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DENISE LINS DE SOUSA

EFEITO ANTIBACTERIANO DO ÁCIDO ANACÁRDICO EM CULTURAS PLANCTÔNICAS E BIOFILMES DE *Streptococcus mutans*

FORTALEZA

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Tese apresentada ao Programa de Pós-graduação em Odontologia da Faculdade de Farmácia, Odontologia e Enfermagem da Universidade Federal do Ceará, como requisito parcial para obtenção do título de Doutor em Odontologia.

Área de concentração: Clínica Odontológica

Orientadora: Profª. Drª. Lidiany Karla Azevedo Rodrigues

Co-orientador: Prof. Dr. José Jeová Siebra Moreira Neto

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"Se as coisas são inatingíveis... ora!
não é motivo para não querê-las.
Que tristes os caminhos se não fora
a mágica presença das estrelas!"
(Mário Quintana)

RESUMO

Ácido anacárdico é um composto extraído do líquido da castanha de caju (LCC) e tem emergido como um composto promissor devido a sua variedade de propriedades biológicas. Este estudo está dividido em três capítulos, cujos objetivos foram: capítulo 1) investigar a atividade antibacteriana de uma emulsão de ácidos anacárdicos, extraídos do LCC, e de uma emulsão sintética do ácido anacárdico, contra culturas planctônicas de *Streptococcus mutans*, bem como avaliar sua citotoxicidade *in vitro*; capítulo 2) avaliar o efeito de diferentes concentrações de uma emulsão de ácidos anacárdicos extraídos do LCC contra biofilmes maduros de *S. mutans*; e capítulo 3) avaliar o efeito da aplicação única *versus* aplicação duas vezes ao dia de diferentes concentrações de uma emulsão de ácidos anacárdicos contra biofilmes de *S. mutans*. A atividade antibacteriana das emulsões foi determinada através da concentração inibitória mínima (CIM) e concentração bactericida mínima (CBM), e a citotoxicidade foi mensurada através do reagente CellTiter Blue® (capítulo 1). Biofilmes foram crescidos em discos de hidroxiapatita imersos em caldo de peptona caseína soja e extrato de levedura com 1% sacarose por cinco dias. Biofilmes foram tratados com a emulsão de ácidos anacárdicos por um minuto no último dia do experimento para avaliar seu efeito em biofilme maduro; viabilidade bacteriana e mensuração de peso seco foram realizadas (capítulo 2). Diferentes concentrações da emulsão de ácidos anacárdicos foram aplicadas apenas no último dia do experimento e duas vezes ao dia durante cinco dias para avaliar o efeito de diferentes aplicações da emulsão de ácidos anacárdicos em biofilmes de *S. mutans;* viabilidade bacteriana, mensuração de peso seco e quantificação de polissacarídeos foram realizados (capítulo 3). A CIM e CBM da emulsão de ácidos anacárdicos (LCC) em cultura planctônica foram 0,48 µg/ml; e a CIM da emulsão sintética do ácido anacárdico foi 4,38 µg/ml, mas sua CBM não pôde ser determinada (> 3.200 µg/ml) (capítulo 1). Observou-se uma redução significante na viabilidade bacteriana de biofilmes maduros após tratamento com as concentrações da emulsão de ácidos anacárdicos, mas estas não alteraram o peso seco do biofilme (capítulo 2). O tratamento diário com diferentes concentrações da emulsão de ácidos anacárdicos reduziu a viabilidade bacteriana do biofilme e modificou os níveis de polissacarídeos intracelular e extracelulares (capítulo 3). Pode-se concluir que a emulsão de ácidos anacárdicos apresenta-se como um promissor agente antibacteriano, tendo a capacidade de reduzir a viabilidade do *S. mutans* tanto em culturas planctônicas quanto em biofilmes.

Palavras-chave: *Anacardium occidentale*. *Streptococcus mutans*. Biofilme.

