL of crude GTF were buffered in PBS, pH 6.0 and different final concentrations ranging from 0 to 1 mg/mL of PBMand PEM extracts were assayed for the inhibition of water-insoluble glucan synthesis. Following incubation at 37ºC for 18h, the water insoluble glucan produced was suspended and after sonication the turbidity of the suspension was measured at 550 nm. A parallel series of mixtures was prepared to see errors due to extracts colour. The formation of water-insoluble glucan was expressed as a percent (i.e. 100xOD_{550nm} of test sample/OD_{550nm}) of control sample without adding extract. All reactions were carried in triplicate.

2.8 Statistical analysis

Each assay was performed in triplicate. The data were analyzed using ANOVA (version 6.22 for Windows). One-way analysis of variance was performed, followed by a post hoc multiple comparisons. The level of significance for statistical analyses was 0.05.

3. RESULTS AND DISCUSSION

Plectranthus species has been used in traditional medicine, due to their pharmacological effects such as antimicrobial and antioxidant properties [2]. In this context, we selected two species of this genus, *P. barbatus* and *P. ecklonii*, in order to evaluated their composition and investigate their anti-caries effects. Both species were selected due to our previous experience in the biological activities of the aqueous extracts of these herbs [6,18,25].

3.1 Extract Chemical Composition

The HPLC analysis of the PBM extract showed two main peaks, with retention times of 14.13 min and 14.65 min (Fig. 1A), while the analysis of PEM extract presented three main peaks, with retention times of 9.41 min, 18.06 min and 19.43 min (Fig. 1B). The peak with retention time 9.41 min showed a UV spectrum with a peak of absorbance at 330 nm and a shoulder at 290 nm, which is characteristic of hydroxycinnamic acids. This peak

exhibited the same retention time and UV spectrum of rosmarinic acid, previously found in a *P. ecklonii* extract by our group [6].

