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Antimicrobial activity of *Piper aduncum* leaf extracts against the dental plaque bacteria *Streptococcus mutans* and *Streptococcus sanguinis*

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***Piper aduncum* has been widely used for medicinal purposes, and it has also been known to possess antimicrobial activity. Dental plaque is a complex ecosystem that harbors benign and pathogenic bacteria. It is desirable that compounds targeted to treat dental plaque-related diseases should be selective in their action, preserving the benign bacteria and inactivating the pathogenic ones. Thus, the study evaluated the antibacterial activity of *P. aduncum* leaf extracts against cariogenic (*Streptococcus mutans*) and health-associated (*Streptococcus sanguinis*) bacterium. For this, ethanol extracts were obtained by decoction, maceration, Soxhlet or turbo-extraction. The minimum inhibitory concentration (MIC) of the extracts was determined using the broth microdilution method. The influence of extracts on virulence traits of *S. mutans* was evaluated by the adherence assay to glass surface and by the glycolytic pH drop assay. *S. mutans* was more susceptible to crude extracts of *P. aduncum* than *S. sanguinis* and the highest activity was obtained with the maceration extract (MAC). Thus, MAC was further fractionated by gel permeation chromatography and the most active fraction against *S. mutans* (MIC of 0.08 mg/mL) had a MIC of 0.62 mg/mL for *S. sanguinis*. In addition, this fraction inhibited sucrose-dependent adherence of *S. mutans* and also reduced the level of acid production by this bacterium. The preferential activity of *P. aduncum* extracts towards *S. mutans* compared with *S. sanguinis*, in addition to their ability to inhibit sucrose-dependent adherence and reduce the level of acid production by *S. mutans*, suggest that this plant may have a potential to prevent dental caries.**

Key words: Plant extracts, *Piper aduncum*, antibacterial activity, *Streptococcus mutans*, caries prevention.

INTRODUCTION

Dental plaque or dental biofilm is a dynamic and complex community of microorganisms found on a tooth surface, embedded in a matrix of polymers of host and bacterial origin. This structure forms via an ordered sequence of

events, resulting in a structurally- and functionally-organized, species-rich microbial community (Marsh, 2004). Under health conditions, dental plaque plays an essential role in natural host defense mechanisms, protecting the host from invasions by exogenous pathogens; however, when the homeostasis is disrupted, changes in the species composition of dental biofilms can lead to diseases such as caries (Filoche et al., 2010).

During caries development, remarkable changes occur in the microbiota on the tooth surface towards the predominance of acidogenic and aciduric bacterial species such as *Streptococcus mutans* (Takahashi and Nyvad, 2011; He et al., 2015). It is widely accepted that *S. mutans* is one of the main caries-related bacteria, since it is responsible for the beginning of the caries process on enamel and root surfaces (Tanzer et al., 2001).

Streptococcus sanguinis is among the most abundant species within the first few hours of biofilm formation of newly cleaned tooth surfaces (Li et al., 2004). This pioneer colonizer is thought to play a beneficial role in the oral cavity (Caufield et al., 2000). It is of interest that both *S. mutans* and *S. sanguinis* have been known for their antagonism at the ecological level (Giacaman et al., 2015). Epidemiological studies showed that early colonization by *S. sanguinis* in infants results in delayed colonization of *S. mutans.* Conversely, high levels of *S. mutans* in the oral cavity correlate with low levels of *S. sanguinis* (Caufield et al., 2000). A possible molecular mechanism underlying these fascinating interspecies interactions relies, at least in part, on antimicrobial compounds such as bacteriocins and hydrogen peroxide, which are produced by *S. mutans* and *S. sanguinis*, respectively, ultimately to create an environment that favors the colonization of one group of organisms over the other (Kreth et al., 2005; Giacaman et al., 2015).

