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Influence of subinhibitory concentrations of extracts from *Psidium cattleianum* (Sabine) and *Myracrodruon urundeuva* (Allemao) on mutans streptococci adhesion to glass and enamel surfaces

Influência de concentrações subinibitórias de extratos de *Psidium cattleianum* (Sabine) e *Myracrodruon urundeuva* (Allemao) sobre a adesão de Streptococcus mutans ao vidro e superfície de esmalte

Influencia de extractos concentraciones subinibitórias de *Psidium cattleianum* (Sabine) y *Myracrodruon urundeuva* (Allemão) sobre la adherencia de *Streptococcus mutans* al vidrio y la superficie del esmalte

Karina Gerhardt **BIANCO**[1]
Luis Fernando **LANDUCCI**[1]
Christiane Marie **SCHWEITZER**[2]
Ellen Cristina **GAETTI-JARDIM**[3]
Elerson **GAETTI-JARDIM JÚNIOR**[1]

[1]*PhD. Department of Pathology and Clinical Propedeutics, School of Dentistry,São Paulo State University (UNESP), Araçatuba, SP, Brazil*
[2]*PhD. Department of Mathematics, School of Engineering, São Paulo State University (UNESP), Ilha Solteira, SP, Brazil*
[3]*PhD. Department of Oral and Maxillofacial Surgery, School of Dentistry, Federal University of Mato Grosso do Sul (UFMS), Campo Grande, MS, Brazil*

Abstract

This study evaluated the influence of subinhibitory concentrations of extracts from *Myracrodruon urundeuva* and *Psidium cattleianum* on the adhesion of cariogenic cocci to the surface of glass and bovine enamel. The aqueous extracts were prepared from leaves by heating in deionized water and sterilized by filtration (22µm). The minimum inhibitory concentration (MIC) of the extracts on *S. mutans* ATCC 1910 and ATCC 35688 was determined by the broth dilution method. In the tests of adhesion to glass surfaces, glass tubes were treated with saliva and sucrose and the bacterial strains were cultivated in concentrations equivalent to 25% of MIC, which were also maintained in the adhesion assay. After the contact between the cells and the glass, the unbound cocci were removed and, finally, it was performed the removal of the cells adhered to the glass by sonication. The percentage of adhesion was determined by comparing the number of bacteria adhered to the total inoculated bacteria, in the presence and absence of the subinhibitory concentrations of the extracts. For enamel adhesion tests, bovine enamel samples were standardized, polished, treated with saliva and, in the test group, received the bacterial inoculum and 25% of the MIC of the extracts. The enamel blocks were removed and the total of adhered cocci was determined by cultivation on BHI agar supplemented with horse blood in anaerobic conditions for 48h at 37°C. The results were evaluated through analysis of variance (ANOVA). The extracts were effective in inhibiting bacterial adhesion to glass and dental enamel surfaces, although inhibition varied according to bacterial strain and extract, producing from 40 to 60% of inhibition. The results reinforce the possibility of the use of extracts of plants of the Brazilian savanna in the control of cariogenic biofilms.
Descriptors: Bacteria; Prevention & Control; Plant Extracts; Dental Caries.

Resumo

Este estudo avaliou a influência de concentrações subinibitórias dos extratos de *Myracrodruon urundeuva* e *Psidium cattleianum* sobre a adesão de cocos cariogênicos à superfície do vidro e esmalte bovino. Os extratos aquosos foram preparados a partir de folhas, por aquecimento em água deionizada e esterilizados por filtração (22µm). A seguir, a concentração inibitória mínima (CIM) dos extratos sobre *S. mutans* ATCC 1910 e ATCC 35688 foi determinada através do método de diluição da droga em caldo. Nos testes de aderência ao vidro, tubos eram tratados com saliva e sacarose e as cepas bacterianas eram cultivadas em concentrações equivalentes à 25% da CIM, que também eram mantidas no ensaio de adesão. Após o contato entre as células o do vidro, fazia-se a remoção dos cocos não aderidos e, posteriormente, por sonicação, a remoção das células aderidas ao vidro. A percentagem de adesão era determinada comparando-se o número de bactérias aderidas em relação ao total, na presença e ausência das concentrações subinibitórias dos extratos. Para adesão ao esmalte, amostras de esmalte bovino foram padronizadas, polidas, tratadas com saliva e, no grupo teste, receberam o inóculo bacteriano e 25% da CIM dos extratos. A seguir, fazia-se a remoção dos blocos de esmalte e a determinação do total de cocos aderidos por cultivo em ágar BHI sangue, em anaerobiose, por 48h, a 37°C. Os resultados foram avaliados através de análise de variância (ANOVA). Os extratos foram efetivos em inibir a adesão ao vidro e esmalte dentário, embora os valores de inibição tenham variado de acordo com a cepa bacteriana e o extrato, produzindo de 40 a 60% de inibição. Os resultados reforçam a possibilidade da utilização de extratos de plantas da savana brasileira no controle de biofilmes cariogênicos.
Descritores: Bactérias; Prevenção & Controle; Extratos Vegetais; Cárie Dentária.

