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Effect of *Psidium cattleianum* leaf extract on enamel demineralisation and dental biofilm composition *in situ*

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ABSTRACT

Objective: Previous evaluations of *Psidium cattleianum* leaf extract were not done in conditions similar to the oral environment. The aim of this study was to evaluate the effect of *P. cattleianum* leaf extract on enamel demineralisation, extracellular polysaccharide formation, and the microbial composition of dental biofilms formed *in situ*.

Design: Ten volunteers took part in this crossover study. They wore palatal appliances containing 4 enamel blocks for 14 days. Each volunteer dripped 20% sucrose 8 times per day on the enamel blocks. Twice a day, deionised water (negative control), extract, or a commercial mouthwash (active control) was dripped after sucrose application. On the 12th and 13th days of the experiment, plaque acidogenicity was measured with a microelectrode, and the pH drop was calculated. On the 14th day, biofilms were harvested and total anaerobic microorganisms (TM), total streptococci (TS), mutans streptococci (MS), and extracellular polysaccharides (EPS) were evaluated. Enamel demineralisation was evaluated by the percentage change of surface microhardness (%ΔSMH) and integrated loss of subsurface hardness (ΔKHN). The researcher was blinded to the treatments during data collection.

Results: The extract group showed lower TM, TS, MS, EPS, %ΔSMH, and ΔKHN values than the negative control group. There were no differences between the active and negative control groups regarding MS and EPS levels. There were no differences in pH drop between the extract and active control groups, although they were significantly different from the negative control group. For all other parameters, the extract differed from the active control group.

Conclusion: *Psidium cattleianum* leaf extract exhibits a potential anticariogenic effect.

1. Introduction

Dental caries is one of the most common chronic and infectious diseases worldwide.[1] This disease results from the interaction of specific bacteria with constituents of a diet in a susceptible host.[2] It is associated with the presence of biofilms, which are microbial communities embedded in a polymeric matrix attached to the surface of the tooth.[1,2] The disease appears as a result of a breakdown of biofilm homeostasis, which is related to frequent sugar exposure and a low biofilm pH.[1]Natural products have been studied for their ability to chemically control dental biofilms. Koo et al.[3] show that a mouthwash with propolis is able to reduce supragingival plaque formation and insoluble polysaccharide formation. Smullen et al.[4] demonstrate the antibacterial

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effects of a wide variety of extracts, including teas, red and green grape extracts, and cocoa on *Streptococcus mutans*. To date, most studies of natural products against oral pathogens attempt to verify their antibacterial properties and mechanisms of action *in vitro*.[4–7]

Once the therapeutic properties of a medicinal plant is empirically known, the World Health Organization initiates the pharmacological evaluation of phytotherapeutic drugs from such sources.[8] Despite current interest for their use in medicine, less than 15% of medicinal plants from tropical areas that have the potential for therapeutic use have been studied.[9]

Psidium cattleianum is commonly known as strawberry guava. It belongs to the family Myrtaceae and is native to tropical America. Plants of *Psidium* species (*Psidium* spp.) are traditionally used to treat several diseases worldwide,[10] and the antibacterial effects of these plants have been tested against bacteria that cause diarrhoea or opportunistic infections.[11,12] Previous studies show that the leaf extracts of plants from this family, such as *P. guajava*, are also able to reduce gingivitis and halitosis.[13,14] Brighenti et al.[15] previously demonstrated the efficacy of *P. cattleianum* leaf extract against *S. mutans* biofilms and elucidated its action mechanism. However, this extract has not been evaluated in conditions similar to the oral environment. The evaluation of dental products using *in situ* caries models helps presume clinical outcomes and allows the evaluation of many features of dental biofilm and hard tissues.

The aim of this study was to evaluate the effect of *P. cattleianum* leaf extract on enamel demineralisation and assess its effect on the biochemical and biological composition of oral biofilm using an *in situ* dental caries model.

2. Materials and methods

2.1. Extract preparation

The harvesting of *P. cattleianum* leaves was authorised by the Brazilian Environment and Natural Resources Institute (IBAMA, permit # 0129208). A voucher herbarium specimen was deposited at the Collection of Aromatic and Medicinal Plants (CPMA: *Coleção de Plantas Medicinais e Aromáticas*) at the University of Campinas (UNICAMP) (voucher #1299).