Based on the notion that dental plaque is a complex ecosystem constituted by both benign and pathogenic bacteria populations, it is desirable that compounds targeted to treat dental plaque-related diseases be selective in their action, preserving the benign bacteria and inactivating the pathogenic ones in order to lead to the re-establishment of a health-compatible species composition (Filoshe et al., 2010). Many studies have shown that plant products can be promising agents for the prevention of dental caries, especially due to their antimicrobial properties against *S. mutans* (Limsong et al., 2004; Yatsuda et al., 2005; Percival et al., 2006; Rukayadi and Hwang, 2006; Tomczyk et al., 2011).

Piper aduncum L. (*Piperaceae*) is a tropical shrub widespread in South and Central America, growing naturally in the Amazon and in the Atlantic Forests of Brazil. This plant has been widely used for medicinal purposes and its antibacterial properties have also been described, including against *Streptococcus* species (Lentz et al., 1998; Kloucek et al., 2005). Thus, the main aim of this study was to evaluate the *in vitro* antimicrobial activity of extracts and fractions of *P. aduncum* on the growth, sucrose-dependent cell adherence and acidogenicity of *S. mutans*. Furthermore, to assess the possible effects of these plant products towards maintaining or restoring the health-compatible state of dental plaque, their inhibitory activity on growth of *S. sanguinis* was also examined.

MATERIALS AND METHODS

Plant material

Leaves of *P. aduncum* (adult plants) were collected between June and July, 2009 in the region of Governador Valadares city, state of Minas Gerais, Brazil. The plant was identified by Dr. Beatriz Gonçalves Brasileiro, formerly from the Faculdade de Ciências Biológicas e da Saúde, Universidade Vale do Rio Doce, where voucher specimen was deposited under the number 423.

Preparation of crude extracts

The powder of the air-dried leaves (15 g) was extracted with 80% ethanol (150 mL) by different extraction techniques:

(i) Maceration for a week at room temperature;
(ii) Soxhlet apparatus for 4 h at 78-80°C;
(iii) Decoction for 6 h at 78-80°C.
(iv) turbo-extraction (2,000 rpm) for 20 min at room temperature

After filtration, the resulting solution was concentrated to dryness under reduced pressure using a rotary evaporator at a temperature lower than 40°C.

Preparation of fractions

The most active antimicrobial extract (5 g) was dissolved in 15 mL of ethanol and further fractionated by gel permeation chromatography (GPC). GPC system was constituted by a glass column of 50 mm diameter and 250 mm length coupled in series to the two other similar columns of 50 mm diameter and 480 mm length, filled with Sephadex^{TM} LH-20 gel (GE Healthcare, USA). The system was pumped by means of a P-500 (Pharmacia, USA) pump. Distilled ethanol was used as mobile phase pumped at 2 mL/min. Two hundred fractions of 20 ml each were collected by SF 2120 collector (Advantec, JP) and grouped according to the profile obtained by thin layer chromatography (TLC), making a total of 17 fractions. TLC was developed using silica 20x20 cm^2 HF_{254} plates (Merck). Ethyl acetate, hexane, dichloromethane and methanol were used as solvents.

The chromatograms were revealed by means of vanillin-sulfuric acid or NP/PEG (diphenylborinic acid ethanolamine ester - polyethylene glycol).

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Table 1. *In vitro* antimicrobial activity of extracts and fractions from *P. aduncum* leaves against *S. mutans* and *S. sanguinis*.

Micro-organisms	Minimum inhibitory concentration (MIC) in mg/ml										
	Crude extracts				Fractions (MAC extract)						
	DEC	MAC	SOX	TUR	G	I	J	L	M	N	Q
S. mutans	0.62	0.16	0.31	0.31	>10.0	1.25	0.08	0.31	5.0	>10.0	>10.0
S. sanguinis	2.5	0.31	0.62	1.25	>10.0	1.25	0.62	0.31	1.25	>10.0	>10.0

DEC: Decoction; MAC: Maceration; SOX: Soxhlet apparatus; TUR: Turbo-extraction.

Microorganisms

The microorganisms used in this study were *S. mutans* IM/UFRJ and *S. sanguinis* ATCC 10557. The culture was grown in Brain Heart Infusion (BHI) broth at 37°C for 24 h, under anaerobic conditions (anaerobic chamber model 1025; Forma Scientific Company, Marietta, OH, USA, containing an atmosphere of 85% N_2, 10% H_2 and 5% CO_2). The stock organism was stored in BHI broth containing 50% glycerol at -80°C.

