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The inhibitory effect of *Plectranthus barbatus* and *Plectranthus ecklonii* leaves on the viability, glucosyltransferase activity and biofilm formation of *Streptococcus sobrinus* and *Streptococcus mutans*

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ABSTRACT

Aqueous extracts of *Plectranthus barbatus* and *Plectranthus ecklonii* are traditionally used as anti-inflammatory and anti-fungal agents. The effect of these extracts and of its main component, rosmarinic acid, on the viability of the cariogenic bacteria, *Streptococcus sobrinus* and *Streptococcus mutans*, was determined by MIC and MBC. The influence of these extracts on the biofilm formation as well as on the inhibition of glucosyltransferase enzyme, produced by these species, was also analysed. Aqueous extracts of *P. barbatus* and *P. ecklonii* were stronger inhibitors than rosmarinic acid. MIC values of 3.8 and 4.7 mg/ml for *S. sobrinus* and 2.9 and 5.0 for *S. mutans* were obtained, while rosmarinic acid presented MIC values of 8.4 and 7.3 mg/ml. *P. barbartus*, *P. ecklonii* and rosmarinic acid presented MBC values of 9.5, 9.0 and 12.0 mg/ml for *S. sobrinus*, and 9.5, 10.0 and 12.5 mg/ml for *S. mutans*. The inhibition of biofilm formation by *P. barbatus*, *P. ecklonii* and rosmarinic acid presented IC_{50} values of, respectively, 0.6, 1.0 and 3.1 mg/ml for *S. sobrinus* and 1.4 and 2.7 and 1.3 mg/ml for *S. mutans*. The glucosyltransferase inhibition activity by theses extracts and rosmarinic acid was calculated and IC_{50} values presented were, respectively, 1.1, *ca* 1.2 and 2.1 mg/ml for *S. sobrinus* and 3.1, 1.6 and 3.9 mg/ml for *S. mutans* were obtained. These extracts may be useful in the prevention of dental carie.

1. Introduction

Nowadays the consumption of herbal teas has been increasing among the general population, either with the objective of ameliorating any physiological problem, or just for the pleasure of having a hot drink. Small bags of medicinal plants are sold in any supermarket. The anti-inflammatory properties of many of these plants are the most reported biological effect. The genus *Plectranthus* is no exception. *Plectranthus* is a large and widespread genus with a diversity of ethnobotanical uses. The aqueous infusions of some *Plectrantrus* leaves contain many antioxidant compounds and exhibit several effects such as anti-inflammatory, antimicrobial and anti-fungal activities (Lukhoba, Simmonds, & Paton, 2006). *Plectranthus barbatus* belongs to the most studied group as a result of its high diversity of uses, in some countries it may be used in food preparations (Lukhoba et al., 2006). This species arising from Africa, Asia and South America is cultivated in Portugal and decoctions of leaves showed high inhibitor activity for acetylcholinesterase and high antioxidant activity. In *P. barbatus* and *Plectranthus ecklonii* aqueous extracts the major compound found was rosmarinic acid. The presence of this compound could explain the biological activity found *in vitro*, the inhibition of acetylcholinesterase and antioxidant activity (Falé et al., 2009). In *P. barbatus* other components as scutellarein 4′-methyl ether 7-O-glucuronide and (16S)-coleon E also may contribute for the extract properties previously found (acetylcholinesterase inhibition and antioxidant activity) (Falé et al., 2009).

Many studies have shown that some natural products can interfere with survival and virulence factors of mutans streptococci (Ooshima et al., 1998; Song et al., 2006; Tsai, Tsai, & Ho, 2007; Xiao et al., 2007; Yu, Lee, Lee, Kim, & You, 2007). Remarkable anticariogenic potency was also observed for natural bioactive compounds (Duarte et al., 2006; Koo et al., 2002).

