UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

JULIANA GONÇALVES PIRES

Effect of *Myracrodruon urundeuva* and *Qualea grandiflora* extracts on viability and activity of microcosm biofilm and prevention of enamel demineralization *in vitro*

Efeito de extratos de *Myracrodruon urundeuva* All. e *Qualea grandiflora* Mart. sobre a viabilidade e atividade de biofilme microcosmo e na prevenção da desmineralização do esmalte *in vitro*

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Thesis presented to the Bauru School of Dentistry of the University of São Paulo to obtain the degree of PhD in Science in the Applied Dental Science Program, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Dr. Ana Carolina Magalhães

Tese apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, área de concentração Estomatologia e Biologia Oral.

Orientadora: Profa. Dra. Ana Carolina Magalhães

Versão Corrigida

BAURU

2018

Pires, Juliana Gonçalves

P666e Effect of *Myracrodruon urundeuva* and *Qualea grandiflora* extracts on viability and activity of microcosm biofilm and prevention of enamel demineralization *in vitro* / Juliana Gonçalves Pires – Bauru, 2018.

132 p. : il. ; 31cm.

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientadora: Prof. Drª Ana Carolina Magalhães

Nota: A versão original desta tese encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

Comitê de Ética em Pesquisa em Seres Humanos e Animais FOB-USP

Protocolo nº CAAE 43948115200005417
Registro nº CEEPA 007/2016
Registro nº CEEPA 008/2017
Data: 29/07/2015, 20/12/2016 e 05/04/2017

FOLHA DE APROVAÇÃO

DEDICATÓRIA

A Deus,

Pela sua bondade indiscutível.

Aos meus pais,

Moacir Duarte Pires e Rosangela Gonçalves Pires,

Pelo amor grandioso, todo apoio, respeito, dedicação e por ensinarem o verdadeiro significado da Família.

Ao meu irmão,

Rafael Gonçalves Pires,

Por todo companheirismo, amor, respeito e por tornar nossas conversas engraçadas e leves.

Aos meus avós,

Mauro Duarte Pires e Teresa Menzatto Pires,

Pessoas especiais com humildade incomparável.

Aos meus avós,

Manoel Jesus Gonçalves (*in memorian*) e Aparecida Leonetti (*in memorian*),

Sinto uma saudade imensa, mas sei que estão zelando por nós.

Aos meus amigos,

Anahi Ricaldes, Daniela Razera, Eduardo Galeskas, Guilherme Fernandes, Paulo Henrique Souza, Leticia Calarga, Karina Tozze, Valéria Guedes, Nadya Melhen, Aline Braga, Daiana Moreli, Vinícius Taioqui e Priscila Salomão.

Verdadeiros amigos que levarei para a vida toda e que sempre terão um espaço em especial no meu coração.

A minha família da Bioquímica e FOB,

Ana Carolina Magalhães, André Luís da Silva, Dalva Ribeiro de Oliveira, Lívia Maria de Melo, Gabriela Neubern, Nádia Amôr, Aline Dionízio, Talita Ventura, Beatriz Souza, Carlos, Even Taira, Luiza, Tatiana Martini, Natália Mello, Tamara, Maria Aparecida, Thelma Silva, Larissa, Sara Zabini, Flávia Levy, Cíntia Tokuhara, Mariana Santesso, Isabela Tomazini, João Paulo Domezi, Adriana Matos, Flávia Amadeu, Mileni Fernandez, Cíntia Souza, Carlos, Zohaib, Polliana Scaffa, Tamara Araújo.

Minha segunda família, por toda paciência e respeito, toda alegria vivida nos dias de laboratório e torcida para os experimentos darem certos.

AGRADECIMENTOS

A Deus,

Só tenho a agradecer, por sempre ter me dado forças nos momentos que achei que iria fraquejar e que não iria conseguir. Por permitir que minha vida seja rodeada de pessoas abençoadas e por eu ter amigos bons.

Aos meus pais **Moacir** e **Rosangela,**

Vocês sempre foram e sempre serão meus exemplos de vida e de pessoas. São meus heróis, amigos e conselheiros. Nossa família já passou por muitas coisas e todas elas fizeram a gente permanecer unidos e fortes. Vocês sempre criaram Rafael e eu com muito amor e carinho, além da vasta educação que nos deram. Tudo isso só me fez tornar uma pessoa madura com muita consciência, respeito ao próximo. Toda essa alegria que carrego dentro de mim, o amor, a paz e sentimento de estar de "bem com a vida" é com certeza devido a vocês. Obrigada por serem tão maravilhosos. O amor e respeito que tenho por vocês é imensurável. Amo vocês!!

Ao meu irmão **Rafael,**

Irmãããõoo, você é a pessoa que mais me faz rir e também a única pessoa que me tira do sério com tanta facilidade, mas você sabe que eu te amo muito!! Obrigada por todo o carinho, amor e respeito que você tem por mim. Obrigada por ser meu companheiro. Lembro de todos os apelidos que colocou em mim e dou risada todas as vezes. Te amo!!

À **Karina Tozze,**

Ka, você é com certeza um dos motivos da minha alegria. Obrigada por ser minha amiga e companheira de todas as horas. Obrigada por ter toda paciência do mundo nos dias que chorei ou que fiquei brava e também por comemorar e sorrir nos dias das vitórias e conquistas. Obrigada por ser essa mulher maravilhosa que você é e por ter aceitado entrar na minha vida.

À **Aline Braga**,

Amiga do céu, quantos perengues passamos, hein?! Mas todos foram superados com muito louvor, alguns foram chorando, outros foram sorrindo. Em todos, no final, estavámos nós lá rindo (as vezes rindo das desgraças, mas rindo!). Todos esses anos vão deixar saudade e vou me recordar só de coisas boas que passamos juntas. Foram muitos dias no fluxo, com muitas placas e as nossas músicas no Fluxo FM, muitas noites no laboratório, pedindo pizza, ou comida japonesa para o jantar. Muitos congressos e histórias para contar e muitos almoços no bandejão. Mas também foram várias conversas, conselhos e opiniões. Além dos trabalhos e perengues passados, foram também muitas jantinhas, algumas baladas (porque não tenho muito pique) e raras caminhadas (porque tenho menos pique ainda). Obrigada por toda paciência e por toda ajuda que me deu em todos esses anos, sem você essas pesquisas não teriam saído com tanta leveza. Nossa amizade levarei por toda a vida!

À **Daiana Moreli**,

Daia, também passamos muitos dias no fluxo, mas eles foram gostosos e divertidos, com várias risadas e conversas. Entrou para o time da Micro, com esse seu jeito todo especial de ser e jeito brava também. Nossa amizade, que começou na FOB, levarei por toda a vida.

Aos **funcionários do Centro Integrado de Pesquisa - CIP**,

Márcia, Rafaela e Marcelo, obrigada por toda ajuda nos experimentos e por serem sempre tão pacientes e prestativos.

As professoras **Profa. Dra. Marília Afonso Rabelo Buzalaf** e **Profa. Dra. Flaviana Bombarda de Andrade**,

Obrigada por compartilharem o conhecimento de vocês, sempre com muita prontidão, carinho, competência e atenção.

À **Dalva Ribeiro de Oliveira,**

Dalva, a sua calma e carinho são contagiosos. Obrigada por toda a dedicação e por toda atenção dada não só para mim, mas para todos os alunos.

Ao **André Silva,**

André, meu amigo! Muito obrigada por toda ajuda que me deu nesses anos todos de FOB. Obrigada por ser prestativo e por sanar todas as minhas dúvidas. Você sempre me recebeu de braços abertos, com um sorriso no rosto, independente da hora ou do lugar que eu te procurava. Sem você os dias de sufoco teriam sido bem piores. Muito obrigada!!

Às secretárias da Pós-graduação **Fátima, Leila, Letícia, Meg e Maristela**,

Ao **Prof. Dr. Rodrigo Cardoso de Oliveira,**

Professor, muito obrigada por ter me concedido os extratos das plantas e por toda paciência e prontidão em me receber.

A **Profa. Dra. Anne L. Dokkedal** e **Leonardo Saldanha,**

Obrigada por terem preparado e cedido todos os extratos utilizados nas pesquisas.

Aos **voluntários** desta pesquisa,

Vocês foram fundamentais para que esse trabalho fosse realizado.

À **Faculdade de Odontologia de Bauru- FOB/USP**, na pessoa da diretora **Prof.ª Drª Maria Aparecida Moreira Machado** e vice-diretor **Carlos Ferreira dos Santos,**
É uma honra realizar minha pós-graduação, em nível de Mestrado e Doutorado, nesta grande instituição, que nos dá total suporte e estrutura indiscutível.

À **Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP),**

Pela concessão da bolsa de Iniciação Científica à aluna *Sara Salustiano Zabini*, relacionada ao 3º artigo desta tese. Esta experiência foi importante para meu aprimoramento profissional e pessoal (Processo 2016/20212-0)

À **Sara Zabini,**

Sara, você chegou tímida, mas logo consegui conquistar sua confiança e entrar um pouquinho na sua vida! Você se mostrou uma pessoa muito responsável, prestativa, esforçada. Uma aluna de Iniciação Científica rara! Agradeço por toda ajuda que me deu, mesmo nos momentos de correria. Obrigada!

À **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES),**

Pela concessão da minha bolsa de Doutorado, a qual foi importante para meu aprimoramento profissional e pessoal.

À **Profa. Dra. Ana Carolina Magalhães**

Carol, agradeço por você ter sempre me recebido tão bem e agradeço pela oportunidade que me deu, sempre confiando no meu trabalho, me abrindo todas as portas, me passando confiança e me deixando voar. Sou muito grata! Te admiro muito como profissional e como pessoa e espero ter um dia pelo menos um pouquinho de toda a sua didática. Você sempre foi muito receptiva com todos os alunos, independente do que está fazendo, você pára e ajuda quem precisa. E dar conta de tudo que você dá, não é para qualquer pessoa não! Porque além de professora e orientadora, você também é mãe, esposa e filha. Te admiro demais por você ser essa super mulher! Pode ter certeza que termino meu doutorado com muitas lições e com um grande conhecimento. A aluna que entrou, não é a mesma que está saindo. Sei que ainda tenho muito a aprender, mas tenho certeza que nesses anos de doutorado, aprendi com a melhor. Muito obrigada!

"A tarefa não é tanto ver aquilo que ninguém viu, mas pensar o que ninguém ainda pensou sobre aquilo que todo mundo vê"

Arthur Schopenhauer

ABSTRACT

Effect of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. extracts on viability and activity of microcosm biofilm and prevention of enamel demineralization *in vitro*

The objective of this study was to evaluate the antimicrobial and anti-caries effects of two plant extracts. The first chapter dealt with a review of the literature whose objective was to discuss the antimicrobial potential of Brazilian natural agents on the biofilm related to dental caries and gingivitis/periodontal disease. The research of the articles was carried out using PubMed. We found a total of 23 papers. Most of the studies were performed using planktonic microorganisms or under clinical trials. Nineteen articles were focused on cariogenic bacteria. From these nineteen articles, eleven were also about periodontopathogenic bacteria. Four studies addressed only periodontopathogenic bacteria. The most tested Brazilian natural agents were green propolis, essential oils of *Lippia sidoides* and *Copaifera* sp. Most of the tested agents showed similar results when compared to positive control (essential oils and extracts) or better effect than negative control (green propolis). More studies involving protocols closer to the clinical condition and the use of response variables that allows understanding the mechanism of action of natural agents are necessary before the incorporation of these natural agents into dental products. The second chapter aimed to test the effect of the hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves on the viability of the microcosm biofilm and on the prevention of enamel demineralization. The microcosm biofilm was produced on bovine enamel, using human saliva pool mixed with McBain saliva (0.2% sucrose) for 14 days. The biofilm was treated daily with the extracts for 1 min. *M. urundeuva* at 100, 10 and 0.1 µg/ml and *Q. grandiflora* at 100 and 0.1 µg/ml reduced cell viability similarly to the positive control and significantly more than negative control. *M. urundeuva* at 1000, 100 and 0.1 µg/ml were able to reduce the counting formation unit-CFU counting of lactobacilli sp. and *Streptococcus mutans*, while *Q. grandiflora* at 1000 and 1.0 µg/ml significantly reduced the *S. mutans* CFU counting. On the other hand, the natural extracts did not reduce the production of extracellular polyssacharides, lactic acid and the development of enamel caries lesions. The third

chapter aimed to evaluate the effect of hydroalcoholic extracts of *M. urundeuva* and *Q. grandiflora* (alone or combined) on the viability of *S. mutans* biofilm and the prevention of enamel demineralization. *S. mutans* strain (ATCC 21175) was reactivated in BHI broth. Minimum inhibitory concentration, minimum bactericidal concentration, minimum biofilm inhibitory concentration and minimum biofilm eradication concentration were determined to choose the concentrations to be tested under the biofilm model. *S. mutans* biofilm ($5x10^5$ CFU/ml) was produced on bovine enamel using McBain saliva with 0.2% sucrose for 3 days. The biofilm was treated daily with the extracts for 1 min. *M. urundeuva* (isolated or combined) at concentrations equal or higher than 0.625 mg/ml was able to reduce the bacteria viability, whereas *Q. grandiflora* extract alone showed antimicrobial effect at 5 mg/ml only ($p<0.05$). On the other hand, none of the extracts was able to reduce the development of enamel caries lesions. Despite the tested natural extracts have antimicrobial effect; they are unable to prevent caries in enamel.

Keywords: Antimicrobial agents; dental biofilm; enamel caries; microcosm biofilm; oral disease; phytotherapy.

RESUMO

Avaliação de extratos de *Myracrodruon urundeuva* All. e *Qualea grandiflora* Mart. sobre a viabilidade e atividade de biofilme microcosmo e na prevenção da desmineralização do esmalte *in vitro*

O objetivo foi avaliar os efeitos antimicrobiano e anti-cárie de dois extratos de plantas. O primeiro capítulo se referiu a uma revisão da literatura cujo objetivo foi discutir o potencial antimicrobiano dos agentes naturais brasileiros sobre o biofilme relacionado à cárie dentária e à gengivite/doença periodontal. A pesquisa dos artigos foi realizada usando o PubMed. Foram encontrados 23 trabalhos. A maioria dos estudos foi realizada utilizando microorganismos na fase planctônica ou ensaios clínicos. Dezenove artigos foram focados em bactérias cariogênicas. Dos dezenove artigos, onze também eram sobre bactérias periodontopatogênicas. Quatro estudos abordaram apenas bactérias periodontopatogênicas. Os agentes naturais brasileiros mais testados foram própolis verde, óleos essenciais de *Lippia sidoides* e *Copaifera* sp. Os agentes testados apresentaram resultados similares quando comparados ao controle positivo (óleos essenciais e extratos) ou melhor efeito que o controle negativo (própolis verde). Mais estudos próximos da condição clínica e o uso de variáveis de resposta que permitam entender o mecanismo de ação são necessários, para permitir a incorporação desses agentes naturais em produtos odontológicos. O segundo capítulo teve como objetivo testar o efeito dos extratos hidroalcoólicos de *Myracrodruon urundeuva* All. e *Qualea grandiflora* Mart. sobre a viabilidade do biofilme microcosmo e na prevenção da desmineralização do esmalte. O biofilme microcosmo foi produzido em esmalte bovino, utilizando pool de saliva humana misturada à saliva de McBain (0,2% de sacarose) durante 14 dias. O biofilme foi tratado diariamente com os extratos durante 1 min. *M. urundeuva* a 100, 10 e 0,1 µg/ml e *Q. grandiflora* a 100 e 0,1 µg/ml reduziram a viabilidade dos microrganismos de forma semelhante ao controle positivo e significativamente maior do que o controle negativo. *M. urundeuva* a 1000, 100 e 0,1 µg/ml foi capaz de reduzir a contagem de Unidade formadora de colônia-UFC para Lactobacilos totais e *Streptococcus mutans*, enquanto a *Q. grandiflora* a 1000 e 1,0 µg/ml reduziu significativamente a contagem de UFC para *S. mutans*. Os extratos naturais não conseguiram reduzir a produção de polissacarídeos extracelulares-PEC, ácido lático

e o desenvolvimento da lesão cariosa em esmalte. O terceiro capítulo teve como objetivo avaliar o efeito dos extratos hidroalcoólicos de *M. urundeuva*. e *Q. grandiflora* (sozinhos ou combinados) sobre a viabilidade do biofilme de *S. mutans* e na prevenção da desmineralização do esmalte. Cepa de *S. mutans* (ATCC 21175) foi reativada em caldo BHI. Concentração inibitória mínima, concentração bactericida mínima, concentração inibitória mínima de biofilme e concentração de erradicação mínima de biofilme foram determinadas para escolher as concentrações a serem testadas sob o modelo de biofilme. O biofilme de *S. mutans* ($5x10^5$ CFU/ml) foi produzido em esmalte bovino, utilizando saliva de McBain com 0,2% de sacarose durante 3 dias. O biofilme foi tratado diariamente com os extratos durante 1 min. *M. urundeuva* (isolada ou combinada) nas concentrações iguais ou superiores a 0,625 mg/ml foi capaz de reduzir a viabilidade das bactérias, enquanto que o extrato da *Q. grandflora* apresentou efeito antimicrobiano somente a 5 mg/ml ($p<0,05$). Nenhum dos extratos reduziu o desenvolvimento da lesão da cárie. Apesar dos extratos naturais terem efeito antimicrobiano, são incapazes de prevenir o desenvolvimento da lesão cariosa em esmalte.

Palavras-chave: Agentes antimicrobianos; biofilme dental; biofilme microcosmo; cárie dentária; doenças orais; fitoterapia.

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1-Introduction

1 INTRODUCTION

Dental caries is a tooth disease related to the presence of supragingival biofilm, whose metabolism is dependent on the frequency of exposure to sugar from diet, especially sucrose (ERIKSSON et al., 2017). It is one of the most important oral health problems (PETERSEN, 2003), since 60-90% of school age children and adults have dental caries experience wordwile (PETERSEN et al., 2005). The first sign of the disease is the appearance of a white-spot lesion in enamel, which may progress to cavitation and reach dentin according to the severity of the acid challenges (CAVALCANTI et al., 2014; FERNANDEZ; TENUTA; CURY, 2016). When caries affects dentin, it can cause negative impact in quality of life due to the consequences such as pain, lack of appetite, weight loss and high cost for treatment (ABANTO et al., 2011; RAMOS-JORGE et al., 2015).

The human oral cavity has more than hundreds microbial species and only some of them take part of dental biofilm (PALMER, 2010). Among the microorganisms present in biofilm, the most known ones are *Streptococcus mutans*, *Streptococcus mitis*, *Streptococcus salivarius*, *Lactobacillus*, *Veillonella*, *Actinomyces*, bifidobacteria and fungi (TAKAHASHI; WASHIO; MAYANAGI, 2010; KOO; FALSETTA; KLEIN, 2013; ABUSLEME et al., 2013). The cariogenic microorganisms have the ability to metabolize sugar, especially sucrose from the diet, producing extracellular polysaccharides (EPS) and acids that alter the biofilm pH, inducing tooth demineralization (KEYES, 1960; MARSH; MOTER; DEVINE, 2011; PITTS et al., 2017). Besides, they are able to survive in acidic environment (TAKAHASHI; NYVAD, 2011; ZHAO et al. 2014).

Streptococcus mutans is considered one of the most important microorganisms involved in the etiology of dental caries (MARSH, 2003). They are known to be acidogenic and aciduric bacteria and highly producers of EPS, which are glycan responsible for the development and protection of dental biofilm (KOO; FALSETTA; KLEIN, 2013; ZHAO et al., 2014).