Only healthy leaves (*i.e.*, those without signs of necrosis and with typical colour and shape) were selected for extract preparation. The leaves were dried at 37 °C for 1 week after being washed in tap and deionised water. The leaves were then ground in a blender until a thin powder was achieved. Aqueous extract was obtained by decoction in deionised water (100 g/600 mL) for 5 min at 100 °C and at 55 °C for an additional 1 h. The solution was then filter sterilised with 0.22 μm mixed cellulose ester membranes (Millipore™; Billerica, MA, USA). The extract was stored in dark bottles at −20 °C until further use.[15]

2.2. Enamel block preparation and analysis

Enamel blocks measuring 4 mm × 4 mm were obtained from bovine incisor teeth previously stored in 2% formaldehyde solution (pH 7.0) for 1 month[16] and had their surfaces serially polished. Measurements of surface microhardness (SMH) and cross-sectional enamel microhardness (kg mm^{-2}) were made using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan). For baseline SMH (SMH_1), 5 indentations spaced 100 μm apart were made with a 25-g load for 10 s in the centre of the enamel block as described by Vieira et al.[17]

After the experimental phase, SMH was measured again (SMH_2). Five indentations spaced 100 μm apart and from the baseline were made. The percentage change of SMH (%ΔSMH) was calculated as follows: $\%\Delta SMH = 100(SMH_2 - SMH_1)/SMH_1$.

To perform cross-sectional microhardness tests, the blocks were longitudinally sectioned through the centre. One of the halves was embedded in acrylic resin with the cut face exposed and gradually polished.

Three rows of 9 indentations spaced 100 μm apart were made 10, 30, 50, 70, 90, 110, 170, 220, and 330 μm from the outer enamel surface under a 25-g load for 10 s. The mean values at all 3 measuring points at each distance from the surface were then averaged.

The integrated hardness (Knoop hardness number; KHN × μm) of the lesion into sound enamel was calculated and subtracted from the integrated hardness of sound enamel to obtain the integrated loss of subsurface hardness (ΔKHN).[18] ΔKHN denotes the enamel subsurface demineralisation area after the *in situ* experiment.

2.3. Experimental design

This study was previously approved by the human ethical committee from Araçatuba Dental School (protocol #2005-02188). Ten healthy volunteers aged 23–34 years were selected. The volunteers used non fluoridated dentifrice 1 week before and throughout the experiment.[19,20]

The crossover *in situ* experiment was composed of 3 phases of 14 days each according to the treatment solution: deionised water (negative control), extract, or Original Listerine Antiseptic™ (Johnson & Johnson, New Brunswick, NJ, USA; active control). The study was not blind during the *in situ* phase because of the physical differences of the solutions used (*i.e.*, colour, smell, and taste). However, it was possible to make the study blind during the analysis of the crossover study. The enamel blocks were coded after the experimental phase, and the researcher who carried out the analysis was blinded to which enamel block belonged to which group.

The volunteers wore palatal appliances containing 4 enamel bovine blocks covered by plastic mesh to enable biofilm accumulation and protect it from disturbances.[21] One millimetre was left between the enamel and the plastic mesh to allow biofilm growth.

Enamel blocks were randomised according to the mean of the SMH from all blocks and its confidence intervals. Microsoft Excel® 2003 was used to calculate the confidence intervals. The level of significance was set at $p < 0.05$ for this experiment. The enamel blocks were distributed amongst the treatment groups such that the mean SMH from each group was within the 95% confidence interval for the total block mean. Thus, the mean of each group was set between 378.1 and 381.1 KHN.

The volunteers removed the palatal appliance from their oral cavity and placed one drop of 20% sucrose in each enamel block 8 times a day. The volunteers waited 5 min before returning the appliance to their oral cavity.[22] During the 4th and 8th sucrose applications, deionised water, extract, or Original Listerine Antiseptic™ was dripped 1 min after sucrose application twice a day and the appliance was replaced in the oral cavity 4 min later. A washout period of 7 days was allowed between each phase.[21,23] The volunteers received oral and written instruction to wear the appliance all the time and remove it during meals or oral hygiene. They were not allowed to use any antimicrobial or fluoride products during the experiment.