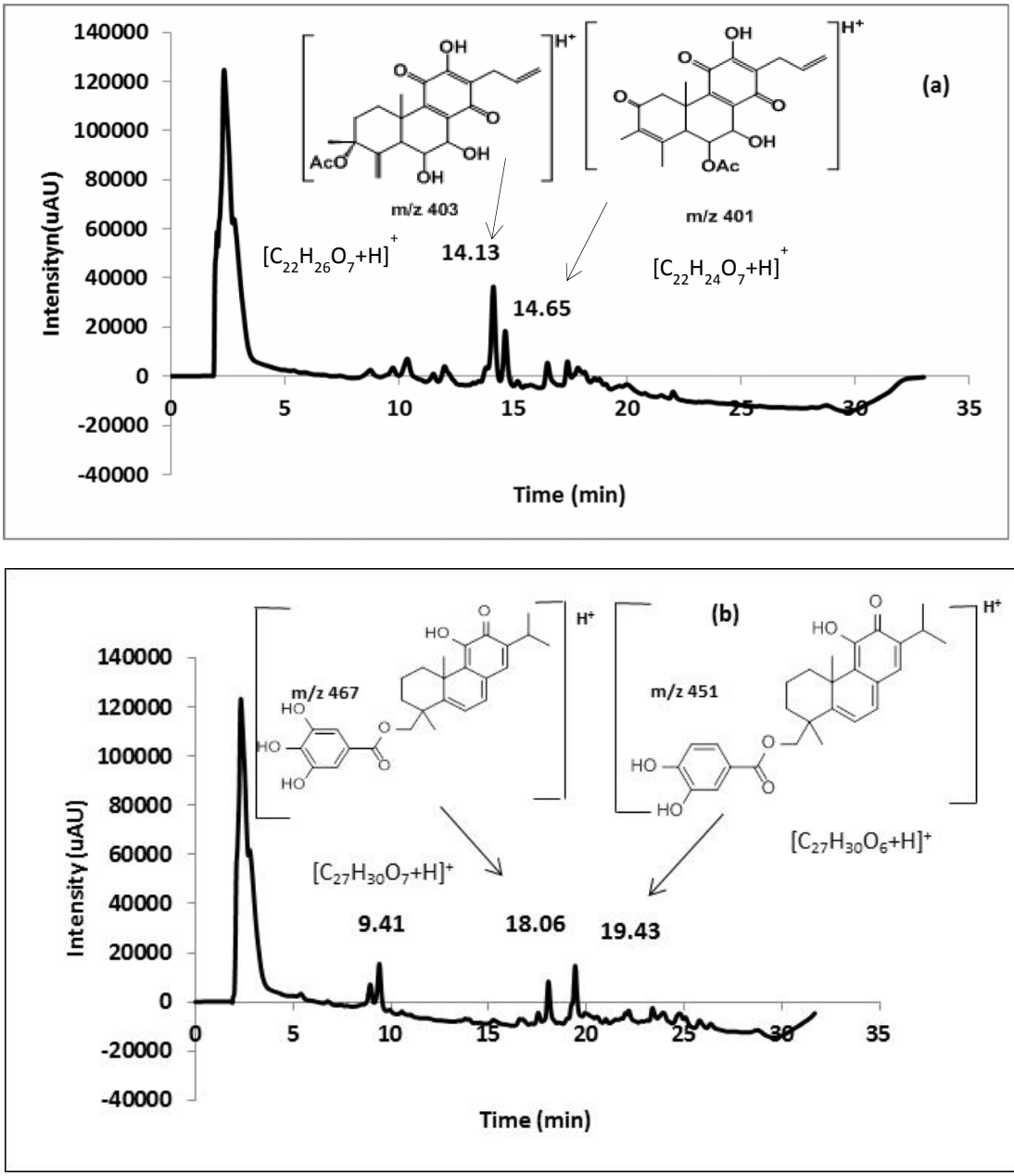


Fig. 1. HPLC chromatograms of methanol extracts: (A) *Plectranthus barbatus* and (B) *Plectranthus ecklonii*.

The others peaks, i.e. 14.13 min and 14.65 min (Fig. 1a) for PBM extract and 18.06 min and 19.43 min (Fig. 1b) for PEM extract, were collected after separation by HPLC and these fractions were infused into the mass spectrometer. For the PBM extract, the compounds with

retention times of 14.13 min and 14.65 min showed a fragmentation pattern similar to those reported in the literature and were attributed to diterpenoids already reported for the genus *Plectranthus,* royleanone type compound and plectranthone J, respectively [3]. For the PEM extract, the compounds with retention times of 18.06 min and 19.43 min, their ESI MS^2 spectra and their fragmentation patterns are quite similar (results not shown). The 19.43 min compound was attributed to a quinone methide already reported in the literature for the genus *Plectranthus*, parviflorone E [3]. The 18.06 min compound seems to have a similar backbone structure with one more oxygen atom. As such, the compound with retention time 18.06 min can be attributed to diterpenoid abietane. The chemical structures of all compounds are shown on the top of each figure. The compounds identified in the PBM extract were quite different from those coming out in the water extract [6]. In the aqueous extract the main component was rosmarinic acid and flavonoid derivatives, although one abietane diterpenoid could be detected (coleon E).

3.2 Antibacterial Activity

The growth and viability inhibition of *S. sobrinus* and *S. mutans*, two representatives of oral microbiota that play an important role in the oral cavity health, were studied in the presence of several concentrations of PBM and PEM extracts. The results showed that both extracts have significant bacteriostatic and bactericidal activity, increasing in an extract concentration dependent way, which allowed the determination of MIC and MBC values (Table 1). For both extracts MIC values against the two bacteria strains were the same (0.3 mg/mL). The MBC values for both extracts were 0.6 mg/mL against *S. sobrinus* and 0.8 mg/mL against *S. mutans* (Table 1). The MBC values were two or three times higher than MIC values, which are in agreement with the literature [9], that referred that in general, MBC values are two for four times higher than MIC values.

Table 1. Values of the evaluated parameters of methanol extracts from *Plectranthus barbatus* and *P. ecklonii* against *Streptococcus sobrinus* and *S. mutans* cells and biofilm: minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and IC_{50} for biofilm removed and biofilm formed.