In order to control dental biofilm and avoid the disease, mechanical practice of brushing and reduction of sugar consume are advised (RUGG-GUNN, 2013). However, for patients under high risk for caries, chemical agents may be needed as co-adjuvant (JAMES et al., 2017). Chlorhexidine (CHX) is known in dentistry as a

gold standard antimicrobial agent (JONES, 1997; PARWANI et al., 2013), however, it may induce undesired side effects (JAMES et al., 2017; ZHENG; WANG, 2011) such as tooth staining, supragingival calculus formation, changes in taste perception, parotid gland swelling and irritation of the oral mucosa, when applied for periods higher than 2 weeks (JAMES et al., 2017). Therefore, inhibition of cariogenic bacteria growth by alternative antimicrobial agents has been extensively investigated in an attempt to obtain an agent with effectiveness and with low incidence of side effects. Accordingly, public and private research institutes are engaged in testing the effect of plant extracts and natural compounds against dental pathogens (PATRA et al., 2014).

Many people believe in the effectiveness of herbal medicines; it is estimated that most population has applied plants or natural agents as the sole source for the treatment of various health problems (WHO, 1998) including oral diseases (PALOMBO, 2011). In dentistry, the phytotherapy is a subject of growing interest (BAKRI; DOUGLAS, 2005; FANI; KOHANTEB; DAYAGHI, 2007). The use of medicinal plants is dated in manuscripts since 1.500 years B.C (BETTEGA et al., 2011). Civilizations throughout history have used plants or parts of plants (roots, stems, leaves and/or bark) to treat toothache, gingivitis, dental abscesses and mouth sores (HENLEY-SMITH; BOTHA; LALL, 2013).

There are about 500.000 species of plants available wordwile, but only 1% has been investigated as phytochemical (PALOMBO, 2011). Brazil houses a larger number of plant species (32,086 native Angiosperms and 23 native Gymnosperms) (ZAPPI et al., 2015) than any other country in the world (MITTERMEIER et al., 2005; FORZZA et al., 2012), allocated mainly in Cerrado and Atlantic Rainforest (FORZZA et al., 2012).

Myracrodruon urundeuva All. (Anacardiaceae Family) and *Qualea grandiflora* Mart. (Vochysiaceae Family) are examples of plants from Cerrado. *M. urundeuva* has antimicrobial (MONTANARI et al., 2012) including against cariogenic bacteria (ALVES et al., 2009; MENEZES et al., 2010), analgesic, hepatoprotective, antidiarrheal, colonic anastomotic wound healing, anti-ulcerogenic effects as well as protective effect on the gastric mucosa (CARLINI et al., 2010). *Q. grandiflora* exhibits anti-ulcerogenic action from the ethanol extract of its bark (HIRUMA-LIMA et al.,

2006). Besides, the ethanolic extract of the leaves has antioxidant effect (SOUSA et al., 2007), analgesic, anticonvulsive potential (GASPI et al., 2006) and antibacterial action (MOURA; NASCIMENTO; PINTO, 2012). However, its antimicrobial effect against cariogenic bacteria is unknown. Furthermore, no information about the anti-caries effect of both plants is available in the literature.

Considering the search for natural agents able to prevent oral diseases and the high prevalence of dental caries in specific populations that are under unfavorable social-economic conditions (MARCENES et al., 2013), this study was divided in three parts. The first chapter is reffered to a review of literature with the aim to discuss the antimicrobial potential of different Brazilian plants with respect to the control of dental caries and periodontal disease. In the second chapter, the antimicrobial and anti-caries effects of the hydroalcoholic extracts of *M. urundeuva* All. and *Q. grandiflora* Mart. leaves were tested under microcosm biofilm (second chapter), while in the third chapter their effects were tested under *S. mutans* biofilm model.

2-Article I

2 ARTICLE I – Review of literature

Article under review in Oral Health & Preventive Dentistry (ANNEX 1).

Brazilian natural antimicrobial agents on caries and periodontitis-related biofilm: a literature review

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Abstract

Purpose: This review aims to discuss the antimicrobial potential of Brazilian natural agents on the biofilm related to tooth decay and gingivitis/periodontal disease.
Methods: The survey was conducted using PubMed for the selection of papers published in English, in journals with impact factor greater than 0.8.
Results: We found twenty-three articles, which tested numerous Brazilian plants, essential oils, propolis or crude extracts. The majority of studies were conducted using planktonic microorganisms or under clinical trial. Nineteen articles were focused on cariogenic bacteria; from these nineteen articles, eleven were also about periodontopathogenic bacteria. Four studies addressed only periodontopathogenic bacteria. The most tested Brazilian natural agents were green propolis, essential oils of *Lippia sidoides* and *Copaifera* sp. Most of the tested agents showed similar results when compared to positive control (essential oils and extracts) or better effect than negative control (green propolis).
Conclusions: More studies involving protocols closer to the clinical condition and using response variables that allow understanding the mechanism of action of the natural agents are needed, to thus allow the incorporation of these natural agents in dental products.

Key words: Antimicrobials; Brazilian natural agents; dental biofilm; dental caries; periodontitis.

Introduction

Worldwide there are about 500.000 species of plants available, but only 1% has been investigated as phytochemical[49]. Brazil houses a larger number of plant species (32,086 native Angiosperms and 23 native Gymnosperms)[69] than any other country in the world[24,40].

Many people believe in the effectiveness of herbal medicines and it is estimated that most population use these plants as the sole source for the treatment of various health problems[47] including oral diseases[49]. In dentistry, the phytotherapy is a study object of growing interest[4,22,27]. Civilizations throughout history have used

plants or parts of plants, such as fresh or dried roots, stems, leaves and/or bark, to treat toothache, gingivitis, dental abscesses and mouth sores[28].

Dental caries and periodontal disease are among the most important oral health problems in the world[54]. Accordingly, 60-90% of school age children and adults have dental caries experience and 5-15% of adults have tooth loss due to periodontitis[53].

Both oral diseases are caused by bacteria. Approximately more than hundreds bacterial species from the oral cavity have been isolated and named and some of them can be organized in supragingival or subgingival biofilm[48]. Dental biofilm is found naturally in health, but in disease there is a shift in its composition inducing damage of the tissues (periodontal inflammation, gingivitis and tooth decay)[39].

The global demand for alternative preventive and treatment measurements of oral diseases with safe, efficiency and low cost has allowed the development of phytotherapy for caries and periodontitis control[11]. Accordingly, plants with medicinal importance have been included as antimicrobial agents into toothpaste, capsules, tablets, gels and ointments[2,21,49].

Therefore, the aim of this review was to highlight the studies about Brazilian natural agents used as antimicrobial agent to promote oral health, under laboratorial and clinical models, focused in the prevention of dental caries and gingivitis/periodontal diseases.

2. METHODS

For the selection, the papers should have been written in English language and published in journals with impact factor higher than 0.8. The search was made using PubMed website, and the key-words applied for the search were: Brazilian plants extracts, Brazilian natural antimicrobials agents, oral biofilm, dental caries, periodontal diseases, gingivitis and oral bacteria. All selected articles involved *in vivo*, *in situ* or *in vitro* (mono or multispecies biofilm or planktonic bacteria) models (Table 1).

3. RESULTS

Twenty-three papers were select for this review. All the selected articles were related with the use of Brazilian native plants against bacteria involved in oral diseases (dental caries, gingivitis and/or periodontitis). Nineteen articles were

focused on cariogenic bacteria, in which one addressed *in situ*[12], five *in vivo*[33,35,41,42,44], eleven *in vitro* models[6-8,12,15,20,25,29,60,61,64] and two studies were *in vitro*/*in vivo*[32,63]. From these nineteen articles, eleven also studied periodontal bacteria, in which four addressed *in vivo*[35,42,44], five *in vitro*[7,14,25,29,64] and two *in vitro*/*in vivo* models[32,63]. Four studies addressed only periodontopathogenic bacteria: three using *in vivo*[51,52,55] and one using *in vitro* models[68].

3.1 Control of dental caries and gingivitis/periodontal disease

Microbial communities in biofilms have been found to be 1000 times more resistant to antibiotics compared to their equivalents planktonic cultures[19,59]. Accordingly, great efforts have been done to find alternative treatments. Several herbal medicines have been tested to treat or prevent oral diseases that are linked with oral pathogens[9,44,51].

The development of dental caries involves gram-positive, acidogenic and aciduric bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Lactobacillus* sp.[36], which accumulate in dental biofilm and grow under high sugar exposure, resulting in tooth demineralization[38]. Periodontitis it is gum infection that damages the soft tissue and the bone that supports the tooth. It involves subgingival biofilm rich in anaerobic gram-negative bacteria including *Aggregatibacter actinomycetemcomitans, Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythia*, *Enterococcus faecalis* and *Treponema denticola*[1]. Gingivitis is a mild and reversible form of periodontal disease (bleeding of gum tissue), while periodontitis causes permanent damage of tooth-supporting tissues and may lead to tooth loss (loss of collagen, tooth insertion and bone mass)[10].

Oral problems can contribute to the development of other serious diseases and, therefore, their prevention is a subject of growing interest. Recent researches suggest that periodontal disease may contribute to the development of heart disease and endanger patients with diabetes, respiratory disease and osteoporosis[5,46].

Nowadays, the demand for plants and extracts that are able to control the pathogens *Porfiromonas gingivalis* and *S. mutans*, are growing exponentially[18,29,50], since the indiscriminate use of antibiotics has led the emergence of multi-resistant pathogens[23,56]. Additionally, the conventional antimicrobial agents (ampicillin, chlorhexidine, sanguinarine, metronidazole, phenolic antiseptics, and quaternary

ammonium-antiseptics) can alter oral microbiota and cause undesirable side effects such as vomiting, diarrhea, taste alteration, teeth and restoration staining, increasing calculus formation and disruption of the oral and intestinal microbiota[26,67]. Chlorhexidine, for example, may cause teeth stain, astringent sensation, changes in taste and mucosal desquamation after 2 weeks of use[23,56]. Therefore, scientists are shifting their attention to folk medicine, in order to find new and better anti-plaque agents against oral microbial infections[28].

3.2 Brazilian plant species used in oral health

Brazil is the country harboring the highest plant diversity, including two (Cerrado and Atlantic Rainforest) of the 34-recognized global hotspots[24,40]. The majority of Brazilian plant species are terrestrial, ranging from 83.7% in the Amazon Rainforest through to 75.6% in the Atlantic Rainforest. The Brazilian states with the largest number of Angiosperm species are: Bahia (1,284 species), Minas Gerais (849 species), Amazonas (733 species), São Paulo (604 species), Rio de Janeiro (586 species), Pará (652 species) and Paraná (629 species). In respect to Gymnosperms, the highest species number is found in the Atlantic Rainforest (15,001 native species), Cerrado (12,097 total species), Amazon Rainforest (11,896 total species), Caatinga (4,657 total species), Pampa (1,685 total species) and Pantanal (1,277 total species)[69].

In their review, Chinsembu[16] documented the potential use of plant extracts and other natural products (poly-herbal and plant compounds) in oral health according to the country (Africa, Asia, Brazil, Mexico, Europe, and the Middle East). In respect to Brazil, the most known natural agents are propolis (green propolis), essential oils (*Lippia sidoides, Mammea americana,* etc*)*, crude extracts (*Schinus terebinthifolius* and *Croton urucurana*), organic extracts (*Cordia* sp., *Psychotria* sp., etc), hydroalcoholic (*Copaifera trapezifolia*, etc) and aqueous extracts (*Psidium cattleianum*), among others.

3.2.1 Propolis

Propolis is a resinous product collected by honeybees from various plant sources[31]. Its composition varies according to the geographic region[37]. The color of propolis (green, red, brown, or almost black) also varies depending on the plants from which the resinous substance is collected[34]. The green propolis, for example, is

derived mainly from *Baccharis dracunculifolia* and is highly applied as antibacterial[30], antifungal[57], antiviral[58], anti-inflammatory[62] and antitumor[65] agent. With all these benefits, green propolis has been incorporated into oral care products (toothpastes, mouthwashes, and prophylactic gels) and tested in dentistry[63,66].

The ethanolic extract of green propolis was tested against *S. mutans*, *Streptococcus sanguinis*, *Staphylococcus aureus*, *Lactobacillus acidophilus*, *P. gingivalis*, *A. actinomycetemcomitans*, *Candida albicans in planktonic phase*[63] and *S. mutans* biofilm[15]. *S. mutans* and *S. sanguinis* were more sensitive than *L. acidophilus* to 50 mg/ml of the extract and *P. gingivalis* and *A. actinomycetemcomitans* were the most resistant species[63]. Cardoso et al[15] determined MIC (2.08 mg/ml) and MBC (8.33 mg/ml) of green propolis for *S. mutans.* The *S. mutans* CFU for green propolis was statistically lower than the negative controls; however, in respect to enamel microhardness, green propolis did not differ from controls[15].

Skaba et al[63] also tested toothpaste containing 3% ethanolic extract of propolis in patients with and without periodontitis, while similar toothpaste was also tested in patients with nonsyndromic complete unilateral or bilateral cleft lip and palate treated with fixed appliances[35] and patients who underwent implant-prosthetic therapy[41]. All authors presented the same research method, in which they evaluated the Approximate Plaque Index (API), Oral Hygiene Index (OHI), Sulcus Blood Index (SBI) or Gingival Index (GI), and isolated and identified, through kits, bacteria from biofilm. In general, API, OHI and SBI/GI values decreased for propolis-treated patients when compared to control. The number of identified strains from the gingival sulcus also decreased for propolis-treated patients[63]. *Streptococcus* spp. and *Neisseria* spp. were the most found bacteria. On the other hand, *Actinomyces israelii*, *Capnocytophaga* spp., *Fusobacterium*, *Bacteroides* and *Eubacterium,* the pathogenic species for periodontal tissue, were found for both groups at the baseline[35]. The number of *C. albicans* did not change with the treatment. The patients who used propolis toothpaste had lower levels of *Actinomyces* spp., *A. israelii* and *Capnocytophaga* spp. at the final compared to the baseline analysis. There was a 10% decrease in *A. israelii* level accompanied by an increase in *Actinomyces* spp. among the control patients[35]. In the study of Morawiec et al[41], the number of microorganisms isolated from dental implants after the use of toothpaste with propolis increased. With bacterial identification, the researchers observed that the

pathogenic bacteria were eliminated, resulting in benefic changes of the microorganisms.

Two years later, Morawiec et al[42], tested gel containing 3% ethanolic propolis extract in patients under postoperative process of oral soft tissue and observed the elimination of *Streptococcus acidominimus*, *Streptococcus oralis*, *Staphylococcus epidermidis*, *Veillonella parvula*, *Bifidobacterium breve* and *L. acidophilus* of mucosal surface of the region that underwent the surgery. In 2016, Niedzielska et al[44], tested the same gel in patients with mandible fractures. They isolated and identified the microorganisms and evaluated the values of API, OHI and SBI. The authors obtained significant reduction of API, OHI and SBI and the elimination of the bacteria *Clostridium perfringens*, *Actinomyces naeslundii*, *Prevotella bivia*, *B. breve* and *S. epidermidis*.

Considering the above studies, we infer that propolis (especially the green one) may be incorporated into toothpaste, gels, mouthwashes and other dental products as promising agent to control oral microorganisms; however, we have few information about its effect on the prevention of tooth decay and the reduction of bone loss under long-term clinical trials, highlighting the need of further studies.

3.2.2 Essential oils

Essential oils (EO) are complex mixtures of low molecular weight compounds extracted in various ways (steam distillation, hydrodistillation or solvent extraction)[43]. They are applied in health, agriculture, cosmetic and food industries, because they are known to have antimicrobial, antiviral, antimutagenic, anticancer, antioxidant, anti-inflammatory, immunomodulatory and antiprotozoal activities[3].

Essential oil of *Lippia sidoides* has been extensively studied, as they present phenolic compounds (carvacrol and thymol) that have antimicrobial activities against yeasts and bacteria[45]. Through a clinical trial, da Silva Pereira et al[17], and Lobo et al[33], evaluated the activities of this essential oil. The EO (10%) was incorporated into the gel and applied 3 times a day for 90 days in healthy volunteers. In one of the cited studies, the authors evaluated Bleeding index (BI) and Plaque index (PI) and, as a result, they observed that the volunteers who applied the gel with essential oil had a significant reduction in BI and PI when compared to the negative control (placebo gel) and similar results when compared to the positive control (2% chlorhexidine gel)[17]. Lobo et al[33], also tested the EO of *Lippia sidoidos*. The

treatment was focused on dental caries in children (aged 6-12 years) and the essential oil was incorporated into toothpaste (1.4%), gel (1.4%) and mouthwash (0.8%). The researchers observed that only the toothpaste showed a protective result, significantly reducing *S. mutans* from the saliva during the first 5 days of use. This result was maintained throughout the study and did not return to the baseline values during the follows up (30, 60, 180 and 365 days). This essential oil was also tested against *E. faecalis* at concentrations of 2.5% and 10% *in vitro*[68]. The biofilm was formed for 3 days in nitrocellulose membrane filters and then, dipped in the treatments for 30 or 60 minutes. *E. faecalis* counts were significantly reduced when the essential oil (2.5%) was compared to the negative control and thymol (positive control). The same results were found for the concentration of 10%[68].

The concentrations of the constituents present in the plants vary according to the season of the year in which they are collected (dry or rainy) and the extracted parts (stem, root, leaves, fruits, bark). These differences were well noted by Furtado et al[25], when authors analyzed the essential oil of leaf and stem bark of *Inga laurina* (dry and rainy season). MIC values obtained were similar for leaf and stem bark (rainy season) with respect to aerobic bacteria (*S. mutans*, *S. sobrinus*, *S. sanguinis*, *S. salivarius* and *S. mitis*, the values ranged from 25 to 50 µg/ml). MIC values increased when essential oils of leaf and stem bark were tested against anaerobic bacteria (*A. naeslundii*, *P. gingivalis*, *Prevotella nigrescens*, *Bacteroides fragilis* and *F. nucleatum*), regardless of the season (50 to> 400 µg/ml). MIC values were higher for anaerobic compared to aerobic bacteria during dry than rainy season (100 to 200 µg/ml). Regarding toxicity, Furtado et al[25] applied VERO cells and observed that the essential oils had low cytotoxic activity at concentrations that were able to inhibit bacterial growth.

EO and ethanolic extract of the fruits of *Mammea americana* were evaluated on *S. mutans* and *P. gingivalis.* For both bacteria, the MIC values for EO were lower when compared to the ethanolic extract. MBC was found only for *S. mutans* and its value was lower for EO than ethanolic extract[29]. Sousa et al[64], tested the EO and the fractions of *Eugenia calycina* Cambess against cariogenic bacteria (*S. mutans*, *S. sanguinis* and *S. sobrinus*) and periodontopathogenic bacteria (*P. nigrescens*, *P. gingivalis*, *A naeslundii* and *B. fragilis*). The lowest MIC values found for cariogenic bacteria were 100 to 200 µg/ml for *S. mutans* treated with fractions F2 to F4. *P. nigrescens* and *P. gingivalis* were more sensitive to the plant, showing MIC values

varying from 50 to 100 µg/ml (EO and fractions). They also tested the cytotoxic potential of the plant and showed low effect on HeLa cells.

Pimentel et al[55], induced experimental periodontitis in Wistar rats and tested the effect of EO of *Cordia verbenacea*. The animals were treated 3 times daily for 11 days. The authors observed that the animals treated had lower loss of alveolar bone, lower IL-1α concentration and higher IL-10 levels compared to the control (without treatment). *A. actinomycetemcomitans* (7% treated rats x 36% control rats) and *P. gingivalis* (13.5% treated rats x 60% control rats) were found in higher percentage in control rats compared to the treated ones. In 2015, Pedrazzi et al[51], formulated a mouthwash containing 0.04% of essential oil and 0.16% of hydroethanolic extract of *Baccharis dracunculifolia* DC and compared them with mouthwash without active component, Plax (Colgate™), and Listerine (Johnson & Johnson™). The volunteers applied the mouthwashes 4 times a day for 4 months. All volunteers completed the study showing similar reduction in dental biofilm among them compared to the baseline. No treatment was able to improve the effect.