Resumen

Este estudio evaluó la influencia de las concentraciones sub inhibitorias de los extractos de *Myracrodruon urundeuva* y *Psidium cattleianum* sobre la adhesión de cocos cariogénicos a la superficie del vidrio y esmalte. Los extractos acuosos se prepararon a partir de hojas de las plantas por calentamiento en agua desionizada y se esterilizaron por filtración (22µm). La concentración mínima inhibitoria (MIC) de los extractos de *S. mutans* ATCC 1910 y ATCC 35688 se determinó mediante el método de dilución de fármaco en caldo. En los ensayos de adhesión a vidrio, tubos fueron tratados con la saliva y la sacarosa y las cepas bacterianas fueran cultivadas en concentraciones equivalentes a 25% de la CIM, lo que también se mantiene en el ensayo de adhesión. Después de que el contacto entre las células y el vidrio, hecha por eliminación de cocos libres y, posteriormente, por sonicación, la eliminación de las células adheridas al vidrio. El porcentaje de adhesión se determinó comparando el número de bacterias adheridas al total, en presencia y ausencia de concentraciones subinibitórias de los extractos. Para la adhesión al esmalte, las muestras de esmalte fueran estandarizadas, pulidas, tratadas con la saliva y el grupo de prueba recibió el inóculo y 25% de la MIC de los extractos. Después, se hizo la remoción de las placas de esmalte y la determinación del total de cocos adheridos mediante el cultivo en agar BHI sangre, incubado anaeróbicamente durante 48 horas, a 37 °C. Los resultados fueran evaluados mediante análisis de varianza (ANOVA). Los extractos fueron eficaces en la inhibición de la adhesión al vidrio y el esmalte, aunque los valores de inhibición han variado de acuerdo con la cepa bacteriana y el extracto para producir una inhibición del 40 al 60%. Los resultados refuerzan la posibilidad de utilizar extractos de plantas de la sabana brasileña en el control de las biopelículas cariogénicas.
Descriptores: Bacterias; Prevención & Control; Extractos Vegetales; Caries Dental.

INTRODUCTION

Dental caries still represents a relevant problem of public health, particularly in low-income areas and distant from the developed and industrialized centers[1,2]. In the caries formation, the acidogenic potential of microorganisms, their

adhesion to dental surfaces and production of intra and extracellular polysaccharides play a key role in the etiopathogenesis[3,4]. Moreover, due to the structural complexity of oral biofilm, microbial physiological features in biofilms[5], horizontal and vertical transference of mobile genetic determinants, the susceptibility of antimicrobial agents are very limited[6]. In addition, the benefits of the chemical control of dental biofilm are also transients, particularly in prolonged usage[7] and the advantages of chemical control of biofilm in caries prevention has been under scrutiny and side effects are significant and cannot be neglected[8].

The mutans streptococci are the most relevant bacterial group in the pathogenesis of dental caries in the smooth enamel surfaces and their relevance is particularly prominent in the initial steps of this disease. The salivary levels of these cariogenic cocci can reflect the caries risk of a subject, what facilitates the evaluation of preventive strategies implemented[9,10].

The adhesion of bacterial cells to tooth enamel is fundamental at the beginning of carious process and such adherence and the subsequent formation of microbial biofilm occur in two steps: the first is the reversible adherence to acquired pellicle on enamel, which is composed mainly of proteins derived from saliva, and the second step is the accumulation of these cocci through the production of extra cellular dextran. The interference in one of these steps could prevent the development of the cariogenic biofilm[11-13]. Thus, inhibition of the adhesion of *S. mutans* to enamel surfaces might interfere in the development of a cariogenic biofilm, reducing the potential for dental caries initiation and progression[14].