2.4. Assessment of biofilm acidogenicity

pH was measured according to Pecharki et al.,[22] using a palladium microelectrode (Beetrode™ NMPH3; World Precision Instruments Inc., Sarasota, FL, USA) with biofilms at overnight fasting to assure that no bacterial carbohydrates were stored[24] and to evaluate the residual effect of the treatments. A salt bridge between the reference electrode and the volunteer's finger was created with 3 mol L^{-1} KCl.[25]

Two conditions were evaluated during pH measurements. First, on the 12th day, only sucrose was applied (''sucrose alone'' measurement). The pH was measured 5 min after sucrose application with the appliances placed in the oral cavity. This was done to evaluate the possible residual effect of the treatment solutions and detect whether pH variations were due to a structural change in the biofilm induced by the treatment solutions. Second, on the 13th day, sucrose was applied and the treatment solutions were dripped (''sucrose + treatment'' measurement) after 1 min. Four minutes later, the appliances were replaced in the mouth and pH was measured. This was done to evaluate the immediate effect of the treatment solutions.

pH was measured twice: before dripping any solutions (baseline measurement) (pH_1) and after dripping sucrose alone (at the 12th day) or sucrose + treatment solution (at the 13th day) (pH_2). Next, pH variation was calculated as follows: $\Delta pH = pH_1 - pH_2$.

2.5. Analysis of dental biofilm composition

At the end of the experiment, the plastic mesh was removed and biofilms were harvested and weighed. Around 5 mg biofilm was diluted in phosphate-buffered saline (PBS: 8.0 g NaCl, 0.2 g KCl, 1.0 g Na_2HPO_4, and 0.2 g KH_2PO_4 per litre, adjusted to pH 7.4; 1 mL mg^{-1} biofilm) and sonicated on ice in an ultrasonic cell disruptor (XL; Misonix Inc., Farmingdale, NY, USA) for 6 × 9.9 s with an amplitude of 40 W.[26] The suspensions were diluted in PBS and plated in duplicate in brain heart infusion agar, Mitis salivarius agar, or Mitis salivarius sucrose bacitracin agar to analyse total anaerobic microorganisms (TM), total streptococci (TS), and mutans streptococci (MS), respectively.[27] The presence of TS and MS was confirmed by Gram staining. Colony-forming units (CFU) were counted after incubation for 72 h at 37 °C in an anaerobic jar (Anaerojar™ + Anaerogen™; Oxoid, Cambridge, UK). The results are expressed in log CFU mg^{-1} wet weight.

The remaining biofilm was dried with phosphorus pentoxide (Vetec Química Fina Ltda., Duque de Caxias, RJ, Brazil).[22] Extracellular polysaccharides (EPS) were extracted by adding 1.0 mol L^{-1} NaOH (10 μL mg^{-1} dry weight) to the biofilm. The samples were vortexed for 1 min; after 3 h under agitation at room temperature, they were centrifuged for 1 min at 11,000 × *g* at room temperature.[28] Supernatants were precipitated overnight with 75% cooled ethanol, centrifuged, and resuspended in 1.0 mol L^{-1} NaOH.[29] Carbohydrate analysis was performed using the phenol–sulphuric acid procedure.[30] The results are expressed as μg mg^{-1} dry weight.

2.6. Statistical analysis

Our hypothesis is that treatment with *P. cattleianum* leaf extract decreases microbial viability in biofilms and enamel demineralisation. The means of the results from the 4 enamel blocks of each participant for each experimental phase were taken. Statistical analysis was carried out using GraphPad Prism Version 3.02 (GraphPad Software Inc., San Diego, CA, USA). Data for %ΔSMH, ΔKHN, EPS, ΔpH and microorganism counts (*i.e.*, TM, TS, and MS) exhibited equality of variances (Bartlett's test) and normal distribution (Kolmogorov–Smirnov test); they were submitted to analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Data of ΔpH between the 12th and 13th days were analysed by two-tailed paired *t*-test. The significance limit was set at 5%. Correlations between the analysed parameters were determined.