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Dental caries are known to be one of the most common oral diseases. Dental plaque is an oral biofilm formed on the tooth surface (Baenhi & Takeuchi, 2003). Although the human oral flora is quite diverse and complex, two species of mutans streptococci, *Streptococcus mutans* and *Streptococcus sobrinus* have been implicated as the primary etiologic agents of dental caries (Loesche, 1986). One of the most important virulence factors of these species is their ability to produce glucosyltransferase (GTF) and synthesise water insoluble glucans from sucrose, which allows bacteria to adhere firmly to the tooth surface and contribute to the formation of dental plaque (Hamada & Slade, 1980; Hamada & Torii, 1978; Schilling & Bowen, 1992). GTF, sucrose 6-glucosyltransferase (E.C. 2.4.1.5), produced by *S. mutans* and *S. sobrinus* has been recognised as a critical virulence factor in pathogenesis of dental caries. GTF is the key enzyme that catalyses the introduction of a glucose moiety from sucrose to the adhesive glucans and contributes significantly to the formation of dental plaque. Inside this dental plaque the accumulation of metabolic acids produced by bacterial colonies leads to local demineralisation of the enamel surface. There are at least three kinds of GTFs which are produced by cariogenic streptococci: one synthesising α-1,3-linked water insoluble glucans, another producing significant amounts of both water-soluble and insoluble glucans, and finally one incorporating glucose in α-1-6-linkages into soluble glucan products. Therefore, the first is more responsible for insoluble glucans synthesis than the other GTFs and it is vital for the progression of dental caries (Huang et al., 2006), this one was used in the present study.

Nevertheless studies on oral diseases, particularly *Plectantrus* activity against the oral pathogen *S. sobrinus* and *S. mutans* were not found in literature and may have important anticaries properties of interest to the dental protection field.

In order to investigate if these plants could inhibit the growth of cariogenic bacteria responsible for the dental plaque formation, while being consumed as a herbal tea, *P. barbatus*, *P. eklonii* water extracts, together with the main compound present in these extracts, rosmarinic acid, were analysed on what concerns the viability of *S. mutans* and *S. sobrinus*, biofilm formation and synthesis of water insoluble glucans by extracellular glucosyltransferase by the two bacteria.

2. Materials and methods

2.1. Materials and microorganisms

S. sobrinus, (CETC 4010) and *S. mutans* (CETC 479) were obtained from Colleccion Española de Cultivos Tipo. Lote 21-10-1998 and cultured in Brain Heart Infusion (BHI-DIFCO Laboratories) and in BHI supplemented with 0.5% yeast extract (DIFCO Laboratories), 0.05% l-cysteine, and 1% sucrose in anaerobic conditions assured by degassing with 0.2 μm sterile-filtered (Acro® 50 Vent Devices, Pall Gelman Corporation) oxygen free nitrogen for 15 min. Rosmarinic acid was purchased from Sigma.

2.2. Plant material

Leaves of *P. barbatus* and *P. ecklonii* cultived in Botanic Garden of the University of Lisbon were collected during summer (June to October) 2008. The vouchers specimens from both species have been deposited in the Herbarium of this Botanic Garden. *P. barbatus* Andr. (LISU:214625), *P. ecklonii* Benth. (LISU:146895).

2.3. Extract preparation

Aqueous plant extracts were prepared as decoctions. Decoctions were prepared using 10 g of freshly ground plant material boiled for 10 min in 100 ml of distilled water, filtered through Whatman paper and lyophilised.

2.4. Determination of antibacterial activity

The bacteriostatic activity of extracts and rosmarinic acid was determined by calculating MIC values. These values were obtained by a slight modification of the dilution method described by Cai and Wu (1996) and correspond to the extract concentration limiting the growth to 90%.

Microorganisms, from overnight cultures, were adjusted to 0.5 (Absorbance Units) at 630 nm and diluted 10 folds in broth. *P. barbatus*, *P. ecklonii* aqueous extracts were serially diluted with broth to give the concentrations ranging from 0 to 5 mg/ml and concentrations ranging from 0 to 10 mg/ml for rosmarinic acid. In sterile 96-well microtitre plates 100 μl of diluted extract samples were added into wells containing 100 μl of bacterial suspension.

To adjust the interference of colour due to extracts, a parallel series of mixtures, with uninoculated broth was prepared. Triplicate samples were taken for each test concentration. A positive control without extract and a negative control, without inoculation, were prepared for all the assays. After incubation for 48 h at 37 °C under anaerobic conditions, *S. sobrinus* and *S. mutans* growth was estimated by cell count using a hemocytometer (Hirschmann Technocolor Neubar improved). The MIC was defined as the minimum concentration of test compound limiting growth to 90%. For the determination of the MBC, aliquots of 50 μl of all the wells after 48 h of growth, diluted 10 fold in broth, were sub-cultured on BHI agar and incubated anaerobically for 48 h. The MBC was defined as the lowest concentration that did not permit any visible growth on the appropriate agar plate after the incubation period.