Species	Evaluated parameters	*P. barbatus* (mg/mL)	*P. ecklonii* (mg/mL)
S. sobrinus (CETC 4010)	MIC	0.30 ± 0.07*	0.30 ± 0.00*
	MBC	0.6	0.6
	Biofilm removed (IC_{50}[a])	1.90 ± 0.2*	0.57 ± 0.06*
	Biofilm formed (IC_{50}[b])	0.63 ± 0.05*	0.07 ± 0.01*
S. mutans (CETC 479)	MIC	0.20 ± 0.01*	0.30 ± 0.01*
	MBC	0.8	0.8
	Biofilm removed (IC_{50}[a])	0.70 ± 0.06*	0.80 ± 0.11*
	Biofilm formed (IC_{50}[b])	0.13 ± 0.02*	0.12 ± 0.00*

**p < 0.05: significantly different from the control.*

[a]IC_{50}: the lowest extract concentration that removed 50% of the biofilm formed.

[b]IC_{50}: the lowest extract concentration that showed 50% of inhibition on the biofilm formation.

Dental caries is one the most common oral diseases worldwide, which results from the interaction of bacteria and diet component, in dental surface. *S. mutans* and *S. sobrinus* are the main pathogens involved in the development of this disease in humans. In this study, methanol extracts from *P. barbatus* and *P. ecklonii* leaves show high inhibitory effect on growth and viability of these two bacteria. Comparing with our previous results for leaves

aqueous extracts of these two *Plectranthus* species (MIC ranging from 3.8 to 5.0 mg/mL) [18], methanol extracts revealed to be 10 time more aggressive against *S. sobrinus* and *S. mutans* than the aqueous ones. This remarkable difference may be due to a different chemical composition of the extracts, being the presence of the abietane diterpenoids and quinone methides in higher quantities in methanol extracts than those observed in the aqueous extracts responsible for the high antibacterial activity reported here. In the *P. ecklonii* aqueous extract the amount of rosmarinic acid was 480 μg/mg extract [26] and in the methanol it was only 4.03 μg/mg of extract. The higher activity found for the methanol extract is probably related with the presence of the diterpenoids and quinone methides detected in these extracts. These types of compounds were not present in the aqueous extract used in our previous study [18].

Comparing with other methanol plant extracts, such as *Rosmarinus officinalis* "rosemary"leaf extract (MIC 4 mg/mL against *S. sobrinus*) [21] and *Polygonum cuspidatum* root extract (MIC 2 mg/mL and MBC 4 mg/mL against *S. mutans* KCTC 3298 and MIC 4 mg/mL and MBC>4 mg/mL against *S. sobrinus* KCTC3288) [9], the antibacterial activity of PBM and PEM extracts against *S. sobrinus* and *S. mutans* was significantly greater. However, the activity of these two *Plectranthus* extracts is lower than the chlorohexidine and antibiotic vancomycin activity (MIC 1 μg/mL), compounds known to induce several adverse effects, such as discoloring of teeth, reducing of immune defense, disrupting of normal ecology of plaque, diarrhea, vomit, etc. [24]. In addition the antibacterial activity of both *Plectranthus* extracts against *S. mutans* is in agreement with the antibacterial activity referred for other plant extracts, such as green tea (MIC 0.25 mg/mL), or even for pure compounds as carvacrol (MIC 0.13 mg/mL) and eucalyptol (MIC 0.50 mg/mL) [27].

Based on the phytochemical analysis in the present study, antibacterial activity of PBM and PEM extracts might be related to the presence of diterpenoids. Some studies report that these compounds are responsible for several plant pharmacological activities, such as antibacterial activity [28,29]. In particular, diterpenoids as coleons [30] and forskolin [31] have been reported as good antibacterial agents. According to Cowan [32], the mechanism responsible for the diterpenoids antibacterial activity could be associated to bacteria membrane disruption by lipophilic compounds.