Determination of minimum inhibitory concentration (MIC)

The MIC values were determined based on the broth microdilution method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2006), with modifications. Briefly, the assays were performed in polystyrene microtiter plates with 96 flat-bottomed wells. Two-fold dilution series of extracts and fractions (concentrations ranging from 10 to 0.08 mg/mL) were tested. Diluted suspensions (100 µL) of each bacterial strain were added to 100 µl of various concentrations of vegetal products diluted with the BHI broth to reach a final bacterial count of approximately 5×10^5 CFU/mL. Growth and sterility controls were included for each assay and tests were performed in triplicate in at least three independent experiments. The vehicle (DMSO) served as negative control and was used at the final concentration ≤ 4%. Chloramphenicol MIC for *S. aureus* ATCC 29213 was included for quality control, and its value (8 µg/mL) was within established ranges as published by the CLSI guidelines. The microdilution plates were incubated at 37°C for 24 h in an anaerobic atmosphere (85% N_2, 10% H_2 and 5% CO_2). The MIC was defined as the lowest concentration of extract that completely inhibited visible growth of microorganisms in the microdilution wells.

Inhibition of bacterial adherence to glass surface

To assess the bacterial adherence of *S. mutans* to a smooth glass surface, the bacteria (approximately 10^6 CFU/mL) were grown at 37°C for 6 h at an angle of 30° in a glass test tube with 1ml of BHI containing 1% (weight by volume (wt/vol)) sucrose plus two-fold dilution series of extracts and fractions (concentrations ranging from 0.62 to 0.04 mg/ml), as described earlier (Limsong et al., 2004), with modifications. After incubation, the tubes were washed three times with 5 ml of saline solution (NaCl 0.85%) and attached cells were stained with 1% crystal violet. The concentration for total bacterial adherence inhibition (TBAI) was defined as the lowest concentration that allowed no visible cell adherence on the glass surface. All determinations were performed in triplicate.

Effect of extracts and fractions on acid production

The assay was performed by standard pH drop with dense cell suspensions, according to Belli et al. (1995), with modifications. *S. mutans* cells from suspension cultures were harvested, washed three times in salt solution (50 mM KCl and 1 mM $MgCl_2$) and resuspended in 5 ml of the same salt solution, which was titrated to pH 7.2 with 0.1 M KOH. Glucose (100 mM final concentration), with and without different concentrations of extracts and fractions was added and the pH fall was assessed with glass electrode over a period of 1 h. Three independent assays were performed, and a statistical analysis was carried out using the Student's *t*-test. Differences between control (no treatment) and treatment with plant products were considered statistically significant if $p < 0.05$.

Time-kill assays

Time-kill curves were obtained according to Santos et al. (2007), with adaptations. Starting inocula of approximately 10^6 CFU/ml of each bacterial strain was grown anaerobically at 37°C in BHI broth until the middle of the exponential growth phase (approx. 3 h for *S. sanguinis* and 6 h for *S. mutans*). Then, the crude ethanol extract (final concentration of 0.31 or 0.16 mg/ml for *S. sanguinis* and *S. mutans*, respectively) or its fractions (final concentration of 0.62 mg/ml for J or 0.31 mg/ml for L) were added to each test vial. No vegetal product was added to the control vial. Cultures were then incubated at 37°C under anaerobic conditions. Samples were removed to determine viable counts every 30 min over a 2.5 h period. Ten-fold serial dilutions were prepared in sterile saline and 0.1 ml of each dilution was plated onto BHI agar. The plates were incubated at 37°C for 24 h, at which time the number of colonies was determined.