It was estimated that one-third to one-half of all Americans practice alternative medicine, with use of natural or herbal health care products, especially dentifrices[15]. In Brazil the biodiversity observed in the tropical forests and savannahs offers a wide variety of pharmacological and therapeutic compounds with intense activity on microbial biofilms, but only a small fraction of their pharmacological potential as sources of drugs has been under evaluation[16,17]. Moreover, since universally effective, low cost and accepted therapeutic approaches to control oral biofilm have not been established for low-income rural or urban populations[7], studies to characterize natural products capable to reduce biofilm accumulation or the presence of targeted microbial species must be stimulated[18-20].

From the lists of plants recommended for control and prevention of dental caries, it is evident the relevance of plants of genus *Psidium*[18,19,21-23], which has been used as toothpaste to maintain the oral hygiene. *M. urundeuva* presents chemical composition similar to *P. cattleianum* and is systematically used as anti-ulcerogenic, analgesic and anti-inflammatory[24,25].

In the oral cavity, mouthrinses and other chemical agents are probably maintained at inhibitory concentrations just for short periods due to salivary flux and the mucosa and muscular structures. Then, it is relevant to evaluate the effects of subinhibitory concentrations of antimicrobial agents on microbial virulence, since morphological and physiological features of oral microorganisms suffers several changes in such circumstances.

Thus, the goal of this study was to evaluate the influence of a subinhibitory concentration of extracts from *M. urundeuva* and *P. cattleianum* on *mutans* streptococci adhesion to glass and bovine enamel surfaces.

MATERIAL AND METHOD

○ Preparation of plants extracts

Myracrodruon urundeuva (Allemao) and *Psidium cattleianum* (Sabine) leaf extract were prepared from plants cultivated at School of Dentistry of Araçatuba UNESP in natural conditions, without addition of any chemical compounds (chemical fertilizers, pesticides and insecticides). All the extracts had presented antimicrobial activity on *S. mutans* ATCC 1910 and ATCC 35688 in vitro through the broth dilution method.

Plants were three-times washed in deionized water and allowed to dry in a dark room, initially at room temperature and then at 37°C for 5 days. The extracts were prepared with 25g of plant powder and mixed with 125 ml of deionized water, and then the mixture was kept 100°C for 5 min., at 55°C for 1 h., and at room temperature for 3 days to the extraction of water-soluble active principles. The extracts were sterilized by filtration through of 0.22µm cellulose membranes (Millipore) and allowing water to evaporate at 37°C, with a final concentration of 15 mg/ml[26]. The extracts were prepared immediately before use to avoid oxidation.

○ Microbial strains and antimicrobial susceptibility

The bacteria used in this study were *Streptococcus mutans* ATCC 1910 and ATCC 35688, which were cultivated in tryptic soy broth (Difco, USA) supplemented with de yeast extract (0.5%), under anaerobiosis (90% N_2 + 10% CO_2), at 37°C, for 24 hours.

The antimicrobial susceptibility tests were carried out using the broth dilution method with Mueller-Hinton broth supplemented with yeast extract (0.5%). Plant extracts were added to the culture medium in concentrations that ranged from 0.125 mg/ml to 128 mg/ml. Then, tubes were inoculated, in duplicate, with a final inoculum of 10^5 CFU/tube and anaerobically incubated at 37ºC, for 48 h.

The minimal inhibitory concentration (MIC) was defined as the lowest plant extract concentration that inhibited totally the bacterial growth. MIC of *M. urundeuva* on *S. mutans* ATCC 1910 and *S. mutans* ATCC 35688 was 4mg/ml, while MICs of *P. cattleianum* on these cocci were 4 mg/ml and 2 mg/ml, respectively.

○ Adherence to glass

Initially, reference strains were cultivated in TSB supplemented with yeast extract (0.5%) and the concentration equivalent to 25% of MIC of the plant extract. The subinhibitory concentrations of extracts were also maintained in contact with microorganisms during the tests of microbial adhesion to glass and enamel surfaces.

Bacterial cells were cultivated in trypticase soy broth (Difco, USA), supplemented with yeast extract (0.5%) and sucrose (10%), for 36h. under anaerobiosis and the turbidity adjusted (OD 550 nm) to about 0.156, corresponding to 10^8 CFU/ml. Then bacterial cells were harvested by centrifugation (5000xg, 8 min.) in phosphate buffered solution (PBS) and resuspended in PBS/plant extract association (giving 25% of MIC of tested extract) to contain 10^8 CFU/ml. Sucrose was added to a final concentration of 10%. Bacterial cells cultivated without addition of plant extracts constituted the control group, in which the bacterial suspensions were prepared with PBS and sucrose only.