2.5. Effect on biofilm formation

To assess the effect of *P. barbatus* and *P. ecklonii* aqueous extracts and rosmarinic acid on biofilm formation, *S. sobrinus* and *S. mutans* were incubated in glass bottles at 37 °C for 18 h anaerobically in supplemented BHI. One millilitre from a bacterial suspension in the exponential phase of growth (optical density of 0.5 AU) was inoculated in small glass bottles with 9 ml of BHI broth plus 1% of sucrose containing concentrations of *P. barbatus* and *P. ecklonii* and rosmarinic acid ranging from 0 to 10 mg/ml for *S. sobrinus* and 0 to 8 mg/ml for *S. mutans*. After incubation at 37 °C for 48 h planktonic cells were removed, sessil cells were re-suspended in 1 ml of water, sonicated (3 × 5 s burst with 15 s intervals) and cells were counted using a hemocytometer. A positive control without extract and a negative control without inoculation were prepared for all the assays. IC_{50} was defined as the lowest agent concentration that showed 50% of inhibition on the formation of a biofilm. Each assay was performed in triplicate.

2.6. Preparation of extracellular glucosyltransferase

A crude glucosyltransferase (GTF) preparation was extracted according to the method described by Koo et al. (2000) with some modifications. *S. sobrinus* and *S. mutans* in exponential phase growth were incubated in 1000 ml of BHI. After incubation for 48 h at 37 °C under anaerobic conditions, these bacteria were removed by refrigerated centrifugation. The pH of culture supernatant was adjusted to 6.8 by addition of 2 M NaOH. The supernatant was treated with ammonium sulfate at 50% saturation and then centrifuged. The precipitate was dialysed against PBS, pH 6.8, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor. The dialysed preparation was used as crude extracellular glucosyltransferase and stored at −20 °C.

2.7. Water insoluble glucan formation by glucosyltransferase

Quantification of glucan synthesis was carried out according to a previous method (Ooshima et al., 2000) with some modification (Tsai et al., 2007). The reaction mixtures contained sterile 0.1 M sucrose as a substrate and 50 μl of crude GTFs were buffered in PBS, pH 6.0 and different final concentrations ranging from (0 to 5 mg/ml) of *P. barbatus* and *P. ecklonii* extracts and rosmarinic acid were assayed for the inhibition of water insoluble glucan synthesis. Following incubation at 37 °C for 18 h, the water insoluble glucan produced by enzyme reaction 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 the extracts colour. The formation of water insoluble glucan was expressed as a percentage (i.e. 100 × $OD_{550\ nm}$ of test sample/$OD_{550\ nm}$) of control sample without adding extract. All reactions were carried out in triplicate.

3. Results

3.1. Antibacterial activity

The growth of *S. sobrinus* and *S. mutans* in the presence of several concentrations of the leaf aqueous extracts of *P. barbatus* and *P. ecklonii* was investigated. In a previous work (Falé et al., 2009) rosmarinic acid was found to be the main compound of these plant extracts so the action of this compound was also evaluated. The results are shown in Fig. 1a and b. It can be seen that both extracts inhibited the bacteria growth in a similar manner (identical curve lines either in Fig. 1a and b). Rosmarinic acid also inhibited bacteria growth with a linear relationship (between 0 and 8 mg/ml), with a correlation (R^2) of 0.981 and 0.979 for *S. sobrinus* and *S. mutans*, respectively. Through the examination of the graphs the minimum inhibitory concentration (MIC) values could be determined. MIC corresponds to the extract concentration limiting growth to 90%. The results are shown in Table 1. Rosmarinic acid presents a lower antibacterial activity than the leaf extracts. In the extract, although rosmarinic acid is the component present in higher quantity, there are other compounds like scutellarein derivative and (16S)-coleon E in *P. barbatus* (Falé et al., 2009) that may contribute to the final antimicrobial activity. The mechanism of antibacterial action of *P. barbatus* and *P. ecklonii* extracts may be related to a synergistic effect of several compounds than to the individual effect of each component.

The minimum inhibitory bactericidal concentration (MBC), values presented in Table 1, were two or three times higher than the MIC values. The MBC values are usually two to four times higher than the MIC values (Song et al., 2006).

The aqueous extract demonstrated similar antimicrobial activity against *S. sobrinus* and *S. mutans* with MIC values of 3.8 and 2.9 mg/ml for *P. barbatus* and 4.7 and 5.0 mg/ml for *P. ecklonii*. Rosmarinic acid exhibits a lower inhibitory effect on the growth of both bacteria with a MIC of 8.4 for *S. sobrinus* and 7.3 mg/ml for *S. mutans*.