3.2.3 Crude, hydroalcoholic, aqueous extracts, fractions and derivatives

Psidium cattleianum, a Brazilian native plant, in previous studies showed activity against oral bacteria[13], however, only in 2012[12], an *in situ* study was carried out, in which the effect of aqueous extract of leaves (100 g/600 ml) was tested against the formation of biofilm on bovine enamel for 14 days. The authors observed that the extract reduced enamel demineralization, acidogenic potential, microorganism viability, and extracellular polysaccharide production.

Silva et al[61], tested the hydroalcoholic extracts of *Acanthospermum hispidum* DC., *Annona coriacea* L., *Schinopsis brasiliensis* Engl., *Ximenia americana* L. and *Hibiscus mutabilis* Briq. (10%, 20%, 30%, 50% and 70%) against *Pseudomonas aeruginosa*, *S. mutans*, *S. salivarius*, *S. oralis*, *Lactobacillus casei*, *E. faecalis*, *S. aureus*, *C. albicans*, *C. tropicalis* and *C. krusei*. The extract of *S. brasiliensis* Engl. inhibited the growth of *S. oralis* and *S. aureus* when compared to chlorhexidine and the MIC values were 0.004 µl/µl (*P. aeruginosa*), 1000 µl/µl (*E. faecalis*), 0.063 µl/µl (*S. aureus*) and 0.500 µl/µl (*S. oralis*). For the extract of *X. americana* L., the MIC was only found for *S. aureus* (0.063 µl/µl). The other extracts had no antimicrobial effect.

The susceptibility of bacteria and fungi (*S. aureus*, *S. epidermidis, S. mutans*, *C. albicans*, *C. tropicalis* and *C. glabrata)* to the extracts of *Equisetum arvense* L., *Glycyrrhiza glabra* L., *Punica granatum* L. and *Stryphnodendron barbatimam* Mart. were evaluated[20]. The authors demonstrated that all extracts possessed bactericidal activity (MBC) against all bacteria; however, the values ranged from 3.13 to 100 mg/ml. There was an increase in IL-1β production in cultures treated with *P. granatum* L. and *S. barbatimam* Mart.; while no difference was found between the cultures treated with the extracts *E. arvense* L. and *G. glabra* L. with respect to the production of TNF-α. The complete inhibition of TNF-α occurred for culture treated with the extract of *P. granatum* L. With respect to cytotoxicity, *G. glabra L.* and *E. arvense L*. were the least and the most cytotoxic agent against mouse macrophage.

In 2014, some aqueous and organic extracts of aerial organs of *Cordia* sp., *Psychotria* sp., *Cordia nodosa* Lam., *Solanum* sp., *Ipomea alba* L., *Casearia javitensis* Kunth, *Dioscorea altissima* Lam., leaves of *Casearia spruceana Benth.* Ex Eichler, *Symphonia globulifera* L.f., *Moronobea coccinea* Aubl., stem of *Zanthoxylum* sp., *Psychotria* sp., *Annona hypogauca* Mart., *Cordia cf.* exaltata, *Gnetum leyboldii* Tul. and flowers of *Moronobea coccinea* Aubl, were evaluated (CFU) against *S. mutans* biofilm cultivated on hydroxyapatite for four days. Only *Dioscorea altissima* and *Annona hypoglaucase* showed to be able to reduce the number of *S. mutans* [8]. In the same year, Silva et al[60], evaluated similar extracts (aerial organs of *Cordia* sp., *Psychotria* sp., *Cordia nodosa* Lam., *Solanum cf. lanceifolium*, *Ipomea alba* L., *Casearia javitensis*, *Smilax sp.,* leaves of *Casearia spruceana Benth.* Ex Eichler, *Moronobea coccinea* Aubl. and stem of *Zanthoxylum compactum*, *Diospyros guianensis, Psychotria* sp., *Annona hypogauca* Mart., *Cordia cf.* exaltata, *Annona hypoglauca, Cordia* sp.) against *S. mutans* in their planktonic form. *Casearia spruceana* and *Psychotria* sp. showed significant activity against *S. mutans* and *Ipomoea alba* L. presented the lowest MIC and MBC values.

Barbieri et al[6], tested methanol fraction, ethyl acetate-methanol fraction and crude methanol extract from the leaves of *S. terebinthifolius* and stem bark of *C. urucurana* against *S. mutans* and *C. albicans*. The researchers diluted the fractions in two different solutions: hydroalcoholic solution-HA (50%) and DMSO solution (10%). The crude extract of *S. terebinthifolius* showed an adherence inhibitory activity against *C. albicans* with a reduction in biofilm formation about 29% using HA and 14% using DMSO. The ethyl acetate–methanol extract and the methanol extract fractions in HA

showed *C. albicans* biofilm reduction of 49% and 47%, respectively. The crude extract of *C. urucurana* (HA) reduced the *C. albicans* biofilm in 35%, while its methanolic fraction (HA) reduced in 46%. With respect to *S. mutans*, the crude extract of *S. terebinthifolius* (HA) showed to reduce biofilm formation in 44%, while the methanolic fraction reduced biofilm formation in 41%. The crude extract of *C. urucurana* (HA) reduced *S. mutans* biofilm formation in 16%, while its methanolic and ethyl acetate–methanol fractions reduced *S. mutans* biofilm in 17% and 34%, respectively. The best solvent for the tested plants was HA. Furthermore, the fractions had antimicrobial effect similar or higher than the crude extract of the tested plants.

Brighenti et al[14], tested different ways for extraction of 10 plants (*Jatropha weddelliana*, *Attalea phalerata*, *Buchenavia tomentosa*, *Croton doctoris*, *Mouriri elliptica*, *Mascagnia benthamiana*, *Senna aculeate, Unonopsis guatterioides*, *Allagoptera leucocalyx* and *Bactris glaucescens*) as following: 70° ethanol 72h/25°C (A), water 5min/100°C (B), water 1h/55°C (C), water 72h/25°C (D), hexane 72h/25°C (E) and 90° ethanol 72h/25°C (F). They observed that only *A. naeslundii* and *S. mitis* were susceptible to the agents by using agar diffusion. Among the tested plants, the only one that showed lower MIC and MBC activity against all microorganisms was *Croton doctoris* (extraction A, E and F). The cytotoxicity of the hydroalcoholic extract of *Croton doctoris* was tested on human epithelial cells, and the results showed no cytotoxic potential at MIC values.

Two species of *Copaifera* sp. were studied against oral bacteria. Bardají et al[7], tested *Copaifera reticulata* (oleoresin) against oral bacteria (11 ATCC and 8 clinical isolated). Lower MIC and MBC values were found against *P. gingivalis* and lower MICB (minimal inhibitory concentration against biofilm) were found against *S. mutans*, *P. gingivalis* and *F. nucleatum*. *S. mitis* and *F. nucleatum* had the best time-kill after 4 h. Cytotoxic effect against human lung fibroblasts was found at concentrations higher than 39 µg/ml[31]. In the same year, Leandro et al[32], tested the hydroalcoholic extract of the leaves of another species, *Copaifera trapezifolia*. MIC and MBC values ranged from 100 to 400 µg/ml for all types of bacteria tested (11 ATCC and 14 clinical isolated). The authors also tested the mutagenic potential of the extract in Swiss mice and cytotoxicity against fibroblasts, showing no dangerous potential at the concentrations lower than 156.2 µg/ml (fibroblasts).

4. DISCUSSION

There are a lot of studies on the use of Brazilian natural agents in dentistry. In general, the effect found is positive against cariogenic and periodontopathogenic bacteria compared to negative control (green propolis) or similar to positive controls (essential oils and extracts). However, the focus of the most studies was the antimicrobial effect rather than the gingival status or caries prevention.

It is also important to keep in mind that there is a wide variety of ways of acquiring the natural agents (essential oils, propolis, crude extracts) varying according to season and the collected parts of the plants (leaves, root) that may contain different fractions of active components (polyphenols, terpene, monoterpene, dimeric chalcones and carvacrol) with antibacterial potential. This review shows a lot of *in vitro* studies and few clinical trials, being most of them about bacteria involved in periodontitis/gingivitis. There is a lack of clinical trials on caries prevention. Unfortunately, the *in vitro* studies are still done using bacteria in planktonic phase and not under more complex environment, as biofilm.

Clinical studies are able to provide information about the periodontal tissues responses, the anti-inflammatory and antimicrobial effects and the prevention of caries lesions by the natural agents. However, before *in vivo* studies, there is a need to ascertain whether natural agents have cytotoxic effects and few of the cited studies (26.10%) attempted to test both antimicrobial and cytotoxic effects.

Finally, studies on this topic should be stimulated, since Brazil is a country of great biodiversity, suggesting a leadership opportunity for the production of new medicines and oral hygiene products from natural sources. The Brazilian plants have awakened interest not only of Brazilian, but also of researchers worldwide, as shown in this review where 26.10% of publications have been produced abroad Brazil.

However, even with the increase in research about Phytotherapy for Dentistry in recent years, we still need to identify new Brazilian species and their fractions and to perform characterization of chemical compounds that have antimicrobial activity on oral pathogens, as well as, to apply better laboratory models to study them.

5. CONCLUSIONS

More studies involving protocols closer to the clinical condition and using response variables that allow understanding the mechanism of action of the natural

agents are needed, to thus allow the incorporation of these natural agents in dental products.

ACKNOWLEDGEMENTS

The authors thank CAPES for the concession of scholarship to the first author. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Table 1. Effect of different Brazilian natural agents against cariogenic and periodontopatogenic bacteria

Authors/Year	Type of medicinal herb	Oral Microorganism	Experimental design	Main results
Brighenti et al, 2012[12]	- Leaves of *Psidium cattleianum* (aqueous extract; 167 mg/ml) NC: deionized water PC: commercial mouthwash	Palatal device (*in situ* biofilm)	CFU, SMH, KHN and EPS analyses	- The numbers of total microorganism, total streptococci and mutans streptococci were reduced when compared with PC or NC. - Decrease in % ΔSMH and ΔKHN when compared with PC or NC. - Decreased the EPS when compared with PC or NC.
Pimentel et al, 2012[55]	- Leaves and stems of *Cordia verbenacea* (essential oil; 5 mg/Kg body wt) NC: non-treatment group	Periodontitis model in rats (*in vivo* biofilm)	Alveolar bone loss, microbiological PCR and cytokine levels	- Significantly lower alveolar bone loss values, lower levels of IL-1α and higher levels of IL-10 in tissues of treated-animals compared to control. - *T. forsythia* was not detected in any ligature biofilm in both groups. In treated group, *A. actinomycetemcomitans* was found in 7.1% (36% control), and *P. gingivalis* detection was 13.5% (60% control).
Silva et al, 2012[61]	- Entire plant of *A. hispidum* DC. - Stem-bark of *A. coriacea* L., *S. brasiliensis* Engl. and *X. americana* L. - Leaves of *H. mutabilis* Briq. (hydroalcoholic extract 10%, 20%, 30%, 50% and 70%; 200 mg/ml) NC: ethanol/water PC: chlorhexidine	*P. aeruginosa* *S. mutans* *S. salivarius* *S. oralis* *L. casei* *E. faecalis* *S. aureus* *C. albicans* *C. tropicalis* *C. krusei* (*in vitro* – planktonic)	MIC and agar diffusion methods	- *S. brasiliensis* Engl. (10%, 20% and 30%) inhibited *S. oralis* growth when compared with PC. - *S. brasiliensis* Engl. (10%, 20%, 30% and 50%) inhibited *S. aureus* growth when compared with PC. - MIC value (*S. brasiliensis* Engl.) against *S. oralis* was 500 µl/µl, against *S. aureus* was 0.063 µl/µl, against *E. faecalis* was 1000 µl/µl and against *P. aeruginosa* was 0.004 µl/µl. - MIC values (*X. americana* L) against *S. aureus* was 0.063 µl/µl and against *E. faecalis* was 1000 µl/µl. - For the others bacteria/fungi, the extracts had

	(bacteria); nystatin (Candida)			no effect.
Machorowska-Pieniazek et al, 2013[35]	- Green propolis (3% Toothpaste) NC: toothpaste placebo	Clinical trial (*in vivo* biofilm)	API, OHI, GI and Isolation and identification of microorganism (gram positive and gram negative, facultative anaerobic and anaerobic bacteria)	- No influence on API. - Decrease of OPI and GI in treated-patients compared to untreated. - Reduction of 15% of *Streptococcus* spp. when compared to NC. - Lower levels of *Actinomyces* spp., *Actinomyces israelii* and *Capnocytophaga* spp. at final analysis compared to the baseline for propolis toothpaste.
Morawiec et al, 2013[41]	- Green propolis (3% Toothpaste) NC: toothpaste placebo	Clinical trial (*in vivo* biofilm)	API, OHI, SBI and Isolation and identification of microorganism (gram positive and gram negative, facultative anaerobic and anaerobic bacteria)	- Decrease of API, OHI, and SBI at final analysis compared to the baseline for propolis toothpaste. - The use of the tested toothpaste increased the number of isolated of 16 for 32; however, the pathogenic bacteria were reduced and the benefic bacteria were increased.
de Oliveira et al, 2013[20]	- *E. arvense* L. - *G. glabra* L. - *P. granatum* L. - *S. barbatimam* Mart. (propylene glycol extract; 0.19 to 100 mg/mL) NC: cell not treated	*S. aureus* *S. epidermidis* *S. mutans* *C. albicans* *C. tropicalis* *C. glabrata* (*in vitro* – planktonic) Cell: Mouse macrophages	MBC, cytotoxicity assay and cytokine levels	- All extracts had a bactericidal effect. - *G. glabra* extract exhibited the least cytotoxicity (100 mg/ml) when compared with NC. - E. arvense extract was the most cytotoxic (50 mg/ml) when compared with NC. - Increase in IL-1β production in cultures treated with *P. granatum* L. and *S. barbatimam* Mart. compared to NC. - Complete inhibition of TNF-α occurred for culture treated with the extract of *P. granatum* L. when compared to NC.
da Silva Pereira	- Leaves of *Lippia*	Clinical trial	BI and PI	- The gel obtained a good acceptance.

et al, 2013[17]	*sidoides* (essencial oil; 10% gel) NC: placebo gel PC: chlorhexidine gel	(*in vivo* biofilm)		- Significant reduction of PI and BI scores in treated (CHX and extract) compared to NC, but no difference was found between the PC and extract groups.
Skaba et al, 2013[63]	- Green propolis (3% Toothpaste and solutions in triptonic water at 10 mg/L, 20 mg/L, and 50 mg/L) NC: toothpaste placebo	Clinical trial (*in vivo* biofilm) *S. mutans* *S. sanguinis* *L. acidophilus* *P. gingivalis* *A. actinomycetemcomitans* (*in vitro* planktonic phase)	API, OHI, SBI (*in vivo*) and CFU (*in vitro*)	- Decrease of API, OHI, and SBI values was observed for patients treated with propolis after the first week. - Concentrations of 20 and 50 mg/l were better that 10 mg/l. - The use of toothpaste removed dental plaque and improved the state of marginal periodontium after the first week. - *S. mutans* and *S. sanguinis* were more sensitive than L. acidophilus to 50 mg/ml. - After 2h, *P. gingivalis* and *A. actinomycetemcomitans* decreases for $3x10^4$ (initial value: $6x10^4$ CFU).
Barnabé et al, 2014[8]	- Aerial organs of *Cordia* sp., *Psychotria* sp., *Cordia nodosa* Lam., *Solanum* sp., *Ipomea alba* L., *Casearia javitensis* Kunth, *Dioscorea altissima* Lam. - Leaves of *Casearia spruceana Benth.* Ex Eichler, *Symphonia globulifera* L.f., *Moronobea coccinea* Aubl. - Stem of *Zanthoxylum* sp., *Psychotria* sp., *Annona hypogauca* Mart., *Cordia cf.* exaltata, *Gnetum leyboldii* Tul.	*S. mutans* (*in vitro* biofilm)	CFU	- *Dioscorea altissima* and *Annona hypogauca* showed to be able to reduce the number of *S. mutans* compared to NC and PC, respectively.

	- Flowers of *Moronobea coccinea* Aubl. (organic or aqueous extract; 0.16 mg/ml, 0.3 mg/ml or 5 mg/ml) NC: Saline solution PC: chlorhexidine (0.12%, 1.0% and 2.0%) and Periogard®			
Barbieri et al, 2014[6]	- Leaves of *S. terebinthifolius* - Stem bark of *C. urucurana* (crude methanol extract, methanol fraction (F-E) and ethyl acetate–methanol fraction (F-D); in 50% hydroalcoholic solution (HA) or 10% DMSO solution; 0.003 to 8 mg/ml) NC: no treatment PC: chlorhexidine (0.12%)	*S. mutans* *C. albicans* (*in vitro* planktonic and biofilm models)	MICA and CFU	- *S. mutans* biofilms adherence was better inhibited by the *S. terebinthifolius* (HA) crude extract (44%) and F-E fraction (41%). - The crude extract of *C. urucurana* extract (HA) had anti-adherent activity on *S. mutans* (16%). The F-E fraction reduced the biofilm in 17% and F-D fraction in 34%. - The inhibition of *S. terebinthifolius* fractions F-E and F-D (HA) on C. albicans were 47% and 49%, respectively. - The inhibition for *C. urucurana* crude extract and F-E fractions (HA) on *C. albicans* were 35% and 46%, respectively.
Brighenti et al, 2014[14]	- Leaves of *Jatropha weddelliana*, *Attalea phalerata*, *Buchenavia tomentosa*, *Croton doctoris*, *Mouriri elliptica*, *Mascagnia benthamiana*, *Senna aculeate, Unonopsis guatterioides*, *Allagoptera leucocalyx* and *Bactris glaucescens*	*A. naeslundii* *L. acidophilus* *S. gordonii* *S. mutans* *S. sanguinis* *S. sobrinus* *S. mitis* (*in vitro* planktonic phase) Human epithelial cells from oral mucosa	Agar diffusion method, MIC, MBC and Cytotoxicity assay	- Extraction methods A and B produced antimicrobial activity for all tested plants against at least one microorganism (agar diffusion). - Only extract of *C. doctoris* showed the lowest MIC (<0.1 µg/ml up to 25 mg/ml) and MBC (<0.1 µg/ml up to >50 mg/ml) against cariogenic bacteria. - Only extract of *C. doctoris* showed the lowest MIC (3.1 µg/ml to 0.1 mg/ml) and MBC (3.1 µg/ml to 0.2 mg/ml) against periodontal bacteria.