In order to prepare glass surfaces to adhesion assays, borosilicate glass tubes were coated with 2ml of clarified saliva, for 2 minutes, and then rinsed with sterile deionized water. In the tests, human saliva was collected through the spitting method from a healthy volunteer (caries free, male)

and stored at -20°C. Then, before use saliva was clarified by centrifugation at 3500x *g* for 5 min.[27].

After that, 2 mL of harvested bacterial cells cultivated in subinhibitory concentrations of plant extracts and control cells suspensions were inoculated into the tubes and incubated for 24 h under anaerobiosis at 37°C. The adhesion tests in the control group were performed without subinhibitory concentrations of plant extracts.

The total free and adherent bacterial cells were determined by spectrophotometer at 550 nm. Determination of the total free bacteria was calculated transferring the suspension from the saliva-coated tube (tube 1) into another glass tube to ensure that all the unbound bacterial cells were collected and the turbidity was measured (first reading). Following that, the now emptied tube was washed with another 2 ml of fresh sterile deionized water and the suspension collected into another tube and the turbidity of the bacterial suspension in this tube determined and referred as the second reading. The total unbound cells in each of the tubes corresponded to the sum of the turbidity from the first reading and second reading.

The adherent cells remained attached to the walls of tube 1 and 2 ml of sterile deionized water was added and submitted to sonication for 10 seconds. The turbidity of the suspension was determined and the percentage of bacterial adhesion was indicated by dividing the amount of the bound cells with the amount of cells (total) and multiplied by 100. The percentage of inhibition of bacterial adhesion in the presence of the subinhibitory concentrations of the extracts was calculated from the proportion between the amount of bound cells in the absence of plant extract and the amount of adhered cells in the presence of extract.

The amount of cells adhering to the saliva-coated glass surfaces in the absence of plant extract represented the total and maximum adherence of *S. mutans*, and was regarded as equivalent of 100% of adherence. Each assay was submitted to 10 repetitions.

○ Preparation of enamel blocks to adherence assay of *S.mutans* of enamel glass

Enamel blocks (5x5 mm) were obtained from bovine incisors and sterilized in autoclave, at 121°C, for 15 minutes. Dentine and enamel surfaces were standardized (APL-4 Arotec), obtaining parallel surfaces between enamel and dentine. Followed that, the polishing and cleaning of the enamel surfaces were performed using ultrasound Branson 2210, in deionized water, for 2 min, with filter papers and polishing discs. Enamel blocks were washed in deionized water and submitted again to ultrasound[28,29].

Each block of bovine enamel was immersed in 2 mL of unstimulated whole saliva with agitation for 2 h., at room temperature, and washed 3 times with Tris-buffered saline (TBS) (10 mmol/L of Tris-HCl, 154 mmol/L of NaCl; pH 7.5). For negative controls, the same procedure was carried out with TBS instead of unstimulated whole saliva. Then, the blocks were washed 3 times in TBS, enamel fragments were added to 10^9 bacteria, with agitation (6rpm) for 24 h., at 37°C. In the test group, bacteria were resuspended in PBS/extract association as described above. The enamel blocks were then washed with TBS.

The bacterial cells were solubilized from the enamel blocks by incubation with 300 µL of 8 M urea, 1M NaCl, and 1% SDS as previously described[30], and submitted to serial tenfold dilutions and plated on brain heart infusion agar supplemented with horse blood (5%). Petri dishes were incubated under anaerobiosis at 37°C, for 48 h. Each assay was repeated 10 times.

○ Statistical Analysis

Influence of sub inhibitory concentrations of plant extracts on microbial adhesion to glass and enamel surfaces was evaluated by mean of Kruskal-Wallis analysis of variance (ANOVA). Differences of $p<0.05$ was considered statistically significant.

RESULTS

M. urundeuva and *P. cattleianum*, in subinhibitory concentrations, are capable to significantly interfere with the adhesion of *S. mutans* reference strains on glass and bovine enamel surfaces (Table 1). In relation to strain *S. mutans* ATCC 1910, extracts from leaves of *P. cattleianum* and *M. urundeuva* were equally effective in reducing adherence to glass and enamel surfaces ($p<0.01$).