RESULTS

All four crude extracts inhibited the growth of both bacteria tested with MIC values ranging from 0.16 to 2.5 mg/mL (Table 1). The MICs for *S. sanguinis* were consistently higher (2 to 4-fold) than those found to *S. mutans,* and the crude extract yielded by maceration technique (MAC) was the most effective. Thus, MAC was fractionated by gel permeation chromatography, yielding 17 fractions, designated as A to Q (data not shown). Only seven of them (G, I, J, L, M, N, Q) were available in sufficient amounts to test their inhibitory effect on bacterial growth (Table 1). The G, N and Q fractions displayed no inhibitory activity, whereas the J and L fractions exhibited the highest activities against *S. mutans* and *S. sanguinis*, respectively. It is noteworthy that the J fraction was approximately eight times more active against *S. mutans* than on *S. sanguinis*, showing also a lower MIC than that of the MAC.

MAC and its J and L fractions had the strongest

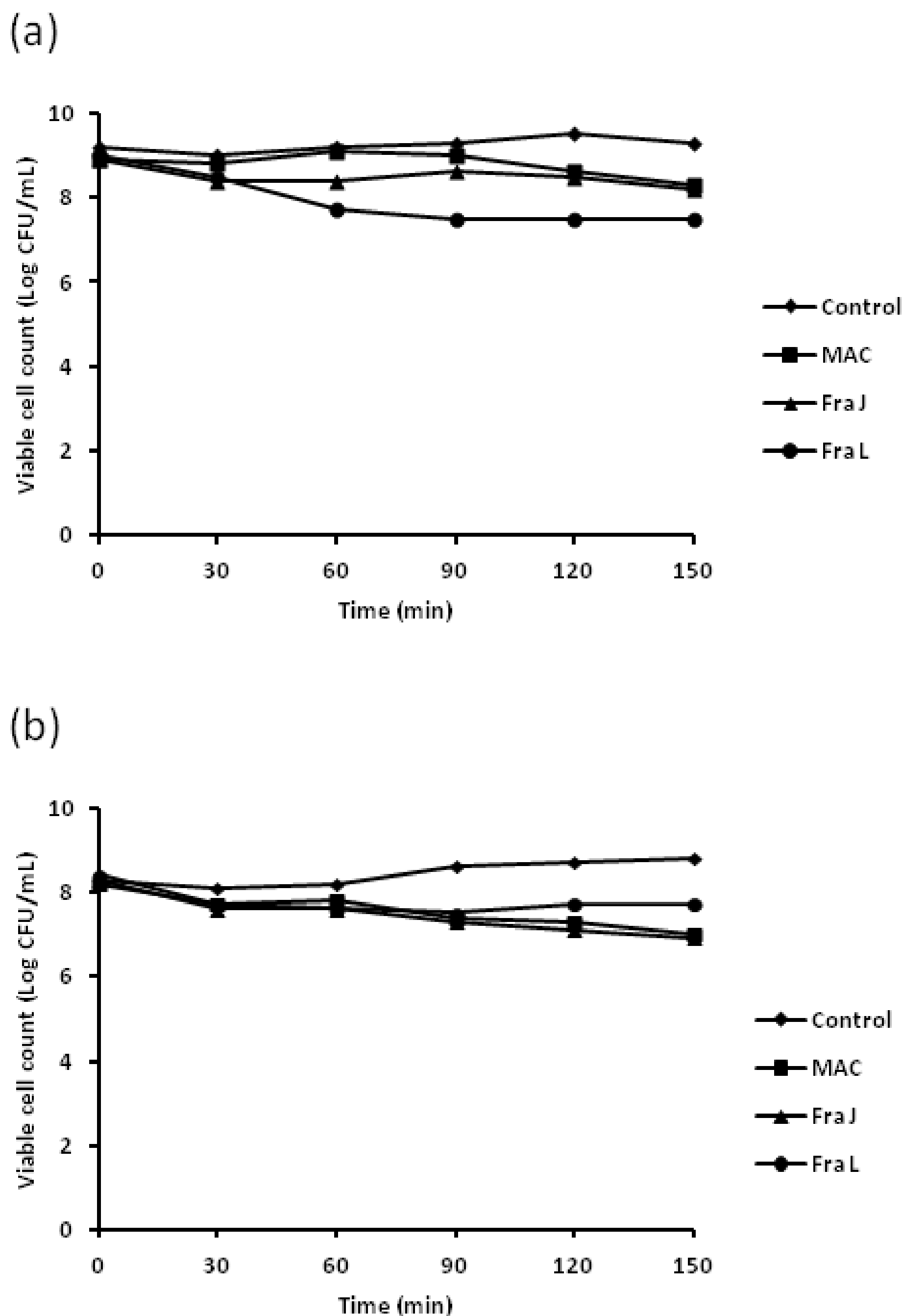

Figure 1. Time-kill curves of maceration extract of *P. aduncum* (MAC) and its both fractions (Fra J and Fra L) against *S. sanguinis* (a) and *S. mutans* (b). A growth control is also shown (Control). The tested concentrations were as follows: (a) MAC 0.31 mg/mL, Fra J 0.62 mg/mL, Fra L 0.31 mg/mL; (b) MAC 0.16 mg/mL, Fra J 0.08 mg/mL, Fra L 0.31 mg/mL. The figure shows representative data from two independent experiments.