3.3 Biofilm Inhibition

In Table 2 it is shown photographs, contrasting a normal biofilm formed by *S. sobrinus* and *S. mutans* after 48h and a biofilm produced by these same bacterial strains in presence of PBM and PEM extracts. The photos show that PBM and PEM extracts markedly inhibit the biofilm formation. The inhibitory effect was dependent on the concentration of PBM and PEM extracts, as shown in Fig. 2. The IC_{50} values could be determined from this figure and values as low as 0.63 mg/mL and 0.07 mg/mL against *S. sobrinus* biofilm and 0.13 mg/mL and 0.12 mg/mL against *S. mutans* biofilm, could be calculated (Table 1). As was observed for antibacterial activity, PBM and PEM extracts revealed to be more effective inhibiting biofilm formation that aqueous extracts, which presented as IC_{50} 1.4 mg/mL and 2.7 mg/mL for biofilm formation by *S. mutans* and 0.6 mg/mL and 1 mg/mL for biofilm formation by *S. sobrinus*, respectively [18]. Comparing with methanol extract of *Polygonum cuspidatum,* a plant species already used to improve oral hygiene, which values of 0.25 mg/mL in sucrose-dependent adherence assay against both *S. mutans* and *S. sobrinus* has been obtained [9], PBM and PEM extracts are more effective to inhibit biofilm formation.

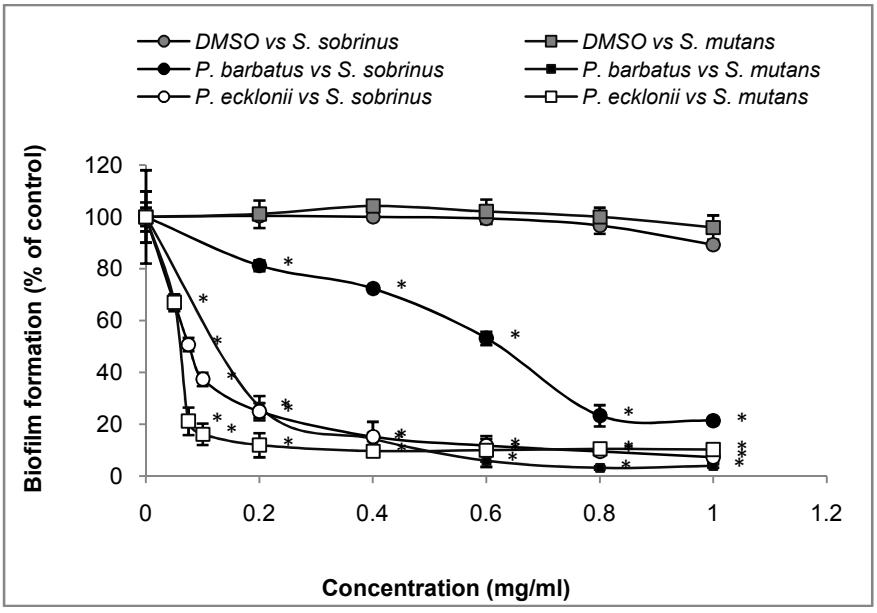
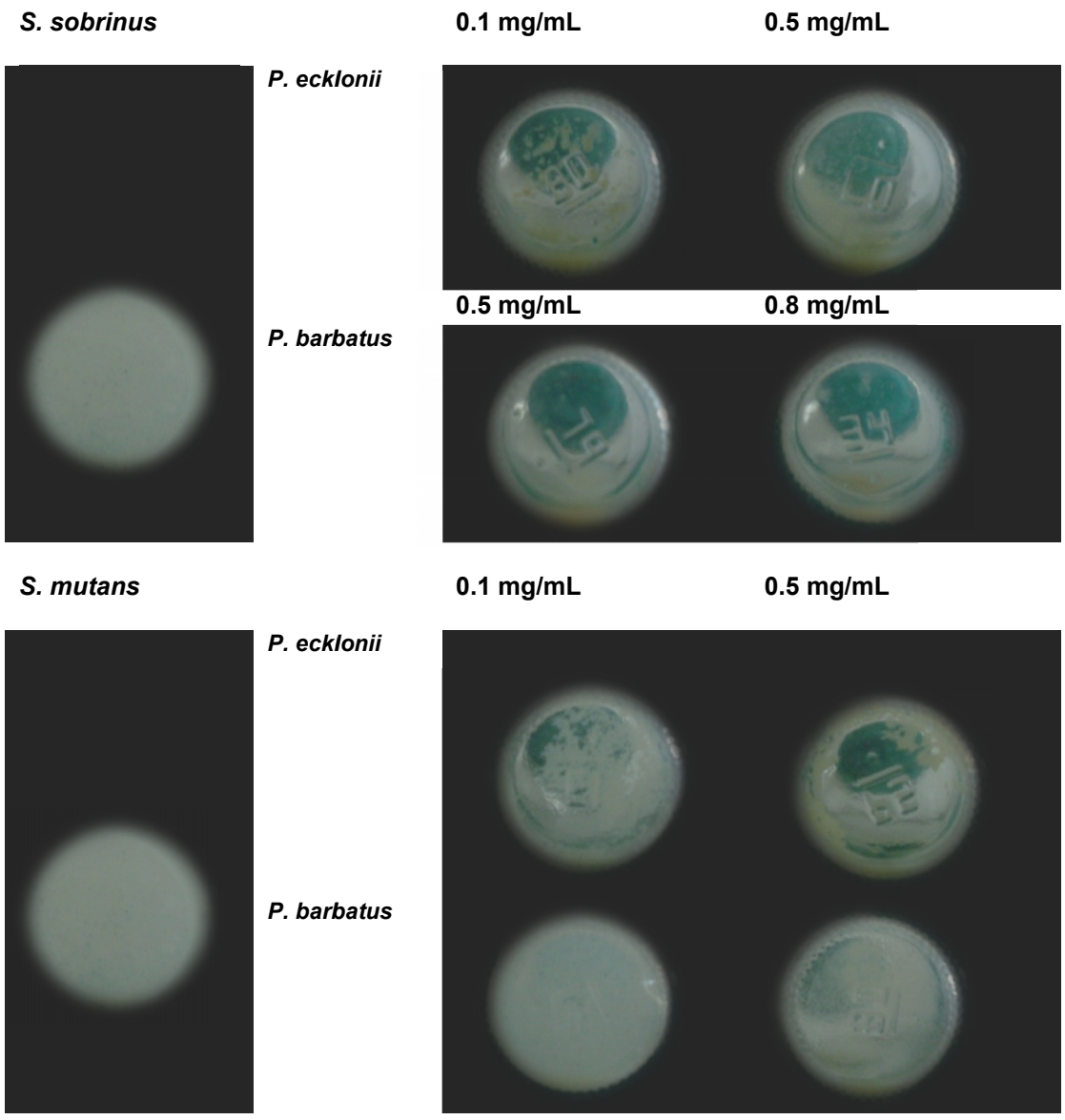