		(A- 70° ethanol 72h/25°C, B- water 5min/100°C, C - water 1h/55°C, D - water 72h/25°C, E - hexane 72h/25°C and F - 90° ethanol 72h/25°C; 100–50 µg/ml) NC: DMSO PC: chlorhexidine (1.2 mg/ml)			- *A. naeslundii* was the most susceptible bacteria (agar diffusion) to all extracts and CHX.
Furtado et al, 2014[25]	-	Leaves and stem bark of *Inga laurina* (dry and rainy season) (Essential oil; 400 to 12.5 µg/ml) NC: no treated PC: chlorhexidine (0.0115 µg/ml to 5.9 µg/ml)	*A. naeslundii* *P. gingivalis* *P. nigrescens* *B. fragilis* *F. nucleatum* *S. mutans* *S. sobrinus* *S. sanguinis* *S. salivarius* *S. mitis* (*in vitro* planktonic phase) Cell: Vero	MIC and Cytotoxic assay	- For cariogenic bacteria, the MIC of dry season ranged between 100 to 200 µg/ml and rainy season ranged between 25 to 50 µg/ml. - For periodontal bacteria, the MIC of dry season ranged between 100 to >400 µg/ml and rainy season ranged between 50 to >400 µg/ml. - The oils have low toxicity at concentrations that inhibited microbial growth.
Herrera Herrera et al, 2014[29]	-	Fruits of *Mammea americana* (essential oil and the ethanolic extract; 500 to 0.06 µg/ml) NC: no treated PC: gentamicin (16 µg/ml)	*S. mutans* *P. gingivalis* *(in vitro* planktonic phase)	MIC and MBC	- For *S. mutans* , the MIC of the essential oil was 15.6 µg/ml and its MBC was 125-500 µg/ml and MIC of the ethanolic extract was 62.5 µg/ml and its MBC was 250-500 µg/ml. - For *P. gingivalis,* the MIC of the essential oil was 250 µg/ml and MIC of the ethanolic extract was 500 µg/ml. The authors did not find MBC values against *P. gingivalis*.
Lobo et al, 2014[33]	-	Leaves of *Lippia sidoides* Cham.	*S. mutans* (*In vi*vo planktonic phase)	CFU	- The mouthwash and gel formulations did not significantly reduce salivary *S. mutans* levels

	(Essential oil; 1.4% toothpaste and gel and 0.8% mouthwash) PC: chlorhexidine gel and mouthwash			in children. - The toothpaste treatment successfully reduced salivary *S. mutans* after 5 days of treatment. - CHX gel and mouthwash treatment demonstrated a higher efficacy in *S. mutans* reduction after 5 days of treatment, but the values returned to baseline on day 30 and remained so until the end of the study.
Silva et al, 2014[60]	- Aerial organs of *Cordia* sp., *Psychotria* sp., *Cordia nodosa* Lam., *Solanum cf. lanceifolium*, *Ipomea alba* L., *Casearia javitensis*, *Smilax sp.* - Leaves of *Casearia spruceana Benth.* Ex Eichler, *Moronobea coccinea* Aubl. - Stem of *Zanthoxylum compactum*, *Diospyros guianensis, Psychotria* sp., *Annona hypogauca* Mart., *Cordia cf.* exaltata, *Annona hypoglauca, Cordia* sp. (organic or aqueous extract and $RCHCl_3$, RBuOH and RH_2O fractions; 250 – 0.8 mg/ml) NC: DMSO PC: chlorhexidine (0.12%, 1% and 2%)	*S. mutans* (*In vitro* planktonic phase)	Disk diffusion method, MIC, MBC and antioxidant analysis	- *Casearia spruceana* (organic extract), showed significant activity against *S. mutans* (20.67 mm). - *Psychotria* sp. (organic extract: 15.04 mm and the $RCHCl_3$ fraction: 17.71 mm) showed significant activity against *S. mutans* . - *Ipomoea alba* had the lowest MIC and MBC against *S. mutans* (0.08 mg/ml<MIC<0.16 mg/ml; MBC=0.16 mg/ml). - Chlorhexidine (0.12%, 1% and 2%) had inhibition halo of 10.12, 12.37 and 19.28 mm, respectively.
Veras et al, 2014[68]	- Leaves of *Lippia sidoides* - Thymol (Drug)	*Enterococcus faecalis* (*in vitro* biofilm)	CFU	- CFU counting of *E. faecalis* from biofilms exposed to essential oil and thymol at 2.5% and 10% were significantly reduced

		(essential oil; 2.5% and 10%) NC: DMSO PC: Sodium hypochlorite			compared to NC.
Morawiec et al, 2015[42]	-	Green propolis (3% ethanolic extract gel) NC: gel placebo	Clinical trial (*in vivo* biofilm)	Isolation and identification of microorganism (gram positive and gram negative, facultative anaerobic and anaerobic bacteria)	- *S. acidominimus, S. oralis, S. epidermidis, V. parvula, B. breve and L. acidophilus* were eliminated in patients treated with propolis compared to those from NC.
Pedrazzi et al, 2015[51]	-	*Baccharis dracunculifolia* DC (mouthwash with 0.04% essential oil and 0.16% hydroethanolic extract) NC: placebo mouthwash PC: Plax (Colgate™) and Listerine (Johnson & Johnson™)	Clinical trial (*in vivo* biofilm)	PI	- There were no differences among the groups.
Sousa et al, 2015[64]	-	Leaves of *Eugenia calycina* Cambess (essential oil and fractions (F1 to F4); 400–0.195 µg/ml or 512–0.25 µg/ml) NC: DMSO	*P. nigrescens* *P. gingivalis* *A. naeslundii* *B. fragilis* *S. mutans* *S. mitis* *S. sanguinis* *S. sobrinus* (*in vitro* planktonic	MIC and Cytotoxic assay	- Fractions F3–F4 showed values of MIC ranging between 100 and 200 µg/ml against cariogenic bacteria. - The essential oil showed strong antimicrobial activity against *P. nigrescens* and *P. gingivalis* (100 µg/ml). - Fractions F2–F4 showed values of MIC ranging between 50 and 100 µg/ml against periodontal bacteria.

	PC: Chlorhexidine (0.115 to 5.9 µg/ml)	phase) Cell: HeLa		- CHX showed MIC values ranging between 0.922 to 3.688 µg/ml. - The essential oil and the fractions presented low toxicity on HeLa cells.
Bardají et al, 2016[7]	- *Copaifera reticulate* (Oleoresin; 0.195-400 µg/ml) NC: DMSO PC: Chlorhexidine	*E. faecalis* *P. gingivalis* *P. intermedia* *F. nucleatum* *A. naeslundii* *A. viscosus* *P. buccae* *P. nigrescens* *S. sobrinus* *S. mitis* *S. mutans* *S. sanguinis* *L. casei* *S. salivarius* (*in vitro* planktonic and biofilm models) Cell: human lung fibroblasts	MIC, MBC, MICB, Time-kill assay, Checkerboard Dilution and XTT assay	- For cariogenic bacteria, the MIC and MBC results ranged from 25 to 50 µg/ml and the MICB ranged from 50 to 200 µg/ml. - The best time-kill assay was shown against *S. mitis* and *F. nucleatum* after 4 h (50 µg/ml). - MIC values of *C. reticulate* combined with CHX showed additive effect between both agents against *S. mutans* and *S. mitis.* - For periodontal bacteria, the MIC and MBC results ranged from 6.25 to >400 µg/ml and the MICB ranged from 50 to 100 µg/ml, - The concentrations above 39 µg/ml significantly reduced cell viability compared to NC.
Cardoso et al, 2016[15]	- Green propolis (ethanolic extract; 266.67 to 0.259 mg/ml or 33.33%) NC: ethanol solution (64 to 0.0625%) PC: Chlorhexidine (0.05% and 0.12%)	*S. mutans* (*in vitro* planktonic and biofilm model)	MIC, MBC, HL and CFU	- MIC and MBC values were 2.08 mg/ml, and 8.33 mg/ml, respectively - The hardness loss for green propolis at 33.33% was 84.41% and for 0.12% CHX was 68.44% - The CFU of *S. mutans* treated with green propolis at 33.33% was 7.26 log_{10} and treated with 0.12% CHX was 6.79 log_{10}
Leandro et al, 2016[32]	- Leaves of *Copaifera trapezifolia* Hayne (hydroalcoholic extract; 0.195 to 400 µg/ml) NC: DMSO	*E. faecalis* *P. gingivalis* *P. intermedia* *F. nucleatum* *A. naeslundii* *A. viscosus* *B. fragilis*	MIC, MBC, time-kill assay, MICB, chequerboard assay and cytotoxicity assay	- MIC and MBC results ranged between 200 and >400 µg/ml against cariogenic bacteria. - MIC and MBC results ranged from 100 to >400 µg/ml against periodontal bacteria. - The best time-kill assay was shown against *P. micros* and *P. gingivalis* after 72 h (100 µg/ml).

	PC: Chlorhexidine	*P. micros* *P. nigrescens* *P. buccae* *S. sobrinus* *S. mitis* *S. mutans* *S. sanguinis* *L. casei* *S. salivarius* (*in vitro* and *in vivo* planktonic and biofilm models, respectively) Cell: fibroblasts from the Chinese hamster lung Mice: Male Swiss		- MICB was 200 µg/ml against *P. micros* and *P. gingivalis*. - The extract mixed with chlorhexidine did not present any synergic effect. - The concentrations higher than 156 µg/ml significantly reduced cell viability. - No mutagenicity was found when the tests were conducted in Swiss rats.
Niedzielska et al, 2016[44]	- Green propolis (3% ethanolic extract gel) NC: gel placebo	Clinical trial (*in vivo* biofilm)	API, OHI, SBI and Isolation and identification of microorganism (gram positive and gram negative, facultative anaerobic and anaerobic bacteria)	- Decrease of API, OHI and SBI was seen for treated compared to NC. - The use of propolis gel reduced the isolated microorganisms from 54 to 48 species.

Abbreviations: EPS = extracellular polysaccharides; CFU = colony forming units; MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; API = Approximate Plaque Index; OHI = Oral Hygiene Index; SBI = Sulcus Blood Index; GI = Gingival Index; BI = Bleeding index; PI = Plaque index; MICB = Minimum Inhibitory Concentration of Biofilm; MICA = Minimal concentration of adherence; SMH = Surface microhardness; KHN = Knoop hardness number; NC = Negative control; PC = Positive control; ΔSMH = delta surface microhardness ; ΔKHN = delta knoop; PCR = Polymerase Chain Reaction ; IL-1α = Interleukin-1alfa; IL-10 = Interleukin-10; IL-1β = Interleukin-1beta; TNF-α = Tumor necrosis factor alpha; CHX = Chlorhexidine; HA = Hydroalcoholic solution; DMSO = Dimethyl sulfoxide; XTT = (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide); HL = Hardness

3-Article II

3 ARTICLE II

Article formatted according to the guideline of Fitoterapia (ANNEX 2).

Effect of hydroalcoholic extract of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves on the viability and activity of microcosm biofilm and on enamel demineralization

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ABSTRACT

The objective of the study was to test the effect of the hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves on the viability of the microcosm biofilm and on the prevention of enamel demineralization. Microcosm biofilm was produced on bovine enamel, using inoculum from pooled human saliva mixed with McBain saliva, under 0.2% sucrose exposure, for 14 days. The biofilm was daily treated with the extracts for 1 min. *M. urundeuva* All. at 100, 10 and 0.1 µg/ml and *Q. grandiflora* Mart. at 100 and 0.1 µg/ml reduced cells viability similarly to positive control and significantly more than the negative control. *M. urundeuva* at 1000, 100 and 0.1 µg/ml were able to reduce both lactobacilli and mutans streptococci CFU counting, while *Q. grandiflora* (1000 and 1.0 µg/ml) significantly reduced mutans streptococci CFU counting. On the other hand, the natural extracts were unable to reduce EPS and lactic acid production and the development of dental caries lesion. The extracts showed antimicrobial properties on the microcosm biofilm, however, they had no anti-caries effect.

Key words: Antimicrobial agent; biofilms; microcosm studies; plants.

1. Introduction

Dental caries involves dental biofilm rich in acidogenic and aciduric bacteria such as *Streptococcus mutans, Streptococcus sobrinus*, *Lactobacillus* sp., *Veillonella*, *Actinomyces*, bifidobacteria and fungi [1], which are metabolically active under high sugar exposure, producing acids that induce tooth demineralization [2]. Mechanical disorganization of the biofilm and the rationale sugar consume are key strategies to prevent the disease. Oral mouthrinses containing antimicrobial agents can be applied, as alternative, for patients at high risk for caries [3]. However, their antimicrobial properties may not reflect in anti-caries effect and, additionally, they may induce some side-effects [4]. Therefore, scientists are shifting their attention to folk medicine, in order to find new and better antimicrobials agents against oral diseases as dental caries [5].

Brazil is the country harboring the highest plant diversity, allocated mainly in Cerrado and Atlantic Rainforest [6]. The majority of Brazilian plant species are terrestrial (83.7% in the Amazon Rainforest and 75.6% in the Atlantic Rainforest) [7].

Myracrodruon urundeuva All. (Anacardiaceae) and *Qualea grandiflora* Mart. (Vochysiaceae) are examples of plants from Brazilian Cerrado. *Myracrodruon urundeuva* has antimicrobial [8], analgesic, hepatoprotective, antidiarrheal, colonic anastomotic wound healing, anti-ulcerogenic effects as well as protective effect on the gastric mucosa [9]. *Qualea grandiflora* exhibits anti-ulcerogenic action from the ethanol extract of its bark [10]. Besides, the ethanolic extract of the leaves has antioxidant effect [11], analgesic, anticonvulsive potential [12] and antibacterial action [13]. However, no information about the anti-caries effect of both plants is available in the literature.

Considering the search for natural agents able to prevent diseases and the high prevalence of dental caries in specific populations that are under unfavorable social-economic conditions [14], the objective of our study was to test the effect of the hydroalcoholic extracts of *Myracrodruon urundeuva* and *Qualea grandiflora* leaves on the viability of a microcosm biofilm and on the prevention of enamel demineralization.

2. Material and methods

2.1 Saliva collection

This study was firstly approved by the local Ethical Committee (CEEA 43948115.2.0000.5417). After sign the informed consent, saliva was collected from 2 healthy donors, who followed the inclusion criteria described by Souza et al. [15]. Prior to the day of collection, the donors did not brush their teeth. Furthermore, they were not allowed to ingest food or drinks 2 h prior the saliva collection. The saliva was collected under stimulation by chewing a rubber material for 10 min during the morning. After collection, pool of saliva was diluted in glycerol (70% saliva and 30% glycerol). Aliquots of 1 ml were stored at -80°C [16].

2.2 Plant material and extraction

Leaf samples of *Myracrodruon urundeuva* and *Qualea grandiflora* were collected in October 2013 at Jardim Botânico Municipal de Bauru (Bauru, Brazil), (22°20'41.4"S - 49°01'45.1"W). Exsiccates were deposited in the Herbarium of UNESP under code numbers HRCB59831 and UNBA6034. The collections have authorization issued by SISBIO under code number 39825-1. The hydroalcoholic extracts were prepared as described by Machado et al. [17]. Briefly, fresh leaves were hot air dried at 45°C and grounded in a knife mill. The extract was obtained with 70% ethanol via percolation at room temperature. The filtrates were concentrated under dryness and reduced pressure at 40°C. They were finally lyophilized, weighed and stored in an amber bottle at room temperature.

2.3 Tooth sample preparation and treatment groups

A three hundred and six enamel samples (4 mm x 4 mm) were prepared from bovine teeth, using a semi-precision cutting machine (Buehler, Enfield, USA). The samples were fixed in acrylic discs with wax and polished in a metallographic polishing machine (Arotec, Cotia, Brazil) using water-cooled silicon-carbide discs (600-grade papers ANSI grit; Buehler, Enfield, USA) to remove grooves and to standardize the surface roughness of approximately 0.153 ± 0.037 μm. The average surface roughness (Ra) was assessed using contact profilometer and Mahr Surf XCR 20 software (Mahr, Göttingen, Germany). Two thirds of the enamel surfaces were protected with nail polish to obtain control areas essential for the TMR analysis. The

samples were sterilized using ethylene oxide [Gas exposure time (30% ETO/70%CO_2) for 4 h under a pressure of 0.5 ± 0.1 kgF/cm^2].

Enamel samples were randomly divided in the treatments groups by using as criteria their Ra means, as following: PerioGard® (Colgate, São Bernardo do Campo, Brazil) – Positive control (pH 5.0); 35% ethanol – Negative/vehicle control (pH 5.7); hydroalcoholic extracts from the leaves of *M. urundeuva* at 0.1 (pH 5.7); 1.0 (pH 5.8); 10 (pH 5.2); 100 (pH 5.2) and 1000 µg/ml (pH 4.8) and *Q. grandiflora* at 0.1 (pH 5.3); 1.0 (pH 5.4); 10 (pH 5.1); 100 (pH 4.9) and 1000 µg/ml (pH 4.5).

2.4 Microcosm biofilm formation and treatments

The human saliva was defrosted and mixed with McBain saliva [18] in the proportion of 1:50. McBain saliva contained 2.5 g/l mucin from porcine stomach (type II), 2.0 g/l bacteriological peptone, 2.0 g/l tryptone, 1.0 g/l yeast extract, 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l cysteine hydrochloride, 0.001 g/l hemin, 0.0002 g/l vitamin K1, at pH 7.0 (Sigma-Aldrich, Missouri, USA). The solution of human saliva and McBain saliva was added to each well containing an enamel sample (v=1.5 ml/well, 24-well plates), which were incubated at 5% CO_2 and 37˚C. After 8h, the medium was removed, the enamel samples were washed using phosphate-buffered saline (PBS) and fresh McBain saliva (with 0.2% sucrose) was added into the wells (v=1.5 ml/well). The microplates were incubated at 5% CO_2 and 37˚C for further 16h, completing the first day.

From the 2nd to the 14th day, the samples were treated once a day with the natural agents or controls for 1 min (1 ml/well). Thereafter, the samples were washed using PBS and fresh McBain saliva containing 0.2% sucrose was added. The microplates were incubated at the same conditions described above [19].

2.5 Biofilm viability analysis

The biofilm was stained using the nucleic acid markers diluted in PBS (1 ml PBS + 1 µl SYTO9 + 1 µl propidium iodide, 10 µl/well) (Kit Live & Dead® cells viability assay, Thermo Fisher Scientific, Waltham, USA) for 15 min in a dark environment. Live bacteria were stained with SYTO9 (480/500 nm) producing a green fluorescence and dead lysed bacteria were stained with propidium iodide (605/635 nm) and SYTO9 producing a red fluorescence [20]. The biofilm was examined using confocal laser scanning microscope-CLSM (Leica TCS SPE, Mannheim, Germany) and Leica

Application Suite-Advanced Fluorescence software (LAS AF, Mannheim, Germany). Three images (275 μm^2) were captured from the center area of each sample surface and analyzed using BioImage L 2.0 software, to quantify the live and dead bacteria (%).

2.6 Microorganism viability analysis

For colony-forming unit- CFU counting, 100 µl of the bacterial suspension obtained for EPS analysis was then diluted to 10^{-4} and spread on petri dishes (25 µl/dish) containing two different types of agar: A) SB-20M [21] containing 15 g bacto-casitone (Difco, Detroit, USA), 5 g yeast extract (Kasvi, Curitiba, Brazil), 0.2 g L-Cysteine hydro-chloride (Sigma, Steinheim, Germany), 0.1 g sodium sulfite (Sigma, Steinheim, Germany), 20.0 g sodium acetate (Synth, Diadema, Brazil), 200.0 g coarse granular cane sugar, 15.0 g agar (Kasvi, Curitiba, Brazil), 1 l distilled water and 0.2 U/ml bacitracin (Sigma, Steinheim, Germany) for determination of mutans streptococci (*S. mutans* and *S. sobrinus*); and B) Rogosa (Kasvi, Curitiba, Brazil) supplemented with 0.13% glacial acetic acid to assess the number of lactobacilli [22].

The plates were then incubated at 5% CO_2 and 37 ºC. After 48h, the CFU numbers were counted and transformed in $\log_{10}$ CFU/ml.