3. Results

P. cattleianum leaf extract had an effect on microorganism counts (Fig. 1). There were significantly less viable bacteria in the biofilms after treatment with the extract than after treatment with water or active control groups ($p < 0.05$) for TM, TS and MS. There were no statistically significant differences with respect to MS viability after treatment with water or the active control ($p > 0.05$). Statistically significant differences were found between water and active control groups for TM and TS. TM was more susceptible than TS or MS, as showed by the difference in log reduction observed (6, 1 and 1 for, respectively TM, TS and MS).

P. cattleianum leaf extract and the active control significantly reduced %ΔSMH and ΔKHN compared to the water group. In addition, the extract exhibited better performance than the active control ($p < 0.05$) (Fig. 2).

Compared to water, ΔpH was significantly lower with the extract and active control in both sucrose alone and sucrose + treatment solutions; however, there were no significant differences in pH drop in these 2 conditions ($p > 0.05$) (Fig. 3). The use of the extract also significantly decreased the amount of EPS compared to the active or negative controls ($p < 0.05$). No significant differences in EPS were observed after treatment with water or the active control ($p > 0.05$).

A positive correlation was found between the amount of formed EPS and the presence of TM and TS ($r = 0.9016$ and 0.9967, respectively) but not regarding MS. Moreover, no significant correlations were found between MS and %ΔSMH, ΔKHN, EPS, ΔpH/sucrose alone, and ΔpH/sucrose + treatments.

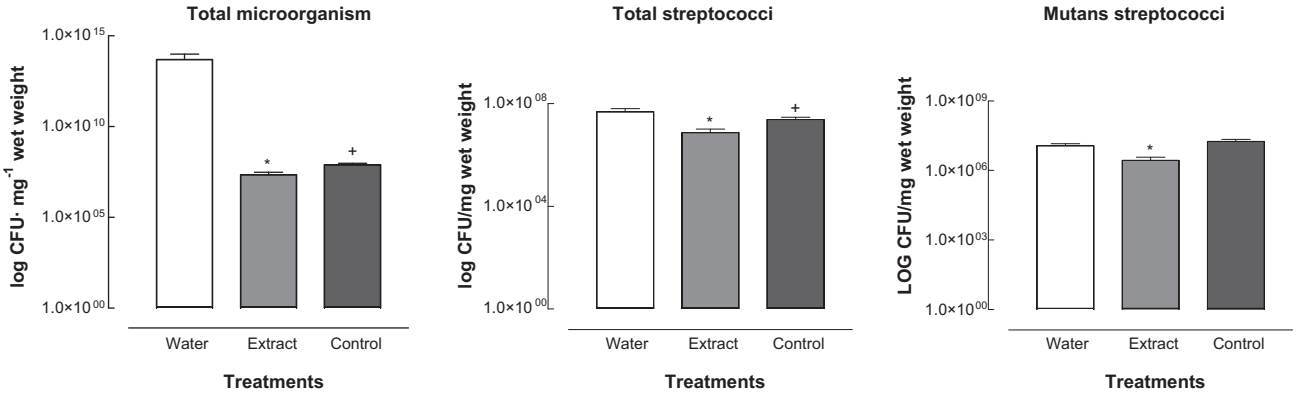

Fig. 1 – Viability of total anaerobic microorganism, total streptococci and mutans streptococci after exposure to different treatments (mean ± SE, $n = 10$). Distinct symbols show statistical difference between treatment groups (ANOVA, $p < 0.05$).

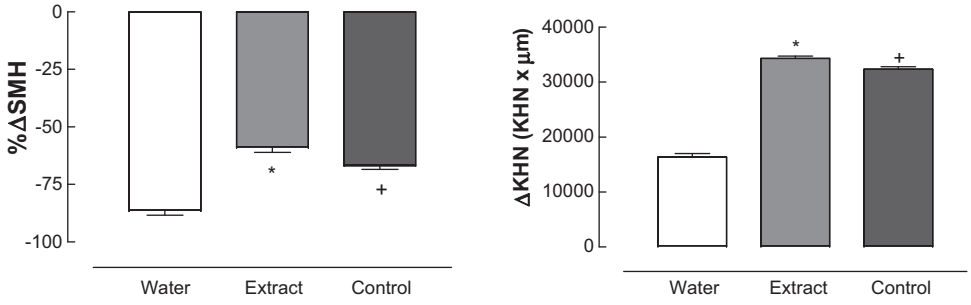

Fig. 2 – Effect of different treatment groups on percentage change of surface microhardness (%ΔSMH) and integrated loss of subsurface hardness (ΔKHN) (mean ± SE, $n = 10$). Distinct symbols show statistical difference between groups (ANOVA, $p < 0.05$).