antibacterial activity against planktonic cells, and were thus selected for the time kill test. The results revealed that these agents at MIC concentrations exhibited a bacteriostatic, but not a bactericidal effect against both *S. sanguinis* and *S. mutans* in exponential growth, since the decrease in the bacterial count for every species, relative to the starting inoculum, was <3 log (Figure 1).

To determine the effect of plant products on bacterial acid production, the pH of dense suspension of *S. mutans* was recorded during 60 min after glucose pulse. The pH of the suspension not exposed to the plant products (control) decreased rapidly from pH 7.20 to pH

Table 2. Inhibitory effects of maceration extract of *Piper aduncum* (MAC) and its both J and L fractions (Fra) on acid production by *Streptococcus mutans* at different time periods.

Treatment (mg/ml)	pH (mean + S.D.)				
	5 min	15 min	30 min	45 min	60 min
None - control	4.22±0.16	3.95±0.09	3.79±0.12	3.65±0.13	3.57±0.15
MAC (0.16)	4.51±0.01*	4.52±0.11***	4.27±0.07***	4.09±0.01***	3.98±0.05**
Fra J (0.08)	4.46±0.08*	4.24±0.16*	4.15±0.10**	3.99±0.11**	3.85±0.14*
Fra L (0.31)	4.39±0.06	4.16±0.01**	3.94±0.01*	3.81±0.02	3.71±0.02

Statistically significant difference between control (no treatment) and treatment with plant products (*p<0.05; **p<0.01; ***p<0.001) assessed by the Student's *t*-test.