2.7 Metabolism analysis:

a) Lactic acid production

For this assay, we only tested the highest and the lowest concentration of each extract. The samples were incubated in buffered peptone water (BPW) (Synth, Diadema, Brazil) supplemented with 0.2% sucrose (1 ml/well) for 3 h, anaerobically, to allow the biofilm produces lactic acid. The anaerobic conditions were obtained using the Whitley A35 Anaerobic Workstation (Don Whitley Scientific, Shipley, UK), maintaining the environment at 80% N_2, 10% CO_2 and 10% H_2 and 37°C.

Lactate concentrations were evidenced by enzymatic method (lactic dehydrogenase method, Boehringer Mannheim, Germany) in the BPW solution according to the manufacture instruction [23]. The absorbance was measured at 340 nm using a microplate reader (Fluorstar Optima- BMG Labtech, Ortenberg, Germany). The values were expressed as mmol lactate/l.

b) Extracellular polysaccharides – EPS quantification

The samples were transferred to microtubes previously weighted containing 1 ml of saline solution (0.89% NaCl) and sonicated for 30 s at 20W (Unique, Indaiatuba, Brazil). The cleaned tooth was removed, the tubes were weighted again and the humidity biofilm weight was calculated from the weight differences.

Four hundred microliters of the saline solution were centrifuged at 10.000 g and 4 °C for 5 min. The supernatants were transferred to other microtube. The microtubes with the sediments were stored for the insoluble EPS analysis. For soluble EPS determination, three volumes of 95% ice-cold ethanol were added into the microtube containing supernatant and stored at -20ºC for 30 min. After precipitation, the microtubes were centrifuged at 10.000 g and 4ºC for 10 min and the supernatant was completely removed. The pellets were resuspended in 1 M NaOH (v = 200 µl) and the total carbohydrates were measured using the phenol-sulphuric acid colorimetric assay. Glucose (mg) curve was done for the EPS quantification. The absorbance was measured at 490 nm using a microplate reader (Fluorstar Optima-BMG Labtech, Ortenberg, Germany).

For the insoluble EPS analysis, the sediments were resuspended in 400 µl of 1 M NaOH, vortexed for 15 s and agitated using a shaker table for 15 min at environment temperature. The samples were then centrifuged at 10.000 g and 4ºC for 5 min. The supernatants were transferred to new microtubes and 3 volumes of 95% ice-cold ethanol were added. The microtubes were stored at -20°C for 30 min. After precipitation, the microtubes were centrifuged at 10.000g and 4ºC for 10 min and the supernatant was completely removed. The remaining pellet in the microtube was resuspended in 1 M NaOH (v = 200 µl). The total carbohydrates were measured as described above [24]. The values for both EPS were expressed as µg EPS/mg (biofilm).

2.8 Transverse microradiography (TMR)

Enamel samples were transversally sectioned and polished to obtain slices with 80-100 µm of thickness. The enamel slices were fixed in a sample-holder together with an aluminum calibration step wedge with 14 steps. Microradiographs were taken using an x-ray generator (Softex, Tokyo, Japan) on the glass plates at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The glass plates were developed

and analyzed using a transmitted light microscope fitted with a 20x objective (Zeiss, Oberkochen, Germany), a CCD camera (Canon, Tokyo, Japan), and a computer. Two images per sample were taken using data-acquisition (version 2012) and interpreted using calculation (version 2006) softwares from Inspektor Research System bv (Amsterdam, The Netherlands). The mineral content was calculated based on the work of Angmar et al. [25], assuming the density of the mineral to be 3.15 kg l^{-1} and 87 vol% of mineral content for the sound enamel. The lesion depth (LD, μm) and the integrated mineral loss (∆Z, %vol. μm) were calculated.

2.9 Statistical Analysis

All experiments were performed in biological triplicate (except the lactate assay, in duplicate) with three data points for each replicate. Data were statistically analyzed using software Graph Pad Instat for Windows (GraphPad Software, San Diego, USA). The normal distribution and homogeneity were checked using Kolmogorov & Smirnov and Bartlett's tests, respectively. The % live and dead microorganisms and ΔZ values were compared using ANOVA and Tukey-Kramer test. For the other analysis (lactic acid and EPS production, CFU counting and LD values), we applied Kruskal-Wallis and Dunn's test. The level of significance was set at 5%.

3. Results

3.1 Biofilm viability

Hydroalcoholic extracts of *M. urundeuva* All. at 100 μg/ml (62.14%), 10 μg/ml (74.59%) and 0.1 μg/ml (59.81%) and *Q. grandiflora* Mart. at 100 μg/ml (67.19%) and 1 μg/ml (64.50%) presented the mean percentage of dead cells similar to the positive control (chlorhexidine, 48.21%), and significantly higher than the negative control (35% ethanol, 33.79%). The other experimental groups did not differ between them and from the positive and negative controls (Figure 1).

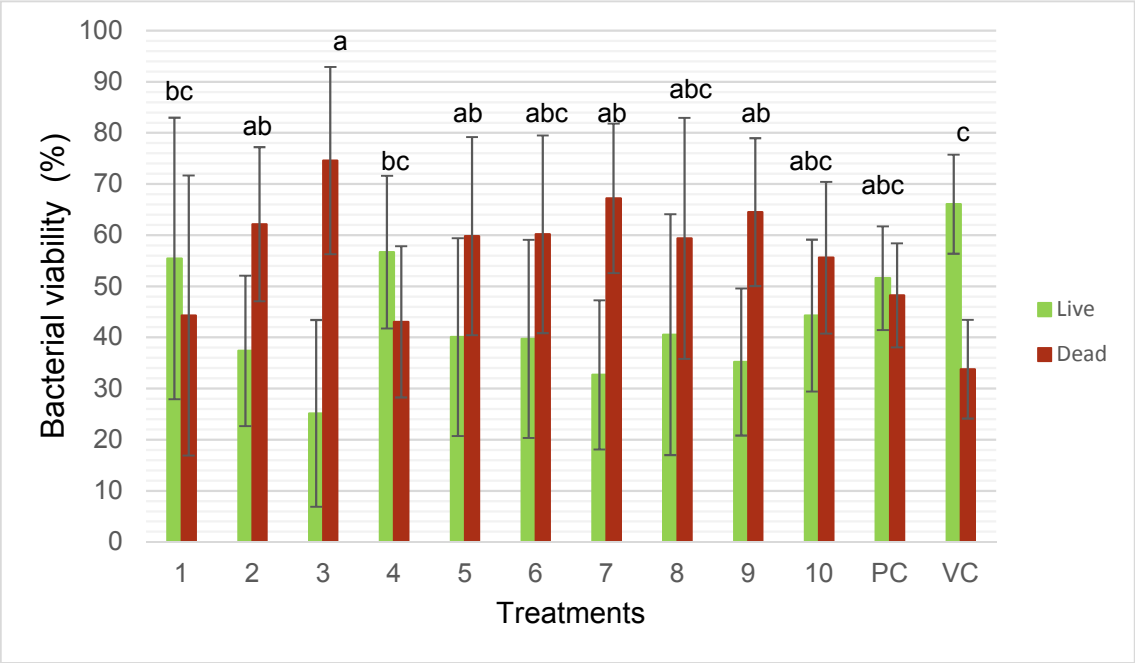

Figure 1. Mean ± SD of the percentage (%) of live (green) and dead (red) microorganisms (viability assay using CLSM) from biofilm treated with hydroalcoholic extracts of *M. urundeuva* All. and *Q. grandiflora* Mart. leaves. 1- 5: *M. urundeuva* from 1000 to 0.1 µg/ml respectively; 6-10: *Q. grandiflora* from 1000 to 0.1 µg/ml respectively; PC: Positive control; VC: Vehicle (negative) control. Different letters show significant differences among the treatments (ANOVA/Tukey-Kramer, $p<0.0001$).

3.2 Microorganism viability

Table 1 shows the CFU counting results. With respect to lactobacilli, only *M. urundeuva* at 1000, 100 and 0.1 µg/ml were able to reduce the CFU counting similarly to positive control and significantly more compared to the negative control. *M. urundeuva* at similar concentrations had the same effect on mutans streptococci. Despite it had no effect on lactobacilli, *Q. grandiflora* at 1000 and 0.1 µg/ml significantly reduced the number of mutans streptococci. Chlorhexidine significantly reduced both microorganisms CFU counting.

Treatments	lactobacilli	mutans streptococci
35% Alcohol (vehicle/negative control)	7.34(0.62)^{c}	7.60(0.61)^{c}
Chlorhexidine (positive control)	6.72(1.09)^{ab}	6.64(1.44)^{ab}
M. urundeuva 1000 µg/ml	6.81(0.54)^{a}	6.75(0.53)^{ab}
M. urundeuva 100 µg/ml	6.78(0.61)^{ab}	6.79(0.90)^{ab}
M. urundeuva 10 µg/ml	7.02(0.60)^{abc}	6.79(0.59)^{abc}
M. urundeuva 1.0 µg/ml	7.51(0.40)^{bc}	7.45(0.75)^{bc}
M. urundeuva 0.1 µg/ml	6.78(0.89)^{a}	6.25(0.54)^{a}
Q. grandiflora 1000 µg/ml	7.19(0.15)^{abc}	6.86(0.97)^{ab}
Q. grandiflora 100 µg/ml	7.43(0.56)^{bc}	7.57(1.06)^{c}
Q. grandiflora 10 µg/ml	6.98(0.52)^{abc}	7.02(1.15)^{abc}
Q. grandiflora 1.0 µg/ml	7.15(0.64)^{abc}	7.16(0.78)^{bc}
Q. grandiflora 0.1 µg/ml	6.88(0.69)^{abc}	6.81(0.51)^{ab}

Table 1. Median (interquartile interval) of CFU counting (log_{10} CFU/ml) for lactobacilli and mutans streptococci. Different letters at the same column show significant differences among the treatments (Kruskal-Wallis/Dunn: $p<0.0001$ for both).

3.3 Metabolism analysis

a) Lactic acid production

None of the extracts were able to significantly reduce lactic acid production compared to the negative control; however, chlorhexidine differed significantly from the negative control (Figure 2).

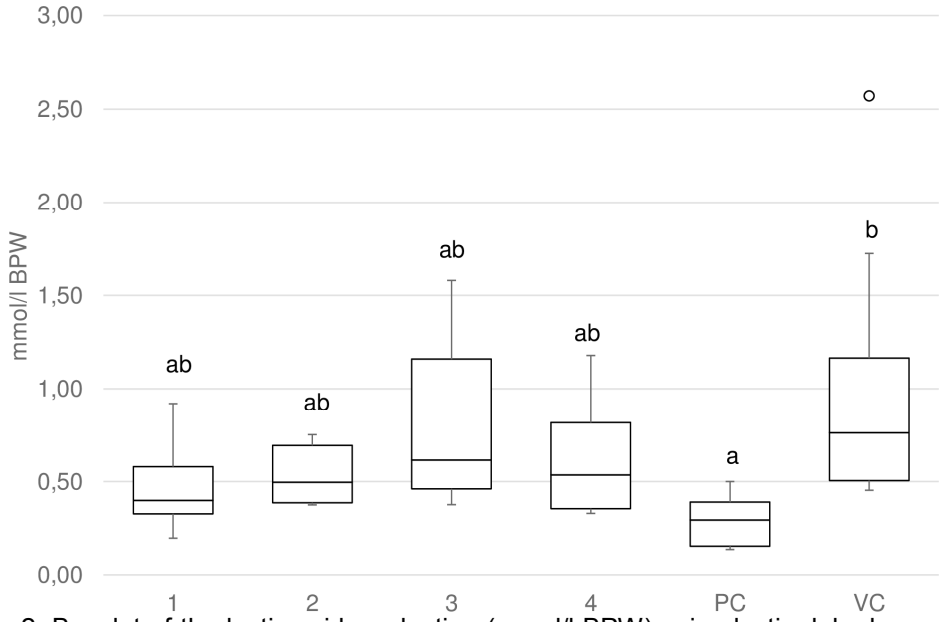

Figure 2. Boxplot of the lactic acid production (mmol/l BPW) using lactic dehydrogenase method. 1-2: *M. urundeuva* at 1000 and 0.1 µg/ml, respectively; 3-4: *Q. grandiflora* at 1000 and 0.1 µg/ml, respectively; PC: Positive control; VC: Vehicle (negative) control; º: Outliers. (Kruskal-Wallis/Dunn, p=0.0121).

b) EPS quantification

Table 2 shows that none of the extracts were able to significantly reduce EPS production compared to the vehicle control, while chlorhexidine significantly reduced the soluble EPS.

Treatments	Soluble EPS (µg/mg)	Insoluble EPS (µg/mg)
35% Alcohol (vehicle/negative control)	0.22(0.06)^{b}	0.32(0.17)^{b}
Chlorhexidine (positive control)	0.07(0.05)^{a}	0.35(0.19)^{ab}
M. urundeuva 1000 µg/ml	0.15(0.10)^{ab}	0.45(0.28)^{ab}
M. urundeuva 100 µg/ml	0.22(0.16)^{ab}	0.79(0.65)^{ab}
M. urundeuva 10 µg/ml	0.12(0.04)^{ab}	0.38(0.24)^{ab}
M. urundeuva 1.0 µg/ml	0.14(0.07)^{ab}	0.59(0.28)^{ab}
M. urundeuva 0.1 µg/ml	0.25(0.15)^{b}	0.50(0.23)^{ab}
Q. grandiflora 1000 µg/ml	0.28(0.13)^{b}	0.87(0.44)^{a}
Q. grandiflora 100 µg/ml	0.16(0.06)^{ab}	0.52(0.19)^{ab}
Q. grandiflora 10 µg/ml	0.17(0.07)^{ab}	0.44(0.33)^{ab}
Q. grandiflora 1.0 µg/ml	0.14(0.08)^{ab}	0.52(0.21)^{ab}
Q. grandiflora 0.1 µg/ml	0.17(0.09)^{ab}	0.74(0.42)^{ab}

Table 2. Median (interquartile interval) of soluble and insoluble EPS (µg/mg biofilm). Different letters at the same column show significant differences among the treatments. Soluble EPS (Kruskal-Wallis/Dunn, $p<0.0001$) and Insoluble EPS (Kruskal-Wallis/Dunn, $p = 0.0082$).

3.4 TMR

No one of the extracts was able to reduce the integrated mineral loss and the lesion depth compared to negative control, while chlorhexidine significantly reduced the enamel caries lesions development (Table 3 and Figure 3). Figure 4 shows TMR pictures of a representative enamel sample from each group.

Treatments	(ΔZ, %vol.µm)
35% Alcohol (vehicle/negative control)	15761.3±3060.2^{bc}
Chlorhexidine (positive control)	11335.0±2832.4^{a}
M. urundeuva 1000 µg/ml	15033.0±3778.2^{bc}
M. urundeuva 100 µg/ml	15384.6±2578.3^{bc}
M. urundeuva 10 µg/ml	13765.0±2524.3^{ab}
M. urundeuva 1.0 µg/ml	17966.5±3010.3^{c}
M. urundeuva 0.1 µg/ml	15791.9±3388.3^{bc}
Q. grandiflora 1000 µg/ml	14522.9±1877.0^{abc}
Q. grandiflora 100 µg/ml	15359.3±2247.7^{bc}
Q. grandiflora 10 µg/ml	15170.6±3117.8^{bc}
Q. grandiflora 1.0 µg/ml	16845.0±3054.2^{bc}
Q. grandiflora 0.1 µg/ml	15645.9±3439.7^{bc}

Table 3. Mean ± SD of the integrated mineral loss (ΔZ, %vol.µm) of the artificial enamel lesions created using microcosm biofilm after applying the tested treatments. Different letters show significant differences among the treatments (ANOVA/Tukey-Kramer, p<0.0001).

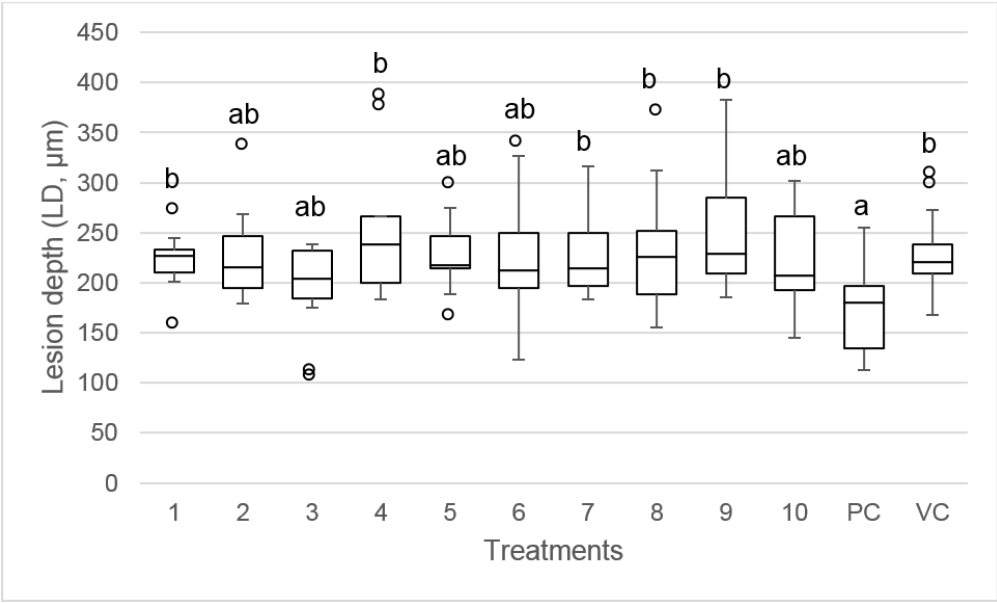

Figure 3. Boxplot of the lesion depth (LD, µm) of the artificial enamel lesions created using microcosm biofilm after applying the tested treatments. 1- 5: *M. urundeuva* from 1000 to 0.1 µg/ml respectively; 6-10: *Q. grandiflora* from 1000 to 0.1 µg/ml respectively; PC: Positive control; VC: Vehicle (negative) control; º: Outliers. Different letters show significant differences among the treatments (Kruskal-Wallis/Dunn, p=0.0012).