Table 1
Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. barbatus*, *P. ecklonii* aqueous leaf extracts and rosmarinic acid against *S. sobrinus* and *S. mutants*.

Compound (mg/ml)	Extract (mg/ml)	Species/strain			
		S. sobrinus (CETC 4010)		*S. mutans* (CETC 479)	
		MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
–	*P. barbatus*	3.8 ± 0.0	9.5	2.9 ± 0.1	9.5
	P. ecklonii	4.7 ± 0.3	9.0	5.0 ± 0.0	10.0
Rosmarinic acid	–	8.4 ± 0.4	12.0	7.3 ± 0.2	12.5

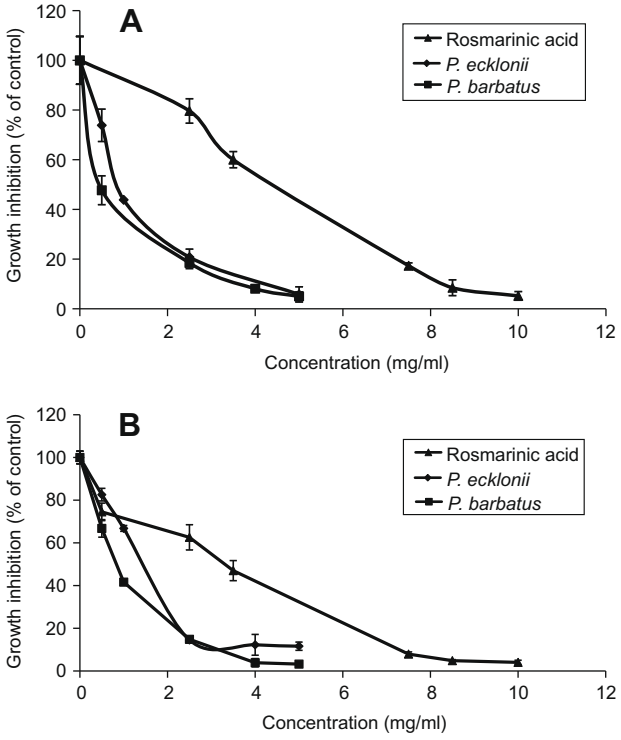

Fig. 1. Growth inhibition by aqueous leaf extracts of *P. barbatus*, *P. eclonii* and rosmarinic acid of: (A) *S. sobrinus* and (B) *S. mutans*. The growth inhibition was express as a percent of control (without sample).

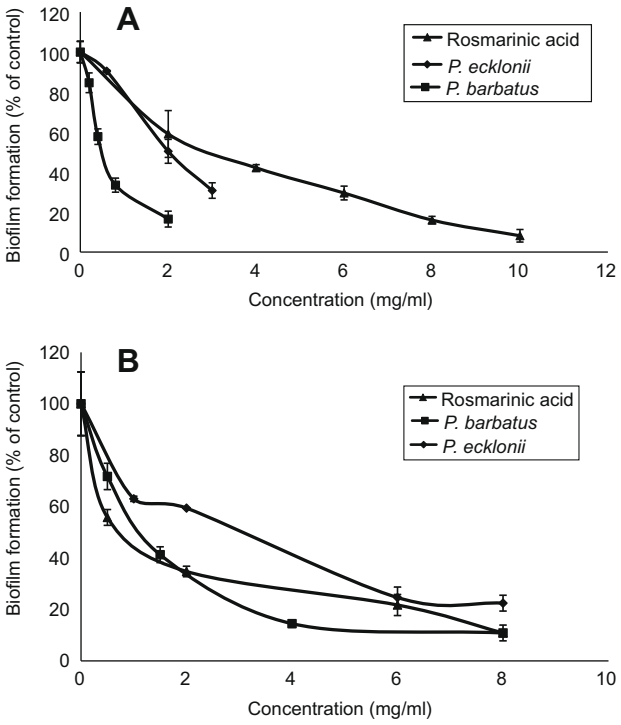

Fig. 2. The inhibitory effect of aqueous leaf extracts of *P. barbatus*, *P. eclonii* and rosmarinic acid on the biofilm formation by (A) *S. sobrinus* and (B) *S. mutans*. The inhibitory effect was express as a percent of control (without sample).