Fig. 2. Inhibitory effects of methanol extracts from *Plectranthus barbatus* and *P. ecklonii* on the biofilm formation by *Streptococcus sobrinus* and *S. mutans*. Inhibitory effects were express as a percent of control (without sample).
**p < 0.05: significantly different from control*

Fig. 3 shows that PBM and PEM extracts have also the ability to remove an old biofilm formed by *S. mutans* and *S. sobrinus* and this capacity increases with the increase in the extract concentration. *S. sobrinus* biofilm was more sensitive to PEM extract, which presented an IC_{50} of 0.57 mg/mL, while a lower effect was noted with PBM extract, which presented an IC_{50} of 1.9 mg/mL (Table 1). *S. mutans* biofilm cells responded also in a dose-dependent manner and were more sensitive to PBM extract than to PEM extract, being the IC_{50} 0.7 mg/mL and 0.8 mg/mL, respectively (Table 1).

In the present study, PBM and PEM extracts showed inhibitory effects on the virulence factors of *S. sobrinus* and *S. mutans*, namely on the formation and viability of biofilm. Microorganisms in biofilms are complex communities known to be much more resistant to antimicrobial agents than planktonic cells (in suspension) and are more similar to biofilms in oral conditions. This proves that the inhibitory effect on the biofilm presented in this investigation can be of great importance for oral health studies, concerning the prevention and treatment of dental caries. The inhibitory effects of PBM and PEM on biofilm formation could result from a decrease in the bacterial growth rate, which was seen with the diminishing of bacteria number with the increase concentration of the extracts (measured for the determination of MIC values) or be also related to the inhibition of adherence by anti-adhesive effect of phenolic acid and diterpenoids present in the extract. It is well known that some molecules like phenolics [33] and terpenoids [34] have the ability to alter the hydrophobic proprieties of a surface by binding their hydrophobic regions to a surface, exposing the hydrophilic side chains to the aqueous environment, thereby blocking hydrophobic interactions between the bacteria and the surface [35].