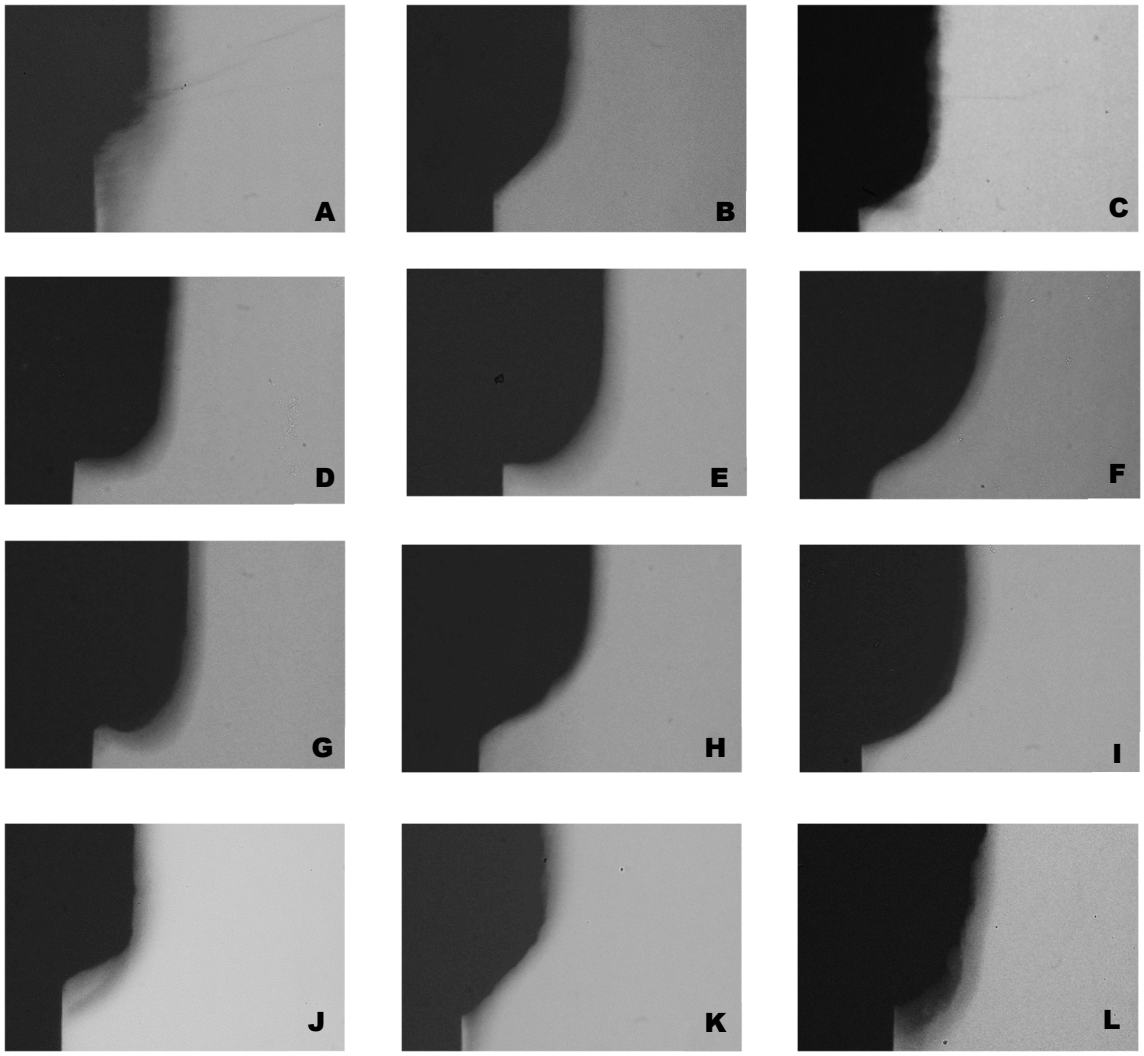

Figure 4. Representative TMR pictures (20x) of the artificial enamel lesions created using microcosm biofilm after applying the tested treatments. A) Positive control (Chlorhexidine, PerioGard®), B) Vehicle (negative) control, C-G) *M. urundeuva* from 1000 to 0.1 µg/ml, respectively; H-L) *Q. grandiflora* from 1000 to 0.1 µg/ml, respectively.

4. Discussion

The use of plant extracts is a common practice for the prevention and treatment of various diseases worldwide. Phytotherapy is considered of low cost and widely accessible [6]. Brazil is one of countries with the greatest biodiversity [7], which in turn has stimulated the use of different type of plants for prevention and treatment of diverse diseases. The most common benefits of the plants in health is their antimicrobial effect, which may be potentially able to control oral diseases as dental caries and periodontitis [26-28].

The experimental model for studying the effect of plants on dental caries must include assays that are capable to show the antimicrobial properties (such as viability, EPS and lactic acid assays) as well as the anti-caries effect (TMR), since one is not synonym of the other. Some antimicrobial agents have no anti-caries potential [4], which unable their use for the prevention of this disease [29]. Accordingly, we have chosen to apply a microcosm biofilm model on enamel, since it is able to simulate the heterogeneity and the variability of *in vivo* biofilm, allowing the analysis of both biofilm and tooth [30].

Myracrodruon urundeuva and *Q. grandiflora* were chosen as they are easily found in Brazilian Cerrado. The ethanolic extracts of *M. urundeuva* leaves and bark have as active components gallic acid, methyl gallate, ethyl gallate, chlorogenic and protocatechuic acid, saponins, flavonoids, tannins and polyphenols [31]. The ethanolic extract of *Q. grandiflora* leaves present gallic and ellagic acids derivatives, galotannins, ellagitannins, triterpenes, flavonoids, benzoquinones and anthraquinones [32]. A previous study showed that the main components of *M. unrundeuva* extract are flavonoids and tannins [17].

Generally, our study showed that *M. urundeuva* has superior antimicrobial effect compared to *Q. grandiflora*. Alves et al. [33] observed antimicrobial effect of *M. urundeuva* (stem bark) against oral bacteria by quantifying inhibition zone (Minimum Inhibitory Concentration-MIC) and Minimum Inhibitory Adhesion Concentration (MICA). They showed MIC and MICA values of 0.125 mg/ml and 0.0625 mg/ml against mutans streptococci, respectively. Their MIC value is in agreement with our results of viability, since we have seen antimicrobial effect with 0.1 mg/ml *M. urundeuva. In addition, M. urundeuva*, as 5% gel or as aqueous solution (7.5 mg/ml),

is also effective against total microorganisms [34] and mutans streptococci [35] presented in biofilm of male Wistar rats, respectively.

With respect to *Q. grandiflora*, there is no study about its effect on cariogenic bacteria, but only on *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Streptococcus pyogenes and Helicobacter pylori* in planktonic phase [13,36,37].

Despite the extracts were able to reduce bacteria viability and the number of lactobacilli and mutans streptococci, they did not interfere in the bacteria metabolism, and, therefore, they were unable to reduce caries lesions development. Despite there were lower number of viable bacteria due to the treatments, they were still able to produce acid and EPS, which in turn induced enamel demineralization. Our work provided support for the statement that no all antimicrobial agents have anti-caries effect [4, 29].

On the other hand, we should take in mind that other bacteria, not analyzed in the present study, could have contributed to enamel caries development (*Scardovia wiggsiae*, *Bifidobacterium* spp. and *Actinomyces* spp.) [38], which shall be further confirmed under this model.

In disagreement with our results, previous work has shown that *M. urundeuva* All. (7.5 mg/ml) protected against enamel surface hardness loss in Wistars rats inoculated with mutans streptococci for 7 days [35]. This difference might be due to the greater concentration of the extract tested in the former study and to the low velocity of caries development *in vivo*.

Chlorhexidine was applied as positive control in agreement with Maske et al. [30], who observed that chlorhexidine solutions at concentrations of 0.06% and 0.12%, applied 2x/day, significantly reduced the counts of mutans streptococci (65%) and the percentage of surface hardness loss compared to control (saline solution). On the other hand, Alcohol (35%) was our negative control, since it was applied as solvent for the leaves extracts. Previous works have shown that 50% or 80% ethanol had no antimicrobial effect against mutans streptococci [39,40].

Further studies shall give attention to test the antimicrobial effect of *M. urundeuva* extracts, varying concentrations, solvents and vehicles, under microcosm biofilm, *in situ* or/and *in vivo* models, to confirm if they really do not have any anti-caries effect. Other important point to consider in future studies is the analysis of the cytotoxic effect of the extracts as well. Some previous studies have shown cytotoxic

effect of *M. urundeuva* and *Q. grandiflora* on fibroblasts, but the extracts were diluted in 80% methanol or 70% ethanol [17,41]. Therefore, this issue should be better addressed using lower alcohol concentrations as solvent.

5. Conclusions

The extracts showed antimicrobial effects (especially *M. urundeuva*) on the microcosm biofilm; however, they had no anti-caries effect under this biofilm model.

Conflict of interest: None

Acknowledgements: The authors thank CAPES for the concession of scholarship to the first author. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical approval: CEEA 43948115.2.0000.5417.

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4-Article III

4 ARTICLE III

Article formatted according to the guideline of Archives of Biology (ANNEX 3).

Hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves on *Streptococcus mutans* biofilm and tooth demineralization

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Abstract

Objectives: This study evaluated the effect of the hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves (alone or combined) on the viability of *Streptococcus mutans* biofilm and on the prevention of enamel demineralization.

Methods: Strain of *S. mutans* (ATCC 21175) was reactivated in BHI broth. Minimum inhibitory concentration-MIC, minimum bactericidal concentration-MBC, minimum biofilm inhibitory concentration-MBIC and minimum biofilm eradication concentration-MBEC were firstly determined in order to choose the concentrations to be tested under biofilm model. *S. mutans* biofilm ($5x10^5$ CFU/ml) was produced on bovine enamel, using McBain saliva under 0.2% sucrose exposure, for 3 days. The biofilm was daily treated with the extracts for 1 min. The biofilm viability was tested by fluorescence and the enamel demineralization was measured using TMR.

Results: Myracrodruon urundeuva (isolated or combined), at the concentrations equal and higher than 0.625 mg/ml, was able to reduce bacteria viability, while *Qualea grandiflora* alone had antimicrobial effect at 5 mg/ml only ($p<0.05$). On the other hand, none of the extracts were able to reduce enamel caries lesion development.

Conclusions: The tested hydroalcoholic extracts had antimicrobial action (especially *Myracrodruon urundeuva* All.); however, they did not have anti-caries effect under *S. mutans* biofilm model.

Keywords: Antimicrobial agents; Biofilm; Dental caries; Plants extracts; Streptococcus mutans.

1. Introduction

Streptococcus mutans is considered the most important microorganism involved in the etiology of dental caries, a tooth disease related to the presence of biofilm, whose metabolism is dependent on the frequent exposure to sugar (Eriksson, Lif Holgerson, Esberg, & Johansson, 2017) *S. mutans* are capable of producing acid from sugar metabolism, surviving in an acidic environment and using sucrose to synthesize extracellular polysaccharide (EPS), which are mostly glycan responsible for the development and protection of dental biofilm (Bowen & Koo, 2011; Koo, Falsetta, & Klein, 2013; Marsh, Martin, Lewis, & Williams, 2009; Takahashi & Nyvad, 2011; Zhao, Li, Lin, Chen, & Yu, 2014).

In order to control dental biofilm and avoid the disease, mechanical practice of brushing and reduction of sugar consume are advised (Rugg-Gunn, 2013). However, for patients under high risk of caries, chemical agents may be needed (James et al., 2017). On the other hand, the conventional chemical agents usually cause a series of undesired side events (James et al., 2017; Zheng & Wang, 2011) such as tooth staining, supragingival calculus formation, changes in taste perception, parotid gland swelling and irritation of the oral mucosa, when applied for periods higher than 2 weeks (James et al., 2017).

Phytotherapy has been developed to overcome this issue (Palombo, 2011; Vieira et al., 2014). Brazil is the home of the largest number of plant species (32,086 native Angiosperms and 23 native Gymnosperms) worldwide (Forzza et al., 2012; Mittermeier, van Dijk, Rhodin, & Nash, 2004; Zappi et al., 2015). *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. are examples of Brazilian plants with anti-inflammatory, antiulcerogenic activity, gastric mucosal protection, analgesic (Carlini, Duarte-Almeida, Rodrigues, & Tabach, 2010; Hiruma et al., 2006; Souza et al., 2007; Viana et al., 1997; Viana, Bandeira, & Matos, 2003), anticonvulsant (Gaspi, Foglio, Carvalho, & Moreno, 2006), antiseptic and antibacterial activities (Alves et al., 2009; Costa et al., 2008).

However, there is no information about their anti-caries potential. Only a study was performed to check the effect of *Myracrodruon urundeuva* All. against *S. mutans* and mineral loss using an animal model (Menezes, Delbem, Brighenti, Okamoto, & Gaetti-Jardim Jr., 2010). Therefore, the objective of this study was to test the effect of the hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora*

Mart. leaves, isolated or combined, on *S. mutans* viability (under planktonic and biofilm form) and on the prevention of enamel caries lesion formation.

2. Methods

2.1 Plant's extracts preparation

Leaf samples of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. were collected in October 2013 at Jardim Botânico Municipal de Bauru (Bauru, Brazil), (22°20'41.4"S - 49°01'45.1"W). Exsiccates were deposited in the Herbarium of UNESP under code numbers HRCB59831 and UNBA6034. The collections have authorization issued by SISBIO under code number 39825-1. The hydroalcoholic extracts were prepared as described by Machado et al. (2016) in the Natural Products Chemistry Laboratory, at the Faculty of Sciences/UNESP-Bauru-SP. Briefly, fresh leaves were hot air dried at 45°C and grounded in a knife mill. The extract was obtained with 70% ethanol via percolation at room temperature. The filtrates were concentrated under dryness and reduced pressure at 40°C. They were finally lyophilized, weighed and stored in an amber bottle at room temperature. The hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. were produced using ethanol at a final concentration of 5%. They were tested isolated and combined.

2.2 Bacterial strain

Strain of *S. mutans* (ATCC 25175) was defrosted and 100 µl were resuspended in tubes containing 3 ml of *Brain Heart Infusion* broth (BHI, Difco, Detroit, USA). The tubes were then incubated for 24 h at 37º C and 5% CO_2 (Hasan, Danishuddin, & Khan, 2015).

2.3 Minimal inhibitory and minimal bactericidal concentrations

The minimum inhibitory concentration (MIC) was determined using microplate serial dilution technique according to the M07-A9 of the manual of *Clinical and Laboratory Standards Institute* with modifications. Briefly, 80 µl of BHI broth and 100 µl of the hydroalcoholic extracts of *M. urundeuva* All. and/or *Q. grandiflora* Mart., at concentrations ranging from 20 mg/ml to 0.00244 mg/ml, were distributed in 96-well plates. Twenty microliters of bacterial suspension ($5x10^5$ CFU/ml) were added in each well (CLSI, 2012). BHI and BHI with 5% ethanol were applied as negative

controls, while Periogard® was used as positive control (0.12% chlorhexidine, Colgate, Brazil, at the dilutions from 0.6 mg/ml to 0.00234 mg/ml).

The plates were incubated for 24 h at 37º C and 5% CO_2. Thereafter, the absorbance was determined using a microplate reader at 540 nm (Fluorstar Optima- BMG Labtech, Germany). MIC was defined as the lowest concentration of the extract capable of inhibiting 100% of bacterial growth compared to the negative control.

For the MBC determination, aliquots (20 µl) from the wells, where no absorbance was detected (values equal or higher than MIC), were spread using the gout technique (Westergren & Krasse, 1978) on petri dishes containing BHI agar (Difco, Detroit, USA). The plates were incubated for 24 h at 37º C and 5% CO_2. MBC was defined as the lowest concentration of the extract capable of inhibiting any visible bacterial growth. All tests were done in biological triplicate (n=3 for each replicate).

2.4 Minimum biofilm inhibition and eradication concentrations

The minimum biofilm inhibition concentration (MBIC) was determined as done for MIC, however, after 24-h incubation, the supernatant were discarded and washed once with phosphate buffered saline (PBS). The viability was quantified using 3-[4, 5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium-bromide (MTT, Sigma-Aldrich, St Louis, MO, USA). Viable bacteria reduce MTT in purple formazan. The absorbance was measured using a microplate reader (Fluorstar Optima- BMG Labtech, Ortenberg, Germany) at 540 nm. The MBIC was defined as the lowest concentration able to reduce in 100% bacterial viability compared to the negative control (Tang et al., 2011; Teanpaisan, Senapong, & Puripattanavong, 2014).

For the minimum biofilm eradication concentration (MBEC) determination, the bacteria was cultivated similarly to MIC assay but without any treatment during the first 24h. The BHI was then removed and the wells washed once with PBS, to remove any bacteria in suspension. The hydroalcoholic extracts were directly added to the wells (200 µl/well), and the plates were incubated again for 24 h at 37º C and 5% CO_2. The viability of the attached bacterial to the wells was measured using MTT assay as described above. The MBEC was defined as the lowest concentration able to reduce in 100% bacterial viability compared to the negative control (Tang et al., 2011; Teanpaisan, Senapong, & Puripattanavong, 2014). All tests were done in biological triplicate (n=3 for each replicate).

2.5 Enamel samples preparation

A one hundred and thirty-five enamel samples (4 mm x 4 mm) were prepared from bovine teeth, using a semi-precision cutting machine (Buehler, Enfield, USA). The samples were fixed in acrylic discs with wax and polished in a metallographic polishing machine (Arotec, Cotia, Brazil) using water-cooled silicon-carbide discs (600-grade papers ANSI grit; Buehler, Enfield, USA) to remove grooves and to standardize the surface roughness of approximately 0.153 ± 0.037 µm. The average surface roughness (Ra) was assessed using contact profilometer and Mahr Surf XCR 20 software (Mahr, Göttingen, Germany). Enamel samples were randomly distributed in the groups according to the Ra mean values, allowing similar enamel surface roughness means among them.

Two thirds of the enamel surfaces were protected with nail polish to obtain control areas for the TMR analysis. The samples were sterilized with ethylene oxide [Gas exposure time (30% ETO/70%CO_2) for 4 h under a pressure of 0.5 ± 0.1 kgF/cm^2].

2.6 3-days S. mutans biofilm formation and treatment

For the formation of the *S. mutans* biofilm on bovine enamel, the strain was reactivated in BHI broth and diluted (concentration of $5x10^5$ CFU/ml) in artificial saliva. The artificial saliva contained 2.5 g/l mucin from porcine stomach (type II), 2.0 g/l bacteriological peptone, 2.0 g/l tryptone, 1.0 g/l yeast extract, 0.35 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l cysteine hydrochloride, 0.001 g/l hemin, 0.0002 g/l vitamin K1, at pH 7.0 (McBain, 2009).

Twenty-four well plates containing enamel samples were filled with inoculum ($5x10^5$ CFU/ml artificial saliva, 1.5 ml/well), which was incubated at 5% CO_2 and 37˚C. After 8h, the inoculum was removed, the samples were washed using PBS and fresh McBain saliva containing now 0.2% sucrose was added into the wells (1.5 ml/well). The microplates were incubated at 5% CO_2 and 37˚C for further 16h, completing the first day of the experiment (24 h).

During the next 2 days, the biofilm was daily treated with the sub-MIC levels of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. extracts, isolated or combined (1x60s/day). After treatment, the samples were washed using PBS and fresh McBain saliva with 0.2% sucrose was added. Periogard® was applied as

positive control (0.12% chlorhexidine, Colgate, Brazil), PBS and PBS with 5% ethanol were used as negative controls. The microplates were incubated at the same conditions described above (Zhang et al., 2013). The experiment was done in biological triplicate (n=3 for each replicate).

2.7 Biofilm viability analysis

The biofilm was stained using the nucleic acid markers diluted in PBS (1ml PBS + 1 µl SYTO9 + 1 µl propidium iodide, 10 µl/well) (Kit Live & Dead® cells viability assay, Thermo Fisher Scientific, Waltham, USA) for 15 min in a dark environment. Live bacteria were stained with SYTO9 producing a green fluorescence and dead lysed bacteria were stained with propidium iodide and SYTO9 producing a red fluorescence (Hannig et al., 2013). The biofilm was examined using confocal laser scanning microscope-CLSM (Leica TCS SPE, Mannheim, Germany) and Leica Application Suite-Advanced Fluorescence software (LAS AF, Mannheim, Germany). Three images (275 μm^2) were captured from each sample surface and analyzed using BioImage L 2.0 software, to quantify the live and dead bacteria (%).

2.8 Analysis of demineralization by transverse microradiography - TMR

After cleaning, all enamel samples were transversally sectioned and polished to obtain slices with 80-100 µm of thickness. The enamel slices were fixed in a sample-holder together with an aluminum calibration step wedge with 14 steps. A microradiograph was taken using an x-ray generator (Softex, Tokyo, Japan) on the glass plate at 20 kV and 20 mA (at a distance of 42 cm) for 13 min. The glass plates were developed for 5 min, rinsed in deionized water, fixed for 8 min in a dark environment, and then rinsed in running water for 10 min and air-dried (all procedures were done at 20°C). The developed plate was analyzed using a transmitted light microscope fitted with a 20x objective (Zeiss, Oberkochen, Germany), a CCD camera (Canon, Tokyo, Japan), and a computer. Two images per sample were taken using data-acquisition (version 2012) and interpreted using calculation (version 2006) softwares from Inspektor Research System bv (Amsterdam, The Netherlands). The mineral content was calculated based on the work of Angmar, Carlstrom & Glas (1963), assuming the density of the mineral to be

3.15 kg l^{-1} and 87 vol% of mineral content for the sound enamel. The lesion depth (LD, μm) and the integrated mineral loss (∆Z, %vol. μm) were calculated.