In relation to *S. mutans* ATCC ATCC 35688, extracts from leaves of *M. urundeuva* was slightly more effective in inhibiting microbial adhesion to glass and enamel ($p<0.01$) in comparison to extract from leaves of *P. cattleianum* ($p=0.01$).

DISCUSSION

The ability to adhere to the acquired pellicle and dental surfaces is a prerequisite for early colonizers of the buccal microbial biofilm[22,31-32]. This adherence is mediated by different types of interactions between microorganisms and their hosts, beginning with more reversible interactions and culminating with the establishment of specific linkage between microbial adhesins and host's receptors[12]. The bacterial adhesion to dental surfaces is a relevant step of biofilm formation and to the maintenance of oral microbial ecology. Moreover, the microbial adhesion is highly influenced by several factors, but in mutans streptococci this phenomenon is mainly related to metabolism of sucrose[31], as well cellular morphology and surface structures, which may be affected by compounds present in leaves of the genus *Psidium*[22,32], particularly rich in polyphenols.

The adherence to glass surfaces is commonly used to evaluate sucrose dependent adhesion mechanisms of *S. mutans*[12,31], due to the production of extracellular glucans, similarly to the mechanism observed in oral biofilm[20,23]. The adherence of both reference strains of *S. mutans* to the glass surface was markedly inhibited by sub-MIC concentrations of the extracts. Limsong et al.[12] reported that the guava extract (*Psidium guajava*) exhibited inhibitory significant effect on adherence of *S. mutans* to glass surface, similar to the results presented in Table 1, and the authors evidenced the adhesion to glass by different strains presented different susceptibilities to inhibition by guava extract, which suggests different mechanisms of microbial adhesion. Since *P. cattleianum* is considered a native and highly invasive species of guava, also named strawberry guava, very similar to *P. guajava* in many morphological and phytochemical aspects, so the phenomenon of interference in the microbial adherence may be similar to that previously reported to guava[12,22,31,32].

The present study evidenced that two plant extracts from plants from Brazilian savannah are capable to inhibit the streptococcal adhesion on glass and enamel surfaces and might play a relevant role in the prevention of adhesion of acidogenic bacteria. Besides antimicrobial activities and antiadhesive properties, these extracts have been used to treat and control infectious and inflammatory pathologies by populations who lived on the border of Amazon forests and in the North-Western Brazilian borders, and some favorable biological properties have been characterized and

described[33-35], although further studies in animal and cellular models are required to confirm and stimulate new clinical trials[36].

Extracts from *P. cattleianum*[38] and *M. urundeuva* present high levels of polyphenols, which are recognized as inhibitors of biofilm[12]. The high concentrations of extracts used in the present investigation in order to inhibit microbial adhesion might be explained by the fact that present investigation used crude extracts in spite of purified preparations[12,13].

Natural products capable of reducing biofilm formation and anti-caries activity seem to act in the synthesis of extracellular polysaccharides[23,38-40], inhibiting the enzyme glycosyltransferase[39], or forming a complex directly involved with the adsorption of oral bacteria to the acquired pellicle, delaying the adhesion of the early colonizers of the oral bacterial biofilm[41]. However, initial data have evidenced that phenols, as tannic acid, are deeply involved in both inhibition of cellular metabolism in cariogenic cocci and in the anti-adhesion properties of these extracts[23].

Since the extracts are capable to act as denaturants, particularly on proteins such as casein or gelatin, it is possible that these products also interfere in the interactions between microbial adhesins and the receptors on the surface of human cells, acquired pellicle, and early settlers involved in the development of aggregation, although the role of aggregation in cariogenicity of *S. mutans* remains to be properly studied [12]. In addition, this ability of *P. cattleianum* and *M. urundeuva* as denaturants of proteins possibly interfere with enzymatic activity of glucosyltransferases at subinhibitory concentrations, and since these proteins constitute relevant virulence determinants of *S. mutans*, furthers studies must be planned to evaluate the influence of such extracts on glucan synthesis and biofilm formation.

CONCLUSION

The results of the present study reinforce that plant extracts of the Brazilian savanna can act reducing *S. mutans* adhesion to smooth surfaces, such as glass and dental enamel.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interests.

CORRESPONDING AUTHOR

Ellen Cristina Gaetti Jardim
ellengaetti@gmail.com

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