These values have significant activity when compared with other aqueous medicinal plant extracts, such as *Rosmarinus officinalis* "rosemary" (16 mg/ml) (Tsai et al., 2007) *Caesalpinia pyramidalis* "Catingueira" (8 mg/ml) and *Ziziphus joazeiro* "juazeiro" (16 mg/ml) which are already used in commercial dentrifices (Alviano et al., 2008). These results together with the previously found low toxicity and good antioxidant activity (Falé et al., 2009), suggest that the leaf plant extracts may be of interest for treatments of oral diseases.

3.2. Effect on biofilm formation

The results of the influence of extracts of *P. barbatus*, *P. ecklonii* and rosmarinic acid on biofilm formation by *S. sobrinus* and *S. mutans* are shown in Fig. 2a and b. The assays were performed at MIC and sub-MIC levels. The inhibitory effect on the biofilm formation was dependent on the concentration of the tested compound. These figures show that both extracts and rosmarinic acid can inhibit 80–90% the biofilm formation. The IC_{50} values, concentration of extracts and tested compound that reduces in 50% the biofilm formation by *S. sobrinus*, were 0.6, 1 and 3.1 mg/ml for *P. barbatus*, *P. ecklonii* extracts and rosmarinic acid, respectively (Table 2). IC_{50} against biofilm formation by *S. mutans* were 1.4, 2.7 and 1.3 mg/ml (Table 2).

It seems that the inhibitory effect of *P. barbatus* and *P. ecklonii* may be related with rosmarinic acid content of the extracts, because this acid also showed inhibitory activity and is the main component of these extracts.

3.3. Water insoluble glucan formation by glucosyltransferase

To study the mechanism responsible for the inhibition of biofilm formation, the effect of *P. barbatus* and *P. ecklonii* extracts and rosmarinic acid on the water insoluble glucans synthesis by cell free extracellular glucosyltransferase (GTF) of *S. sobrinus* and *S. mutans* were examined. *P. barbatus*, *P. ecklonii* aqueous extracts together with rosmarinic acid significantly decreased the water insoluble glucans synthesis by *S. sobrinus* and *S. mutans* glucosyltransferases, Fig. 3a and b. There was a linear relationship between the inhibition activity of rosmarinic acid with the concentration, $R^2 = 0.964$ for *S. sobrinus*, Fig. 3a. No linear relationship was obtained for *S. mutans*, Fig. 3b. The IC_{50} values are indicated in Table 3. Analysis of this table shows that rosmarinic acid markedly inhibited glucosyltransferase activity, which suggests that the bioactive compound that mediate the inhibitory effect of the extracts against glucosyltransferase activity may be related to the presence of rosmarinic acid in both extracts.

4. Discussion

The use of agents that reduce the viability but also control the colonisation on the tooth surface by inhibiting the biofilm formation could be a promising approach for the prevention of dental caries. Many studies (Ooshima et al., 2000; Taguri, Tanaka, & Kuono, 2004; Moreno, Scheyer, Romano, & Vojnov, 2006) have identified phenolic compounds isolated from medicinal plants, like teas and other beverages with antimicrobial properties against mutans streptococci. In this context and taking into account that previous studies reported that *P. barbatus*, *P. ecklonii* aqueous extracts and rosmarinic acid, have shown a wide variety of pharmalogical effects on enzymes such as acetylcholinesterase (Falé et al., 2009) and gastric H^+, K^+-ATPase (Schultz et al., 2007). In this study we examined the potential activity of these plants extracts and of rosmarinic acid against *S. mutans* and *S. sobrinus*. The extracts as well as rosmarinic acid inhibited the biofilm formation (Table 2), as they were able to inhibit the activity of glucosyltransferase (Table 3). This fact indicates that the inhibition of the enzyme is one of the important factors in preventing the bacterial cellular adherence and consequently the biofilm formation.

Our data suggest that the inhibitory effect on biofilm formation depends on rosmarinic acid present in the extract. This compound has a significant inhibitor effect in glucosyltransferase activity. This enzyme causes *S. sobrinus* and *S. mutans* to adhere to the tooth

Table 2
The inhibitory effect of aqueous leaf extract of *P. barbatus*, *P. ecklonii* and rosmarinic acid on biofilm formation by *S. sobrinus* and *S. mutants*.