2.9 Statistical Analysis

Data were statistically analyzed using software Graph Pad Instat for Windows (GraphPad Software, San Diego, USA). The normal distribution and homogeneity were checked using Kolmogorov & Smirnov and Bartlett's tests, respectively. The % live and dead microorganisms were compared using Kruskal-Wallis followed by Dunn test and the TMR data were analyzed using ANOVA followed by Tukey test. The level of significance was set at 5%.

3. Results

Table 1 shows the antimicrobial concentrations values found for the extracts against *S. mutans* (planktonic phase). The lowest MIC, MBC, MBIC and MBEC values were seen for *M. urundeuva*; effect that was not improved by the combination with *Q. grandiflora*. However, no extract reached the lowest antimicrobial concentration found for chlorhexidine.

With respect to the 3-days *S. mutans* biofilm, similar antimicrobial effect was found. *M. urundeuva* at the concentrations equal and higher than 0.625 mg/ml were able to reduce bacteria viability, while *Q. Grandflora* had antimicrobial effect only at 5 mg/ml. The combination of the extracts was effective in reducing bacterial viability at concentrations equal and higher than 0.625 mg/ml, similarly to *M. urundeuva* alone (Table 2). Figure 1 shows representative CLSM pictures of the biofilms treated with *M. urundeuva* (1.25 mg/ml), *Q. grandiflora* (5 mg/ml), chlorhexidine (0.12%) and PBS.

None of the plant's extracts were able to reduce the enamel caries lesion development. Only chlorhexidine significantly reduced mineral loss, but not lesion depth. Table 3 shows the TMR data and Figure 2 shows representative TMR pictures of enamel samples from the same groups displayed in Figure 1.

4. Discussion

Considering the important role of *S. mutans* in dental caries etiology (Eriksson, Lif Holgerson, Esberg, & Johansson, 2017), we chose to apply this bacteria specie under planktonic and biofilm models. *Myracrodruon urundeuva* All. and *Qualea*

grandiflora Mart. were selected as representative of plants typical from Brazilian "cerrado". Our results showed that *M. urundeuva* had a better antimicrobial effect against *S. mutans* than *Q. grandiflora*; however, it did not reach the antimicrobial effect of chlorhexidine. The combination of the extracts had no further antimicrobial effect, differently from the findings of Shafiei, Haji Abdul Rahim, Philip, & Thurairajah (2016), who observed a synergic effect of three natural extracts (*Psidium* sp., *Magifera* sp. and *Mentha* sp.) on *S. mutans* .

M. urundeuva leaves and barks extracts have as active components: gallic acid, methyl gallate, ethyl gallate, chlorogenic and protocatechuic acid, saponins, flavonoids, tannins and polyphenols (Mota et al., 2015), while *Q. grandiflora* Mart. leaves' extract presents gallic and ellagic acids derivatives, galotannins, ellagitannins, triterpenes, flavonoids, benzoquinones and anthraquinones in its composition (Ayres, Escórcio, Costa, & Chaves, 2008). Therefore, the best antimicrobial performance of *M. urundeuva* All. may be due to the presence of polyphenols.

Alves, Queiroz, Pereira, & Pereira (2009) determined the MIC and MIAC (Minimum Inhibitory Adherence Concentration) of hydroalcoholic extracts of *M. urundeuva* (stem bark) against *S. mutans.* The MIC and MIAC values were 0.125 mg/ml and 0.0625 mg/ml, respectively. Their MIC value was lower than our MIC value, which might be justified by the process of extraction and the part of the plant tested as well as due to the method applied to determine MIC and the bacteria strain used for that.

With respect to *Q. grandiflora* extract, the studies have been done using *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa, Streptococcus pyogenes* and *Helycobacter pylori* strains (Alves et al., 2000; Bonacorsi, Da Fonseca, Raddi, Kitagawa, & Vilegas, 2013; Moura, Nascimento, & Pinto, 2012). This is the first study about its effect against cariogenic bacteria.

In 2010, Menezes, Delbem, Brighenti, Okamoto, & Gaetti-Jardim Jr evaluated the action of aqueous extracts of *Psidium cattleianum* and *M. urundeuva* (7.5 mg/ml) in Wistar rats under cariogenic diet for 7 weeks. They observed that both extracts reduced the cariogenic biofilm (level of *S. mutans* by RT-PCR) and had a protective effect on enamel surface hardness loss. Oppositely, our study did not show any protective effect of the extracts against enamel demineralization. The reason to

explain the different findings might be related to the fact that the tested concentrations in the present study were lower than those applied in the former work.

On the other hand, we should take in mind that high extracts concentrations could induce some side effects. Cytotoxic potential is an important aspect that should be considered before testing the effect of a new antimicrobial agent in clinical trials. A recent study has shown that *M. urundeuva* leaves' extracts at concentrations higher than 100 µg/ml could reduce the viability of fibroblasts; however, the authors applied 80% methanol as solvent (Machado et al., 2016). Therefore, further studies on this issue are needed to better establish the cytotoxic potential of the hydroalcoholic plants extracts containing 5% ethanol. Furthermore, the lack of anti-caries effect shall be confirmed under more complex biofilm models in the future.

5. Conclusion

The hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves (isolated or combined, especially *M. urundeuva*) had antimicrobial action against *S. mutans*; however, they did not have anti-caries effect under 3-days *S. mutans* biofilm model.

Acknowledgments

The authors thank FAPESP (2016/20212-0) for the concession of financial support to the second author and Prof. Dr. Anne L. Dokkedal (Department of Biological Sciences Unesp/Bauru-SP) for the donation of the plants' extract. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical approval: Not applicable.

Declarations of interest: none

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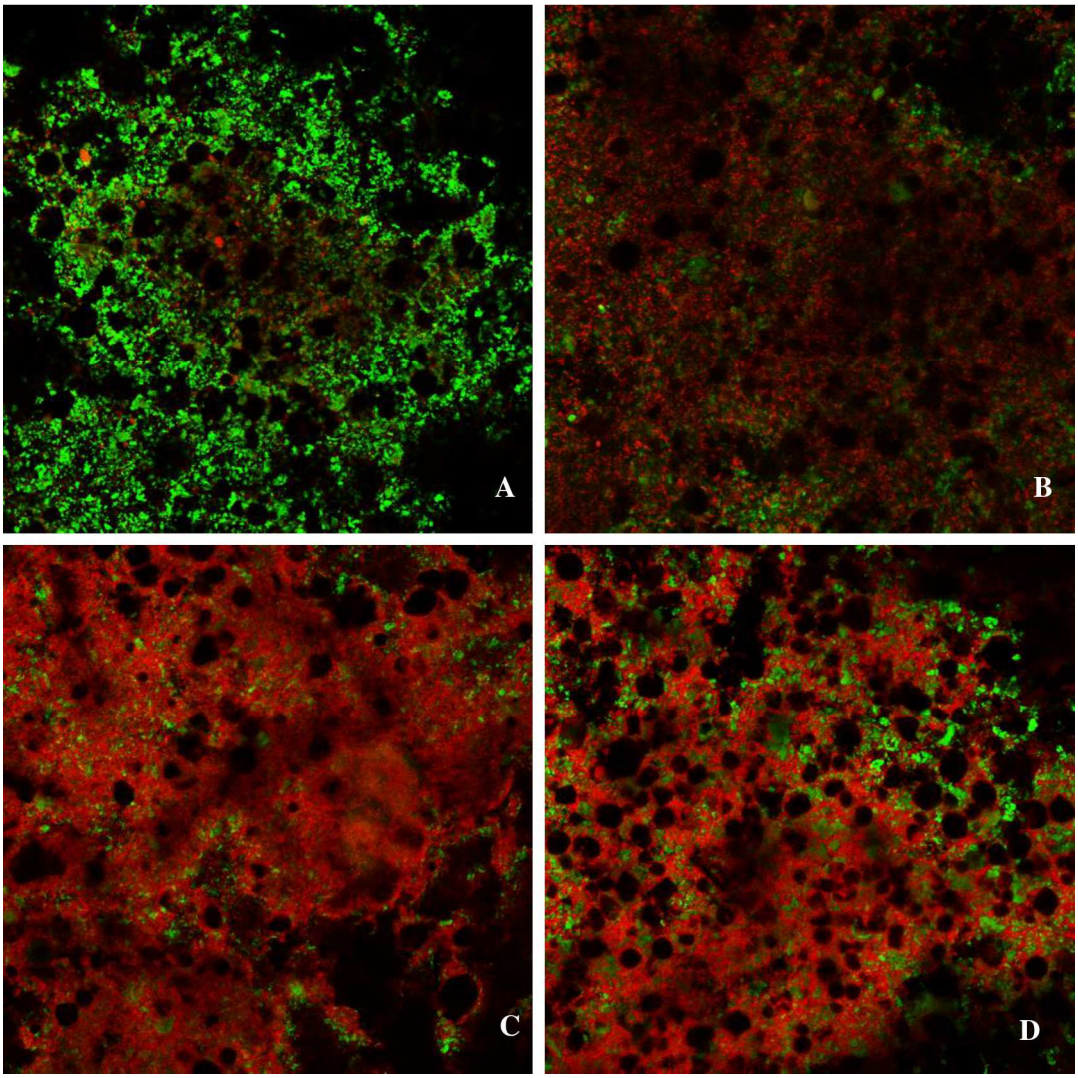

Figure 1. Representative CLSM pictures of the biofilms treated with: A) PBS (Negative control). B) *M. urundeuva* All. extract at the concentration of 1.25 mg/ml. C) *Q. grandiflora* Mart. extract at the concentration of 5.0 mg/ml. D) 0.12% Chlorhexidine (Positive control)

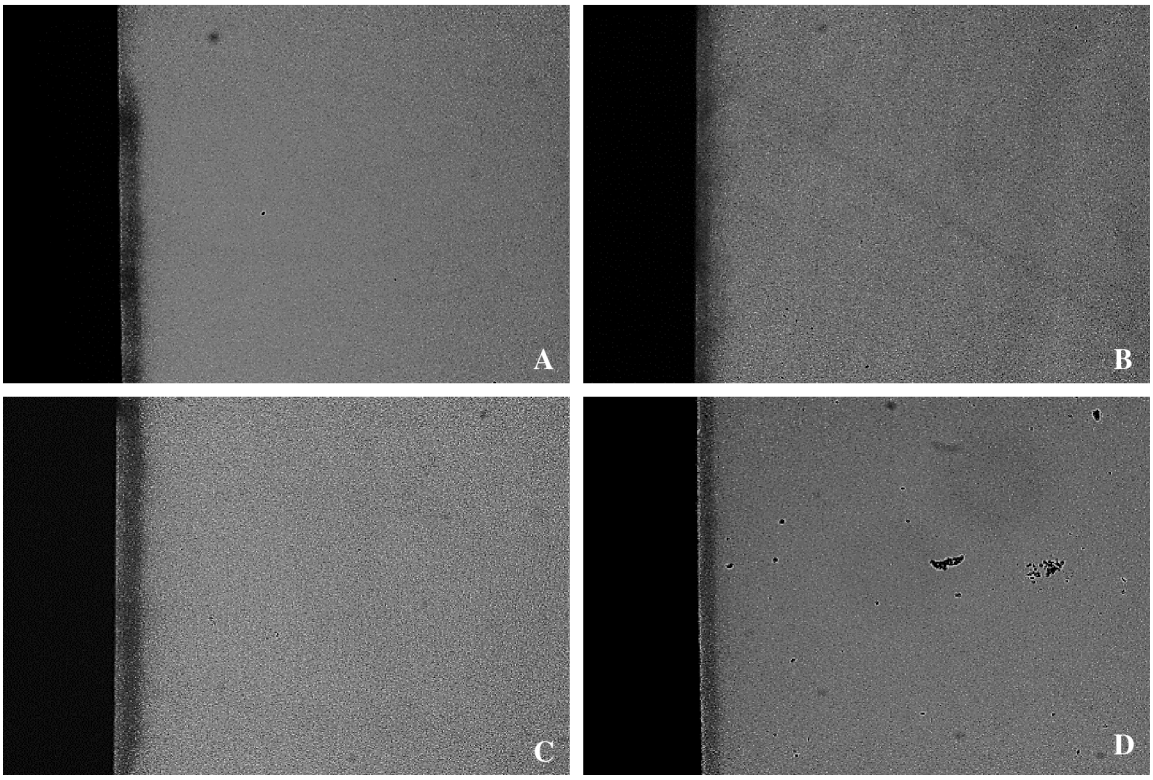

Figure 2. Representative TMR pictures of the enamel samples treated with: A) PBS (Negative control). B) *M. urundeuva* All. extract at the concentration of 1.25 mg/ml. C) *Q. grandiflora* Mart. extract at the concentration of 5.0 mg/ml. D) 0.12% Chlorhexidine (Positive control)

Table 1. Antimicrobial concentrations of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart., isolated or combined (mg/ml), on *S. mutans*

Hydroalcoholic extracts	*S. mutans* (ATCC 25175)			
	MIC	MBC	MBIC	MBEC
Myracrodruon urundeuva All.	2.5	2.5	1.25	2.5
Qualea grandiflora Mart.	5.0	-	5.0	10
Myracrodruon urundeuva All. + *Qualea grandiflora* Mart.	2.5	2.5	2.5	5.0
Chlorhexidine	0.004	0.009	0.004	0.0187

Table 2. Median (interquartile interval) of the percentage (%) of live and dead microorganisms (viability assay using CLSM)

Treatments	**% Live**	**% Dead**
PBS (negative control)	84.10(10.00)^{a}	15.60(9.80)^{a}
5% Ethanol (negative control)	70.30(9.70)^{ab}	29.30(9.60)^{ab}
Chlorhexidine (positive control)	54.40(11.10)^{bc}	45.80(12.10)^{bc}
M. urundeuva 2.5 mg/ml	54.70(4.30)^{bc}	44.90(4.40)^{bc}
M. urundeuva 1.25 mg/ml	49.90(4.70)^{c}	49.60(4.60)^{c}
M. urundeuva 0.625 mg/ml	50.20(10.00)^{bc}	49.00(10.00)^{bc}
M. urundeuva 0.312 mg/ml	63.80(8.20)^{ab}	35.40(8.40)^{ab}
Q. grandiflora 5.0 mg/ml	29.30(13.50)^{c}	70.40(13.60)^{c}
Q. grandiflora. 2.5 mg/ml	57.10(17.30)^{abc}	42.50(17.30)^{abc}
Q. grandiflora 1.25 mg/ml	60.60(19.20)^{abc}	38.40(18.80)^{abc}
Q. grandiflora 0.625 mg/ml	60.50(16.40)^{abc}	38.90(16.00)^{abc}
M. urundeuva + *Q. grandiflora* 1.25 mg/ml	55.10(12.30)^{bc}	44.10(12.40)^{bc}
M. urundeuva + *Q. grandiflora* 0.625 mg/ml	52.40(11.70)^{bc}	47.10(11.60)^{bc}
M. urundeuva + *Q. grandiflora* 0.312 mg/ml	71.90(11.30)^{ab}	27.70(11.20)^{ab}
M. urundeuva + *Q. grandiflora* 0.156 mg/ml	77.50(15.00)^{ab}	22.10(14.60)^{ab}

Different letters in the same column show significant differences among the groups (Kruskal-Wallis/Dunn, $p<0.0001$).

Table 3. Mean ± SD of the integrated mineral loss (ΔZ, vol% μm) and the lesion depth (LD, μm) of enamel lesions provoked by 3-days *S. mutans* biofilm

Treatments	**ΔZ**	**LD**
PBS (negative control)	1745±706^{bc}	55±5^{ab}
5% Ethanol (negative control)	1536±354^{abc}	57±18^{ab}
Chlorhexidine (positive control)	812±438^{a}	37±15^{b}
M. urundeuva 2.5 mg/ml	1994±540^{c}	77±26^{a}
M. urundeuva 1.25 mg/ml	1113±661^{abc}	46±22^{ab}
M. urundeuva 0.625 mg/ml	954±373^{ab}	43±10^{ab}
M. urundeuva 0.312 mg/ml	912±281^{ab}	35±16^{b}
Q. grandiflora 5.0 mg/ml	1401±511^{abc}	54±16^{ab}
Q. grandiflora. 2.5 mg/ml	1193±469^{abc}	55±17^{ab}
Q. grandiflora 1.25 mg/ml	1116±675^{abc}	49±20^{ab}
Q. grandiflora 0.625 mg/ml	1498±478^{abc}	63±23^{ab}
M. urundeuva + *Q. grandiflora* 1.25 mg/ml	1246±556^{abc}	54±22^{ab}
M. urundeuva + *Q. grandiflora* 0.625 mg/ml	1673±334^{abc}	63±19^{ab}
M. urundeuva + *Q. grandiflora* 0.312 mg/ml	1240±575^{abc}	60±22^{ab}
M. urundeuva + *Q. grandiflora* 0.156 mg/ml	1633±414^{abc}	56±9^{ab}

Different letters in the same column show significant differences among the groups (ANOVA/Tukey test, p = 0.001 for ΔZ, and p = 0.008 for LD).

5- Discussion

5 DISCUSSION

Brazil is one of countries with the greatest biodiversity wordwile (SILVA et al., 2014), which in turn has stimulated the use of different type of plants for prevention and treatment of diverse diseases. The most common benefit of the plants in health is related to their antimicrobial effect, which may have potential to control oral diseases as dental caries and periodontitis (BERSAN et al., 2014).

Therefore, the present thesis started with a review of literature about this topic and, thereafter, tested the effect of two plants extracts (*Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart.) against enamel caries under a microcosm biofilm model and *S. mutans* biofilm, respectively. Microcosm biofilm is a model able to induce biofilm formation using inoculum from pooled human saliva or biofilm mixed with nutrients, being able to mimic the heterogeneity and variability of the supragingival biofilms (RUDNEY et al., 2012; AZEVEDO et al., 2014; MASKE et al., 2016). We also included a monospecie biofilm test, allowing the detection of the effect of the extracts on specie; in this case *S. mutans*, which is one of the most important bacteria involved in the etiology of dental caries (ERIKSSON et al., 2017).

We have chosen two plants extracts available in the Brazilian Cerrado. No previous study has compared these extracts against dental caries using a biofilm model. According to the literature review, there are a lot of studies on the use of Brazilian natural agents in dentistry, however, most of them are focus on periodontitis/gingivitis, while few clinical trials have been done about caries prevention. Unfortunately, the *in vitro* studies have still been done using bacteria in planktonic phase and not under more complex environment, as biofilm. It is also important to keep in mind that there is a wide variety of ways of acquiring the natural agents (essential oils, propolis, crude extracts) varying according to season and the collected parts of the plants (root or leaves) that may contain different fractions of active components (polyphenols, terpene, monoterpene, dimeric chalcones and/or carvacrol) with antimicrobial potential. In the present study, we have applied hydroalcoholic extracts of the leaves, since these extracts have been produced by collegues from FC-UNESP/Bauru and tested with respect to biological properties by a co-partner research group (MACHADO et al., 2016).

Other important point is that the experimental model for studying the effect of plants on dental caries must include assays that are capable to show the antimicrobial properties (such as viability, EPS and lactic acid assays) as well as the anti-caries effect (TMR), since one is not synonym of the other. Some antimicrobial agents have no anti-caries potential (BRAGA; PIRES; MAGALHÃES, 2017), which unfeasible their inclusion in oral products and their use for the prevention of this disease.