Table 2. Effect of methanol extracts from *Plectranthus barbatus* and *P. ecklonii* on biofilm formation by(a) *Streptococcus sobrinus* and (b) *S. mutans*. Both methanol extracts showed an inhibitory effect against biofilm formation on glass surface



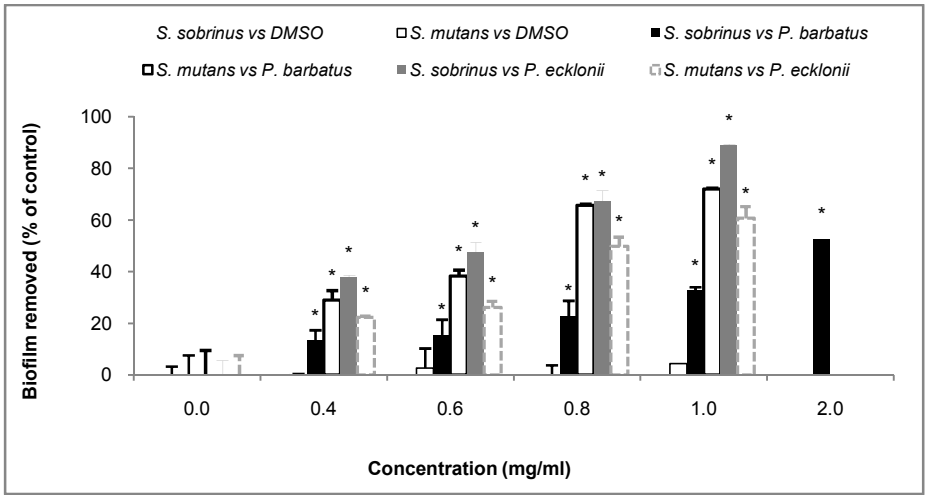


Fig. 3. Effect of methanol extracts from *Plectranthus barbatus* and *P. ecklonii* on the 48 hours biofilm formed by *Streptococcus sobrinus* and *S. mutans*. The effect was express as a percent of biofilm removed (percentage of control).
**p < 0.05: significantly different from the control.*

3.4 Antibacterial Activity in the Biofilm Cells

Microorganisms growing in biofilm are physiologically distinct from their planktonic counterparts; they are more resistant and less susceptible to chemical agents [7,36]. In order to study the inhibitory effect of PBM and PEM extracts on the viability of *S. sobrinus* and *S. mutans* biofilm cells, the bactericidal kinetic assay in a 24 h old biofilm was performed. As shown in Fig. 4, these plant extracts at concentrations 3xMBC reduced viable counts of *S. sobrinus* by more than 80% after 2 h of exposure. *S. mutans* was less sensitive to PBM extract; viable counts were reduced about 50% by the same concentration of extract and the same exposure time.

3.5 Water-insoluble Glucan Formation by GTF

To study the mechanism behind the inhibition of biofilm formation, the effect of PBM and PEM extracts on the water-insoluble glucans synthesis by cell free extracellular GTF of *S. sobrinus* and *S. mutans* were examined. GTF, sucrose 6-glucosyltransferase (E.C. 2.4.1.5), produced by *S. mutans* and *S. sobrinus* has been recognized as a critical virulence factor in pathogenesis of dental caries. GTF is the key enzyme that catalyzes the introduction of a glucose moiety from sucrose to the adhesive glucans and contributes significantly to the formation of dental plaque.