4. Discussion

The present study evaluated the anticaries effect of *P. cattleianum* leaf extract. The compounds present in *P. cattleianum* with antibacterial activity are all phenolic compounds: 3 are flavonoids (kaempferol, quercetin, and cyanidin) and 1 is a tannin (ellagic acid).[31] The studied extract does not contain considerable amounts of fluoride, calcium, or phosphate (data not shown because the levels were below the detection limit). The aim of using fluoride-free dentifrice was not to overestimate the efficacy of the tested extract but to evaluate the potential effect of the extract alone. This allowed a better scientific control of the study and is concordant with the literature.[32,33] Further studies are needed to evaluate the antibacterial effect of the extract in compared to fluoride products.

Although this is the first time an antibacterial substance has been tested using an *in situ* caries model, this model has been extensively studied to evaluate the microbiological and

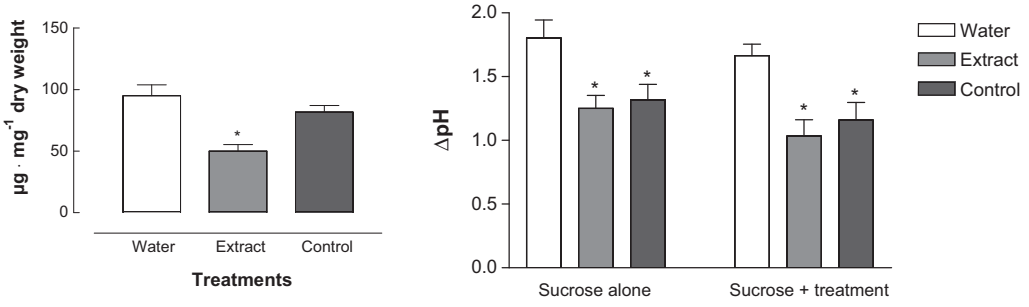

Fig. 3 – Effect of different treatment solutions on alkali-soluble carbohydrates (μg mg^{-1} dry weight) and pH variation (ΔpH) (mean ± SE, $n = 10$). Distinct symbols show statistical difference between treatment groups (ANOVA, $p < 0.05$) and between differences on the pH measurements (sucrose alone or sucrose + treatment) (unpaired *t*-test, $p < 0.05$). "Sucrose alone" stands for ΔpH after dripping only sucrose, without any treatment. "Sucrose + treatment" represents ΔpH after dripping sucrose and the treatment solutions.

biochemical composition of biofilms[34–36]; microbial virulence factors[37,38]; the cariogenicity of foods, sugars, and other substances[39–41]; and the protective effects of milk products and chewing gum.[42–44] These studies suggest that this model is suitable for evaluating antibacterial substances. Moreover, this *in situ* model was chosen because it promotes a high cariogenic challenge, leading to enamel demineralisation; this was very useful for testing the extract after the previous *in vitro* study[15,45] highlighted the tendency of the extract to decrease acid production.

A previous study by our research group shows that *P. cattleianum* leaf extract can inhibit the expression of proteins involved in general metabolism, especially those involved in the carbohydrate metabolism of *S. mutans* biofilms.[15] More recently, Menezes et al.[45] confirmed the anticariogenic action of the extract using a caries rat model. The present study represents an intermediate stage between *in vitro* and *in vivo* studies. *In situ* experiments allow the exploration of laboratory findings in a situation that mimics the development of natural caries whilst using sensitive detection methods.[46]