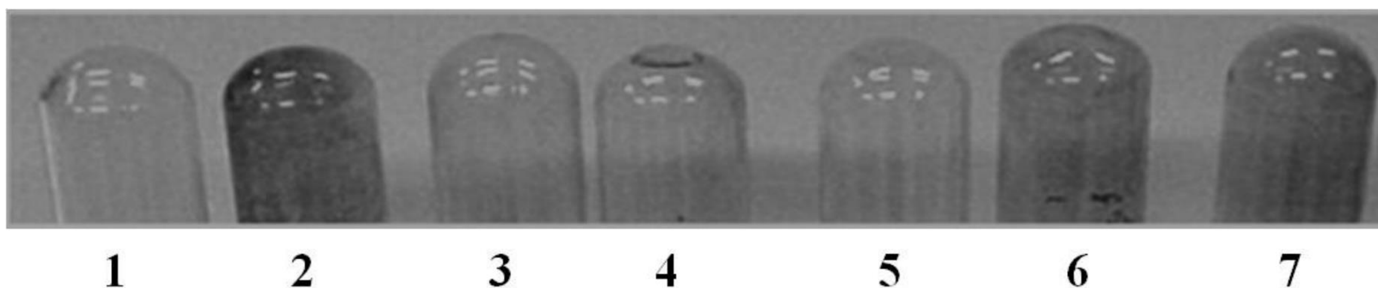

Figure 2. Inhibitory effect of maceration extract of *P. aduncum* (MAC) on sucrose-dependent adherence to glass surface of *S. mutans. S. mutans* were grown at 37°C for 6 h at an angle of 30° in a glass test tube with 1ml of BHI broth containing 1% [weight by volume (wt/vol)] sucrose plus two-fold dilution series of MAC. After incubation attached cells were stained with 1% crystal violet. (1) Negative control: bacteria alone; (2) Positive control: bacteria plus sucrose; (3-7) Bacteria plus sucrose with MAC at (3) 0.62 mg/mL, (4) 0.31 mg/mL, (5) 0.16 mg/mL, (6) 0.08 mg/mL e (7) 0.04 mg/mL.

4.22 and 3.57 after 5 min and 60 min, respectively (Table 2). However, the presence of both MAC and J fraction, at MIC concentrations, significantly reduced the rate of acid production by *S. mutans*, when compared to the control, at all the time intervals tested (5 to 60 min). MAC was actually somewhat more potent than its J fraction. The L fraction showed the weakest inhibition of acid production, significantly reducing the pH drop only at 15 and 30 min after glucose pulse.

In order to study the effect of the plant products on sucrose-dependent adherence of *S. mutans*, the study determined their concentration for total bacterial adherence inhibition (TBAI) to a glass surface. MAC (Figure 2) and its L fraction (data not shown) had a similar inhibitory activity (TBAI = 0.16 mg/ml). However, the J fraction was the most effective agent, showing inhibitory effect at a concentration of 0.08 mg/mL (data not shown).

DISCUSSION

Dental caries remains the most prevalent dental disease in many countries therefore being one of the greatest challenges in oral health care (Bagramian et al., 2009). Although the oral microbiota is quite diverse and complex, *S. mutans* has been recognized as an important etiological agent in human dental caries (Loesche, 1986; He et al., 2015).

It is widely accepted that this disease appears as a result of the breakdown of the microbial homeostasis due to a more frequent exposure of plaque to low pH following an increased frequency of sugar intake. This acidic condition provides a selective pressure that allows overgrowth of acidogenic and acid-tolerant species, such as *S. mutans*, whereas at the same time suppressing acid-sensitive bacteria such as *S. sanguinis* (Marsh, 1994).

Consequently, caries control can involve direct use of antibacterial agents to suppress bacterial overgrowth. Nevertheless, the major drawback is that antibacterial products currently in use are not selective in their action, affecting both pathogenic and beneficial bacteria (Marsh, 2010). Thus, the searches continue to find an ideal chemical agent that could control the levels of pathogenic bacteria while preserving the beneficial properties of the resident oral microbiota.

In the present study, *S. mutans* was more sensitive to *P. aduncum* extracts than *S. sanguinis.* Among all of the tested extracts, the J fraction yielded by gel permeation chromatography from MAC was the most effective, inhibiting the growth of *S. mutans* at MIC value

approximately eight times lower than that of *S. sanguinis* (0.08 and 0.62 mg/ml, respectively). In an oral environment in which *S. mutans* is dominant, this differential action, in addition to well-known molecular mechanisms of interspecies competition between *S. sanguinis* and *S. mutans* (Kreth et al., 2005), would result in microbial shifts, and *S. sanguinis* would take the place of *S. mutans* in the ecosystem, restoring the health-compatible state of dental plaque.

According to Ríos and Recio (2005), a significant problem in many studies is to claim positive activity for excessively high concentrations. They consider that plant extracts that are active at concentrations lower than 0.1 mg/ml have a good potency level. Thus, based on this criterion, the study showed that J fraction has promising activity against *S. mutans*. It is possible to speculate that there are specific compounds in this fraction that can inhibit the growth of *S. mutans* at low concentrations. In addition, the study fractionation process had good result towards enhancing the antibacterial activity against *S. mutans*, since the J fraction showed higher activity than its corresponding crude extract (MAC) only on this bacterium.