Generally, our study showed that *M. urundeuva* has a superior antimicrobial effect compared with *Q. grandiflora* under both microcosm biofilm and *S. mutans* biofilm; however, it did not reach chlorhexidine action. With respect to microcosm biofilm, we observed that despite the extracts were able to reduce bacteria viability and the number of lactobacilli and mutans streptococci, they did not interfere in the bacteria metabolism, and, therefore, they were unable to reduce caries lesions development. On the other hand, we must consider that other bacteria present in microcosm biofilm, but not analyzed in the present study, could have contributed to enamel caries development (*Scardovia wiggsiae, Bifidobacterium* spp. and *Actinomyces* spp.) (HENNE et al., 2016), which shall be further confirmed in the future.

Considering the lack of anti-caries effect of the extracts under microcosm biofilm, we decided to test higher concentrations of the extracts and combined both plants on *S. mutans* biofilm viability and the enamel caries lesions development. Considering the agressivity of this specie, the biofilm was produced only for 3 days, as tested in a pilot study, to produce non-cavitated enamel lesions. Similar results, as reported in the previous article (2nd chapter), were found under *S. mutans* biofilm model. *M. urundeuva* had a better antimicrobial effect than *Q. grandiflora,* but it did not reach the action of chlorhexidine. The combination of the extracts did not enhance their antimicrobial effect, at least not compared to *M. urundeuva* alone. None of the extracts were able to reduce the development of enamel caries lesions. In this 3rd chapter, we only applied two response variables, since it involved an undergraduate student research. Besides, the lack of anti-caries effect (demineralization prevention) justifies not including other response variables.

M. urundeuva leaves extract has as active components gallic acid, methyl gallate, methyl gallate, ethyl gallate, chlorogenic and protocatechuic acid, saponins, flavonoids, tannins and polyphenols (MOTA et al., 2015), while *Q. grandiflora* leaves

extract presents gallic and ellagic acids derivatives, galotannins, ellatannins, triterpenes, flavonoids, benzoquinones and anthraquinones in its composition (AYRES et al., 2008). Therefore, the best antimicrobial performance of *M. urundeuva* might be due to the presence of polyphenols.

With respect to the antimicrobial effect of *Q. grandiflora* extract, previous studies have been done using *S. aureus, E. coli, B. cereus, P. aeruginosa, S. pyogenes* and *H. pylori* strains (ALVES et al., 2000; MOURA; NASCIMENTO; PINTO, 2012; BONACORSI et al., 2013), but not species involved in caries etiology.

On the other hand, *M. urundeuva* has been tested against cariogenic species. Alves et al. (2009) observed antimicrobial effect of *M. urundeuva* (stem bark) against oral bacteria by quantifying inhibition zone (MIC) and Minimum Inhibitory Adhesion Concentration (MICA). They showed MIC and MICA values of 0.125 mg/ml and 0.0625 mg/ml against *S. mutans*, respectively. Their MIC value is in agreement with our viability results under microcosm biofilm but not under planktonic phase (MIC for *S. mutans*), which might be due to the bacteria strain used, the method applied to determine MIC and the preparation of the extract.

M. urundeuva, as 5% gel or as aqueous solution (7.5 mg/ml), has been also tested against total microorganisms (BOTELHO et al., 2007) and *S. mutans* (MENEZES et al., 2010) presented in biofilm of male Wistar rats, respectively, with positive results compared to saline-based gel and water, respectively. Menezes et al. (2010) evaluated the action of aqueous extracts of *Psidium cattleianum* and *M. urundeuva* (7.5 mg/ml) in Wistar rats under cariogenic diet for 7 weeks. They observed that both extracts reduced the cariogenic biofilm (level of *S. mutans* by RT-PCR) and had a protective effect on enamel surface hardness loss. Oppositely, our study did not show any protective effect of the extracts against enamel demineralization. The reason to explain the different findings might be related to the fact that the tested concentrations in the present study were lower than those applied in the former work.

Chlorhexidine was applied as positive control in both articles in agreement with Maske et al. (2016), who observed that chlorhexidine solutions at concentrations of 0.06% and 0.12%, applied 2x/day, significantly reduced the counting of *S. mutans* (65%) and the percentage of surface hardness loss compared to control (saline solution). On the other hand, alcohol was used as negative control, since it was applied as solvent for the extracts leaves. In the 2nd article, we applied 35% ethanol

as solvent, while we decided to reduce its concentration in the 3rd article, to avoid questioning about a possible antimicrobial effect of a high ethanol concentration. Previous works have shown that 50% or 80% ethanol had no antimicrobial effect against *S. mutans* (BARBIERI et al., 2014; CARDOSO et al., 2016).

Further studies shall give attention to test the antimicrobial effect of *M. urundeuva* extracts, varying concentrations, solvents and vehicles, under microcosm biofilm, *in situ* or *in vivo* models, to confirm if they really do not have any anti-caries effect. Other important point to consider in the future is the analysis of its cytotoxic effect. Cytotoxic potential is an important aspect that should be considered before testing the effect of a new antimicrobial agent in clinical trials. A recent study has shown that *M. urundeuva* leaves' extracts at concentrations higher than 100 µg/ml could reduce the viability of fibroblasts; however, the authors applied 80% methanol as solvent (MACHADO et al., 2016). Therefore, further studies on this issue are needed to better establish the cytotoxic potential of the hydroalcoholic plants' extracts containing 5% or 35% ethanol.

In conclusion, the extracts showed antimicrobial effects (especially *M. urundeuva*) on microcosm and *S. mutans* biofilms, which was not improved by the increasing concentration or combining the extracts. Unfornately, none of the extracts, under both tested conditions, was able to reduce the development of enamel caries lesions, unfeasible their use in the present form. It would be interesting to isolate their active components in fractions and test their anti-caries potential.

6-Conclusions

6 CONCLUSIONS

According to chapter 1, more studies involving protocols closer to the clinical condition and using response variables that allow understanding the mechanism of action of the natural agents are needed, to thus allow the incorporation of these natural agents in dental products.

Under microcosm biofilm model (2nd chapter), the tested extracts showed antimicrobial effects (especially *M. urundeuva*); however, they had no anti-caries effect on enamel.

Under *S. mutans* biofilm model (3rd chapter), the tested extracts showed antimicrobial effects (especially *M. urundeuva*); however, they had no anti-caries effect on enamel.

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APPENDIX

APPENDIX 1

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article "Brazilian natural antimicrobial agents on caries and periodontitis-related biofilm: a literature review" will be included in Thesis of the student Juliana Gonçalves Pires was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, January 15, 2018.

Juliana Gonçalves Pires _____
Author Signature

Aline Silva Braga _____
Author Signature

Ana Carolina Magalhães _____ _____
Author Signature

APPENDIX 2

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article "Effect of *Myracrodruon urundeuva* and *Qualea grandiflora* extracts on viability and activity of microcosm biofilm and prevention of enamel demineralization in vitro" will be included in Thesis of the student Juliana Gonçalves Pires was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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APPENDIX 3

DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN DISSERTATION/THESIS

We hereby declare that we are aware of the article "Hydroalcoholic extracts of *Myracrodruon urundeuva* All. and *Qualea grandiflora* Mart. leaves on *S. mutans* biofilm and tooth demineralization" will be included in Thesis of the student Juliana Gonçalves Pires was not used and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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Annex

ANNEX 1

Oral Health and Preventive Dentistry - OHPD-2017-535 - (4752): Co-author manuscript submission confirmation

Oral Health and Preventive Dentistry <ohpd@manuscriptmanager.com>
para mim

inglês > português Traduzir mensagem

Manuscript: OHPD-2017-535 - (4752) - Brazilian natural antimicrobial agents on caries and periodontitis-related biofilm: a literature review

Dear Ms Pires

Automatic notification:

The above manuscript has been submitted online to Oral Health and Preventive Dentistry. You have been included as a contributing author for this manuscript.

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ANNEX 2

Your co-authored submission Entrada ×

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para mim

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Dear Miss. Pires,

You have been listed as a Co-Author of the following submission:

Journal: Fitoterapia

Title: Effect of hydroalcoholic extract of Myracrodruon urundeuva All. and Qualea grandiflora Mart. leaves on the viability and activity of microcosm biofilm and on enamel demineralization

Corresponding Author: Ana Carolina Magalhaes

Co-Authors: Juliana Pires, Aline Braga, Flaviana Andrade, Rodrigo Oliveira

Ana Carolina Magalhaes submitted this manuscript via Elsevier's online submission system, EVISE®. If you are not already registered in EVISE®, please take a moment to set up an author account by navigating to http://www.evise.com/evise/faces/pages/navigation/NavController.jspx?JRNL_ACR=FITOTE

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ANNEX 3

Archives of Oral Biology
Ref: AOB-D-17-00767
Title: Hydroalcoholic extracts of Myracrodruon urundeuva All. and Qualea grandiflora Mart. leaves on S. mutans biofilm and tooth demineralization
Authors: Juliana Pires; Sara Zabini; Aline Braga; Rita Fabris; Flaviana Andrade; Rodrigo Oliveira; Ana Carolina Magalhaes
Article Type: Research Paper

Dear Dr. Ana Carolina Magalhaes,

Your submission entitled "Hydroalcoholic extracts of Myracrodruon urundeuva All. and Qualea grandiflora Mart. leaves on S. mutans biofilm and tooth demineralization" has been assigned the following manuscript number: AOB-D-17-00767.

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Thank you for submitting your work to this journal. Please do not hesitate to contact me if you have any queries.

Kind regards,

Administrative Support Agent [30-Mar-11]
Archives of Oral Biology

ANNEX 4

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Departamento de Ciências Biológicas

Termo de Consentimento Livre e Esclarecido

Através deste termo, lhe convidamos para participar da pesquisa **"Avaliação dos extratos de *Myracrodruon urundeuva* e *Qualea grandiflora* sobre a viabilidade e atividade de biofilme microcosmo e na prevenção da desmineralização do esmalte *in vitro*".**

A nossa pesquisa tem como objetivo testar o efeito de dois agentes naturais, extratos de *Myracrodruon urundeuva* e *Qualea grandiflora*, sobre a viabilidade, contagem de UFC, produção de ácido e PEC/PIC por um biofilme microcosmo submetido ao desafio cariogênico (cárie) e o consequente efeito na desmineralização do esmalte.Para isso, gostaríamos de pedir autorização ao senhor (a) para o uso da saliva. Esta pesquisa será feita pela Juliana Gonçalves Pires (eu) e pela Profa. Dra. Ana Carolina Magalhães (FOB – USP).

Assim, se o(a) Sr.(a) concordar, terá que mastigar uma parafina plástica- *Parafilm* (que é uma película plástica, sem cheiro, sem cor, resistente à água) com o objetivo de aumentar a quantidade de saliva para que então seja realizada a coleta que não é invasiva. Durante a coleta você cuspirá toda a saliva em um recipiente plástico. A saliva será coletada no período da manhã. Para a coleta você não poderá escovar os dentes, nem fazer uso de nenhum tipo de bochecho com fluoreto/agente antimicrobiano por um período de 24h, sendo que 2h antes da coleta, você não poderá fazer a ingestão de nenhum tipo de alimento. Após a utilização da sua saliva para este estudo, toda ela será descartada em local apropriado. A sua saliva será utilizada para a formação do biofilme microcosmo no Laboratório.

O(a) sr(a) não precisarão passar por nenhum outro tipo de procedimento adicional. Isso quer dizer que iremos utilizar apenas a saliva. Queremos deixar claro que não existe a menor obrigação do(a) sr.(a) aceitar doar parte da saliva retirada para a pesquisa. Isso é totalmente voluntário.

Esta pesquisa gera o benefício do aumento do conhecimento sobre o funcionamento dos extratos das plantas sobre as bactérias presente na boca e na formação da cárie, podendo, futuramente, auxiliar no tratamento de doenças. Não há benefícios imediatos para os participantes da pesquisa. 1

A sua participação neste trabalho acarretará em risco mínimo, que acontecerá no caso de você ter alergia ao plástico utilizado para mastigação ou se você apresentar enjoos na hora da coleta. Nestes casos, o voluntário deverá comunicar o responsável pela pesquisa, que estará presente no momento da coleta, e o voluntário será liberado imediatamente da participação na pesquisa, sem penalização alguma. Os gastos que forem gerados por este trabalho ficará a cargo da responsável pelo projeto. Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela participação do sujeito neste estudo. Você terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação acarretou algum problema a você.

Mesmo após assinar este termo, caso o senhor(a) mude de idéia e queira sair da pesquisa, poderá fazê-lo. Todo o trabalho será feito sem a sua identificação, preservando completamente sua identidade. Ao concordar em participar desta pesquisa, o senhor(a) receberá uma via, igualmente válida deste termo. O direito á indenização lhe será permitido, caso ocorra algum dano decorrente da sua participação nesta pesquisa. Caso você necessite de ajuda financeira de transporte para participar desta pesquisa ela poderá ser ressarcida pelo pesquisador.

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-901 – C.P. 73
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Universidade de São Paulo
Faculdade de Odontologia de Bauru

Departamento de Ciências Biológicas

Qualquer dúvida ou maiores esclarecimentos o sujeito da pesquisa poderá recorrer a qualquer um dos membros da equipe do projeto (Laboratório de Bioquímica 14-3235-8247) ou a pesquisadora responsável Juliana Gonçalves Pires (telefone 14 32358497/ 988085557, e-mail jugpires@gmail.com). Caso possua preocupações quanto aos seus direitos como participante deste estudo, ou queira fazer denúncias quanto a condução do mesmo, sinta-se a vontade para procurar *o Comitê de Ética em Pesquisa, da Faculdade de Odontologia de Bauru/USP, Alameda Dr. Octávio Pinheiro Brisolla, 9-75, telefone (14)3235-8356 ou e-mail:* cep@fob.usp.br

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o participante da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d. 2

Bauru, SP, _____ de _____ de _____.

_____ _____
Assinatura do Participante da Pesquisa Nome/Assinatura do Pesquisador(a)

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.
Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

Horário e local de funcionamento:
Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **13h30 às 17 horas**, em dias úteis.
Alameda Dr. Octávio Pinheiro Brisolla, 9-75
Vila Universitária – Bauru – SP – CEP 17012-901 Telefone/FAX(14)3235-8356 e-mail: cep@fob.usp.br

ANNEX 5

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

Uso exclusivo da CEEPA/FOB/USP
Registro número: **007/2016**
Recebido em: 20/12/2016
Maristela Petenuci Ferrari
Secretária da CEEPA -- SRTE 53052

REGISTRO DE PESQUISA E/OU ENSINO, COM UTILIZAÇÃO DE CADÁVERES DE ANIMAIS, OU PARTE DELES

Finalidade:	Pesquisa
Período:	Abril/2015 à Fevereiro/2017
Título da pesquisa:	Avaliação dos extratos de *Myracrodruon urundeuva All.* e *Qualea grandiflora* Mart. Sobre a viabilidade e atividade de biofilme microcosmo e na prevenção da desmineralização do esmalte *in vitro*
Pesquisador Responsável:	Profa Dra. Ana Carolina Magalhães
Pesquisador Executor:	Juliana Gonçalves Pires
Colaboradores:	Prof. Dr. Rodrigo Cardoso de Oliveira e Profa. Dra. Flaviana Bombarda de Andrade
Dados Nota Fiscal/ Termo de Doação:	Frigol S/A (Doação)
Quantidade de Dentes Bovinos:	200 (utilizados na pesquisa = 96 dentes/16 grupos)

Al Dr Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C P 73
e-mail ceepa@fob usp br – Fone/FAX (0xx14) 3235-8356
http //www fob usp br

ANNEX 6

Universidade de São Paulo
Faculdade de Odontologia de Bauru

Comissão de Ética no Ensino e Pesquisa em Animais

Uso exclusivo da CEEPA/FOB/USP
Registro número: **008/2017**
Recebido em: 05/04/2017
Maristela Petenuci Ferrari
Secretária da CEEPA – SRTE 53052

REGISTRO DE PESQUISA E/OU ENSINO, COM UTILIZAÇÃO DE CADÁVERES DE ANIMAIS, OU PARTE DELES

Finalidade:	Pesquisa
Período:	Janeiro/2017 à Janeiro/2018
Título da pesquisa:	Avaliação dos extratos hidroalcoólicos das folhas de *Myracrodruon urundeuva All.* e *Qualea grandiflora Mart.* sobre a viabilidade de biofilme de Streptococcus mutans e na prevenção da desmineralização do esmalte in vitro
Pesquisador Responsável:	Profa. Dra. Ana Carolina Magalhães
Pesquisador Executor:	Sara Salustiano Zabini
Colaboradores:	Juliana Gonçalves Pires, Prof. Dr. Rodrigo Cardoso de Oliveira, Profa. Dra. Flaviana Bombarda de Andrade
Nota Fiscal/Termo de Doação:	Termo de Doação Frigol S.A. **Total adquirido/doado:** 200 dentes incisivos inferiores
Nº Lote: 4 e 5	**Data do abate:** 06/12/2016
Nºde dentes bovinos utilizados:	20

Al. Dr. Octávio Pinheiro Brisolla, 9-75 – Bauru-SP – CEP 17012-101 – C.P. 73
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ANNEX 7

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DA EMENDA

Título da Pesquisa: Avaliação dos extratos de Myracrodruon urundeuva e Qualea grandiflora sobre a viabilidade e atividade de biofilme microcosmo e na prevenção da desmineralização do esmalte in vitro.

Pesquisador: Juliana Gonçalves Pires

Área Temática:

Versão: 4

CAAE: 43948115.2.0000.5417

Instituição Proponente: Faculdade de Odontologia de Bauru

Patrocinador Principal: Universidade de São Paulo - Faculdade de Odontologia de Bauru

DADOS DO PARECER

Número do Parecer: 1.959.539

Apresentação do Projeto:

Trata-se de uma Emenda para anexar Comprovante de Registro da CEEPA, devido a utilização de dentes bovinos no estudo.

Objetivo da Pesquisa:

Idem ao Parecer 1.172.925

Avaliação dos Riscos e Benefícios:

Idem ao Parecer 1.172.925

Comentários e Considerações sobre a Pesquisa:

A pesquisa foi aprovada em 29/07/2015, essa Emenda refere-se a inclusão do Comprovante de Registro da CEEPA.

Considerações sobre os Termos de apresentação obrigatória:

Foi anexado o Comprovante de Registro da CEEPA.

Recomendações:

Sem recomendações

Conclusões ou Pendências e Lista de Inadequações:

Sem pendências

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Considerações Finais a critério do CEP:

A EMENDA encaminhada pela pesquisadora responsável foi analisada por um relator e considerada APROVADA na reunião ordinária do CEP de 18.03.2017. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_870903_E1.pdf	21/02/2017 11:26:39		Aceito
Outros	oficio_animais.pdf	21/02/2017 11:24:23	Juliana Gonçalves Pires	Aceito
Outros	comite_animais.pdf	21/02/2017 11:22:22	Juliana Gonçalves Pires	Aceito
Outros	carta FINAL.pdf	03/06/2015 10:57:38		Aceito
Projeto Detalhado / Brochura Investigador	Projeto doutorado Comite de etica Corrigido.pdf	03/06/2015 10:56:08		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE Juliana Corrigido.pdf	11/05/2015 10:21:20		Aceito
Folha de Rosto	Folha de rosto.pdf	19/02/2015 15:32:41		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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USP - FACULDADE DE ODONTOLOGIA DE BAURU DA USP

Continuação do Parecer: 1.959.539

BAURU, 10 de Março de 2017

Assinado por:
Ana Lúcia Pompéia Fraga de Almeida
(Coordenador)

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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