A concentration of 0.3 mg/mL PBM and PEM extracts inhibit 8% and 13% of water insoluble glucans synthesis by *S. mutans* GTF, respectively (data not showed). 30% and 5% of enzymatic activity of *S. sobrinus* GTF was inhibited by 0.3 mg/mL of PBM and PEM extract, respectively (data not showed). Due to precipitate formation, when concentrations higher than 0.3 mg/mL of PEM and PBM extracts were added in the water insoluble glucans synthesis assay, higher concentrations of these extracts were not evaluated.

High concentrations of PBM and PEM extracts (≥ MIC) had the ability to remove and reduce biofilm viability. These effects could result from bactericidal activity of these extracts. The capacity to remove biofilm could also be related to inhibition of glucan synthesis, affecting intracellular or extracellular polysaccharide accumulation. The diterpenoids present in these extracts could alter the bacteria cell membrane integrity, which may affect the secretion of GTFs and thereby diminish the synthesis of glucans. In addition, these compounds may be also responsible for disruption of the glycolytic pathway, disturbing the synthesis of intracellular polysaccharides. The extracellular matrix formation, which is responsible for the biofilm integrity is also affected by glucan and polysaccharide synthesis and accumulation.

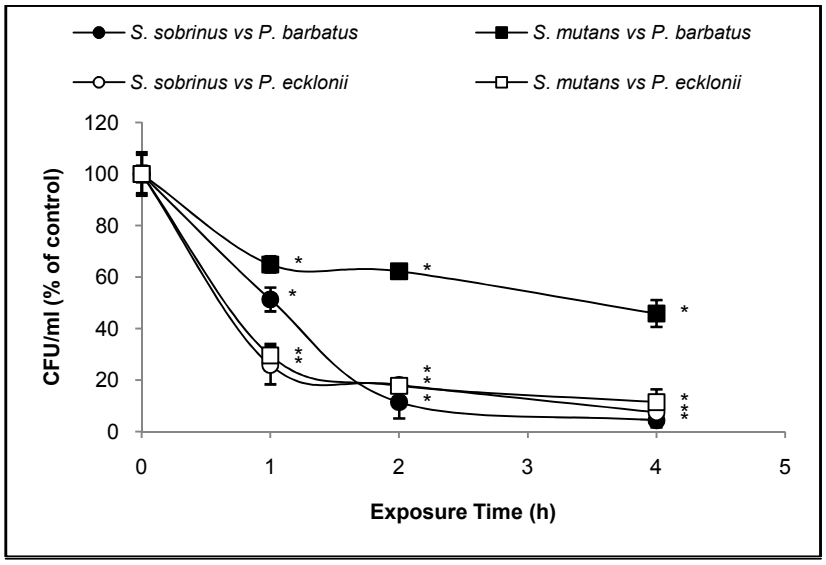


Fig. 4. Results of bactericidal kinetic assays for methanol extracts of *Plectranthus barbatus* and *P. ecklonii* against *Streptococcus sobrinus* and *S. mutans* in 24 hours biofilms. Data are expressed as CFU/ml recovered on BHI agar at each time point. Vehicle control was 3× MBC% DMSO (v/v, final concentration) in the absence of extract.

**p < 0.05: significantly different from the control.*

4. CONCLUSION

The present study demonstrated that crude of PBM and PEM extracts can inhibit the growth and viability of *S. mutans* and *S. sobrinus*. In addition to the high antibacterial activity against cells in suspension, a remarkable inhibitory activity against biofilm cells was also observed. The study also revealed that these extracts were effective in inhibiting biofilm formation and consequently promising in the prevention of oral diseases, since they can inhibit bacterial colonization and growth on teeth and can also interrupt the formation of mature biofilms. The presence in the extracts of abietane diterpenoids for *P. barbatus* and two quinone methides for *P. ecklonii*may explain the anticariogenic activity observed in this study.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

ACKNOWLEDGEMENTS

The authors thank the Portuguese Foundation for Science and Technology (FCT) for financial support (REDE/1501/REM/2005) and the Project POCTI/CMT/56029 for providing the streptococcal strains used. P. L. V. Falé and P. J. A. Madeira acknowledge FCT for the financial support, PhD grants SFRH/BD/37547/2007 and SFRH/BD/27614/2006, respectively.

COMPETING INTEREST

All authors declare that no competing interests exist.

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