Compound (mg/ml)	Extract (mg/ml)	IC_{50} (mg/ml) Species/strain	
		S. sobrinus (CETC 4010)	*S. mutans* (CETC 479)
–	*P. barbatus*	0.6 ± 0.1	1.4 ± 0.7
	P. ecklonii	1.0 ± 0.2	2.7 ± 0.6
Rosmarinic acid	–	3.1 ± 1.2	1.3 ± 0.7

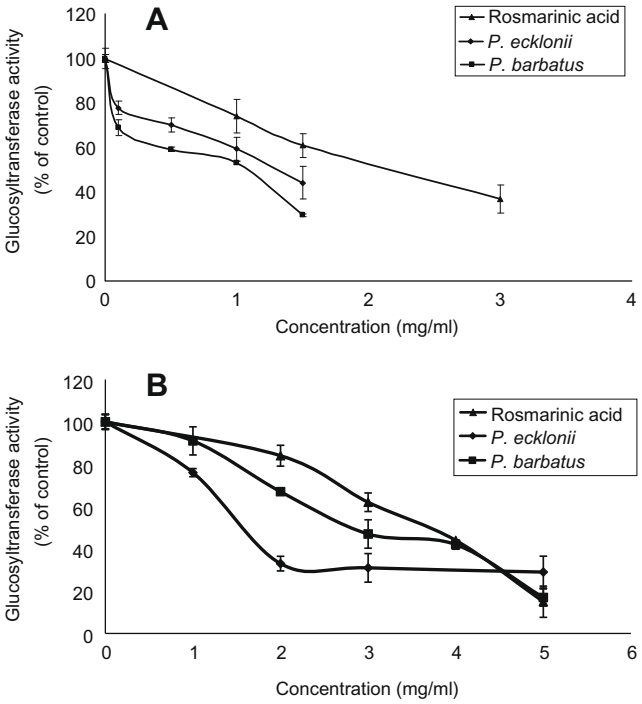

Fig. 3. Effect of *P. barbatus*, *P. eclonii* aqueous leaf extracts and rosmarinic acid on water insoluble glucan formation by glucosyltransferase of (A) *S. sobrinus* and (B) *S. mutans* from sucrose. The formation of water insoluble glucan was express as a percent of control (without sample).

Table 3
Effect of *P. barbatus*, *P. ecklonii* aqueous leaf extracts and rosmarinic acid on water insoluble glucan formation by glucosyltransferase of *S. sobrinus* and *S. mutants* from sucrose. The formation of water insoluble glucan was express as a percent of control (without sample).

Compound (mg/ml)	Extract (mg/ml)	IC_{50} (mg/ml) Species/strain	
		S. sobrinus (CETC 4010)	*S. mutans* (CETC 479)
–	*P. barbatus*	1.1 ± 0.0	3.1 ± 0.5
	P. ecklonii	1.2 ± 0.1	1.6 ± 0.0
Rosmarinic acid	–	2.1 ± 0.1	3.9 ± 0.3

surface, being one of the virulence factors contributing to the formation of the biofilm. There were no reports on the effect of rosmarinic acid on the activity of glucosyltransferase, although Tsai et al. (2007) have already reported that the main phenolic compound in organic extracts of *Rosmarinus officinalis* L. are diterpenes derivatives such as rosmarol and rosmarinic acid (Zheng & Wang, 2001).

The values found for the MIC, MBC are within the ranges published. Besides the standard rosmarinic acid, both extracts have the capacity to inhibit the biofilm formation. *P. barbatus* showed higher activity than *P. ecklonii*, what may be due to the fact of containing, besides rosmarinic acid, a flavonoid derivative (scutellarein 4′-methyl ether 7-O-glucuronide) and a diterpenoid (16S-coleon E) (Falé et al., 2009) that may contribute to the global activity found. The values found are within those reported in the literature.

S. sobrinus was more sensitive than *S. mutans* to all the compounds tested. This fact was also reported by Song et al. (2006) when evaluating the effect of the root of *P. cuspidam* on bacterial viability and virulence factors of *S. mutans* and *S. sobrinus*.

5. Conclusions

In conclusion, our studies showed that leaf water extracts of *P. barbatus*, *P. ecklonii* and rosmarinic acid may prevent dental caries, since they demonstrate significant inhibition of water insoluble glucans synthesis and reduce biofilm production, critical factor for dental plaque formation. These results also indicate that this activity may be related to the presence of rosmarinic acid in the extracts. Taking into account the low toxicity and potent antioxidant properties of both extracts found in our previous studies, together with the results obtained in the present work, these herbal teas may be useful in the prevention or controlling several oral diseases, even while being consumed as a hot beverage.

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