The low toxicity of *Psidium* spp. extract is described in the literature. The LD_{50} of *P. guajava* leaf extract is more than 5 g kg^{-1}.[47] Teixeira et al.[48] demonstrate that *P. guajava* infusion does not alter chromosomes or the cell cycle both *in vitro* and *in vivo*. Costa et al.[49] show that *P. cattleianum* leaf extract does not have any genotoxic or mutagenic effects on some cell types of mice, suggesting that this extract can be used as a safe therapeutic agent.

pH expresses the acidity or alkalinity of a solution and is widely used to evaluate the acidogenicity of biofilms.[22,36] The lower ΔpH values and lower enamel demineralisation found in this *in situ* experiment are concordant with previous *in vitro* and *in vivo* observations. Brighenti et al.[15] found a decrease in *S. mutans* proteins from carbohydrate metabolism and a reduction in pH drop after exposure to the extract. Menezes et al.[45] observed that the extract is able to reduce *S. mutans* viability and enamel demineralisation in rats submitted to a cariogenic challenge.

On the other hand, the decrease in enamel demineralisation observed in the extract group may possibly be attributed to membrane-associated proteins such as glycosyltransferases (GTF) in addition to the inhibition of these proteins.[5] Thus, the decrease in extracellular polysaccharide formation observed after sucrose application may also be related to the inhibition of GTF activity in dental biofilms as was demonstrated by Al-Hebshi et al.[50] after testing a tannin-containing extract. However, the effect of *P. cattleianum* leaf extract on GTF activity should be evaluated in further detail.

Besides the reduction in bacterial viability, the control of biofilm matrix formation found after the use of the extract may also control the pathogenicity of dental biofilm by altering its thickness and porosity.[21,51] In the present study, the positive correlations found between EPS and both TM and TS, which are associated with the reduction on enamel demineralisation found with the extract and active control, demonstrate the importance of controlling not only the presence of microorganisms but also polysaccharide accumulation, which may be an important approach for the prevention of biofilm-associated diseases. The lack of correlation found between MS and the analysed parameters is corroborated by the literature.[33,52]

The numbers of TM, TS, and MS were significantly reduced after the use of the extract compared to the active or negative controls. However, the extract's effect on biofilm ecology should be evaluated in greater detail using molecular methods. The reduction in TM also indicates that beneficial species may also be reduced. This suggests that the extract should be used with caution and should not be incorporated into oral hygiene products on a daily basis. The effect of the extract on beneficial species should be evaluated in further detail.

A higher proportion of MS in biofilm was found in the group treated with the active control followed by the group treated with the extract compared with the water-treated group. Al-Hebshi et al.[53] also found similar selective antimicrobial properties after testing a tannin-containing plant extract. On the other hand, both the extract- and active control-treated biofilms showed lower EPS and lower enamel demineralisation than the water group, suggesting that these solutions are able to reduce the metabolism of MS; this is also evidenced by the lack of correlation between EPS and MS, which means that an increase in MS viability does not necessarily mean an increase in EPS formation. The extract also reduced biofilm acidogenicity compared to the active control. Al-Hebshi et al.[50] previously demonstrated an inhibitory effect on cell metabolism before cell viability is affected in which GTF activity is inhibited with low concentrations of a tannin-containing extract whilst biofilm formation is minimally influenced by such concentrations.

The active control was composed of a combination of purified essential oils and has the American Dental Association seal of approval.[54] It contains cleaning agents, surfactants, and preservatives that synergistically inhibit microorganism growth. In the present study, the extract exhibited either the same (*i.e.*, ΔpH) or better effects (*i.e.*, TM, TS, MS, %ΔSMH, ΔKHN, and EPS) compared to the active control. The fact that the extract used in the present study is a crude concentrate obtained by decoction of the leaves makes the findings even stronger. This may be due to synergistic effects between different compounds found in the plant extract as described previously.[55,56]

In conclusion, the results of the present study show that *P. cattleianum* leaf extract might reduce enamel demineralisation, acidogenic potential, microorganism viability, and extracellular polysaccharide production. However, it is important to note that since this *in situ* study was done with only 10 volunteers, the results should be interpreted with care. Having clearly demonstrated the potential activity of *P. cattleianum* leaf extract to interfere in *in situ* biofilm pathogenicity, the effect of this plant extract on biofilm formation *in vivo* should be evaluated in the future.

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