Results of a previous study have shown that there were differences between the chromatographic profiles of the *P. aduncum* extracts yielded by different extraction methods. The MAC had the highest content of sesquiterpenes, which accounted for more than 97% of the total identified compounds (Santos et al., 2013). Sesquiterpenes have been extensively described in the literature for their antibacterial properties (Paduch et al., 2007; Saleem et al., 2010), including against *S. mutans* (Kubo et al., 1992). These latter authors reported that nerolidol, which was one of the most abundant sesquiterpenes present in MAC (Santos et al., 2013), has potent activity against *S. mutans*, with MIC of 0.025 mg/ml (Kubo et al., 1992). Thus, the good performance of the MAC and its J fraction can be related to their high content of sesquiterpenes. The mechanism of action of terpenes is not fully under-stood, but it is thought to involve membrane disruption associated with their lipophilic character (Cowan, 1999; Paduch et al., 2007). However, the reason for which MAC and its J fraction inhibited *S. mutans* and *S. sanguinis* to varying extent remains to be clarified.

It is important to note that the present results are different from those of Lentz et al. (1998). They reported that ethanol extract from *P. aduncum* collected in Honduras had no activity against *S. mutans* but revealed measurable antibiosis on *S. sanguinis*. Since in this study the agar well test was employed, MIC values were not determined. These divergent results can be explained by many factors, including differences in the geographical locations of the plants (Honduras × Brazil), extraction methods (percolation × maceration) and antibacterial assays (agar-diffusion method x dilution method). According to Cos et al. (2006), several methods to detect the extract activity are available; however, since they are not equally sensitive or not based upon the same principle, results may be profoundly influenced by the chosen method. The diffusion method is not appropriate for testing non-polar samples or samples that do not easily diffuse into agar. Contrarily, in general, dilution methods are appropriate for assaying polar and non-polar samples. Kubo et al. (1992) showed that some active sesquiterpenes against *S. mutans* by broth dilution method did not have any activity by an agar-diffusion method.

Besides inhibiting bacterial growth, J fraction at a concentration of 0.08 mg/ml had also biological activity against two of the main virulence traits of *S. mutans*: sucrose-dependent adherence and acidogenicity. Since the concentration that inhibited bacterial growth was the same as that affected the virulence factors, it can be suggested that the anti-virulence effect of this fraction may be due to its antibacterial activity rather than due to a direct effect on specific virulence traits. However, at least for acidogenicity, the effect of J fraction occurred at early times (5 to 60 min) in which it has not bactericidal activity, according to the time kill assays.

The time kill assays were carried out for the maximum length of time of 2.5 h because, based on the present results, *P. aduncum* extracts could be thought to be used in oral care products. Thus, whether in this exposure time a bactericidal effect was not yet achieved *in vitro*, where there is fixed concentration of agent, probably under the *in vivo* conditions these extracts also will fail to show bactericidal activity because of their lack of persistence in the mouth. Chemical antimicrobial compounds used as a mouthwash tend to be rapidly dislodged, diluted or removed (Addy, 1994; Marsh, 2010).

In order to analyze the inhibitory effect of the extracts on adherence of *S. mutans*, we performed the adherence assay to glass surface. According to Limsong et al. (2004), in this assay, the adherence is mediated by glucan as well as the *in vivo* situation. This is an interesting issue because the establishment of *S. mutans* on the tooth surface is rendered irreversible only after the synthesis of sticky water-insoluble glucan from sucrose by enzymatic action of glucosyltransferase and the subsequent cell-to-cell aggregation (Nostro et al., 2004). Thus, the effect of the extracts from *P. aduncum* can be of value to prevent both adherence and accumulation of *S. mutans* on the tooth surface.

Conclusion

The findings that *P. aduncum* extracts exhibit a preferential antimicrobial activity towards *S. mutans* compared with *S. sanguinis*, in addition to their ability to inhibit sucrose-dependent adherence and reduce the level of acid production from *S. mutans*, suggest that this plant may have a potential for further exploitation in

dentistry to prevent dental caries. Future studies should be conducted in order to find the compounds of *P. aduncum* responsible for its anti-*S. mutans* properties.

Conflict of Interests

The authors have not declared any conflict of interests.

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