ABSTRACT

Anacardic acid is an extract from processing of cashew nut shell liquid (CNSL) and it has been recognized to have several biological activities. This study is divided into three chapters, whose aims were: chapter 1) to investigate the antibacterial activity of an anacardic acids emulsion, from CNSL, and a synthetic emulsion of anacardic acid against planktonic cultures of *S. mutans* as well as to evaluate its cytotoxic effect *in vitro*; chapter 2) to evaluate the effect of different concentrations of an anacardic acids emulsion against *Streptococcus mutans* mature biofilm; and chapter 3) to evaluate the effect of a single and daily treatment of an anacardic acids emulsion against *Streptococcus mutans* biofilm. The antibacterial activity of the emulsions was determined using the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), and the cytotoxicity was evaluated using CellTiter-Blue® cell viability (chapter 1). The biofilms were grown on hydroxyapatite discs and immersed in tryptone yeast-extract broth containing 1% (w/v) sucrose for 5 days. The biofilms were exposed to anacardic acids emulsion for 1 min on the last day of experiment to evaluate its effect on mature biofilm; bacterial viability and dry weight were analyzed (chapter 2). Different concentrations of anacardic acids emulsion were applied on the last day of the experiment and twice daily until the fifth day to evaluate the effects of different treatments of anacardic acids emulsion on *S. mutans* biofilms; bacterial viability, dry weight and polysaccharides were analyzed (chapter 3). The MBC and MIC of the anacardic acids emulsion (from CNSL) on planktonic culture were 0.48 μg/ml; the MIC of the synthetic emulsion of anacardic acid was 4.38 μg/ml, but the MBC could not be determined (> 3,200 μg/ml) (chapter 1). Significant decreases in the viability of mature biofilms were observed after anacardic acids emulsion treatment, but they did not change the amount of dry weight (chapter 2). The daily treatment with different concentrations of anacardic acids emulsion decreased the bacterial viability and modified the polysaccharides levels on biofilm (chapter 3). We concluded that anacardic acids emulsion is a promising antibacterial agent, and it can decrease *S. mutans* viability in planktonic cultures and in biofilms.

Keywords: *Anacardium occidentale*. *Streptococcus mutans*. Biofilm.

SUMÁRIO

1 INTRODUÇÃO GERAL

A cárie dentária é a doença oral mais prevalente mundialmente e ainda é considerada um grande problema de saúde bucal, afetando 60-90% das crianças em idade escolar e a grande maioria dos adultos (OMS, 2014). A cárie é uma doença biofilme-açúcar-dependente que, na ausência de tratamento, progride, podendo afetar desde os tecidos mineralizados do órgão dentário até a polpa (FEJERSKOV, 2005).

O *Streptococcus mutans* é o principal agente associado ao início e desenvolvimento da lesão cariosa. Esta bactéria sintetiza polissacarídeos intracelulares (PI) e extracelulares (PE), principalmente do tipo glucano, a partir da sacarose da dieta, e possui características acidogênicas e acidúricas, as quais são considerados fatores de virulência críticos envolvidos na patogênese da cárie (LOESCHE, 1986; YAMASHITA *et al.*, 1993; PAES LEME *et al.*, 2006; DUARTE *et al.*, 2008).

A produção de PE pelo *S. mutans* desencadeia a formação de biofilme, pré-requisito fundamental para ocorrência de lesões cariosas (BOWEN e KOO, 2011). Os biofilmes são comunidades de microrganismos que se aglomeram dentro de uma matrix de PE, sendo considerados importantes fatores de virulência, a medida que melhoram e promovem a aderência e co-aderência de diferentes microrganismos, atuam como reserva de energia, afetam a difusão de substâncias para dentro e fora do biofilme, e ajudam a concentrar íons metálicos e outros nutrientes fisiológicos dentro de um microambiente (HAYACIBARA *et al.*, 2003; PAES LEME *et al.*, 2006; ARPANA e YADAV, 2008; FLEMMING e WINGENDER, 2010; KOO *et al.*, 2010; BOWEN e KOO, 2011). Além disso, biofilmes podem proteger os microrganismos de condições adversas, e o estado fisiológico das células bacterianas no biofilme confere um alto nível de resistência aos agentes antimicrobianos (COSTERTON; STEWART; GREENBERG, 1999; ARPANA e YADAV, 2008).

O tratamento de doenças relacionadas à presença de biofilme bacteriano envolve sua remoção mecânica e uso de antissépticos e antibióticos. Na Odontologia, a clorexidina desponta como um potente agente antimicrobiano e tem sido estudada exaustivamente. Ela tem se mostrado com um amplo espectro de ação, sendo altamente eficaz para uma grande variedade de microrganismos, e tem sido utilizada como padrão ouro de tratamento e como grupo controle de experimentos contra a qual é medida a potência de outros agentes. No entanto, embora seja um excelente antimicrobiano, devido a efeitos colaterais, como sabor desagradável e capacidade de pigmentação das estruturas dentárias, o seu uso prolongado não

é recomendado. Logo, torna-se necessária a descoberta de outras substâncias que tenham eficácia, porém sem tantos efeitos colaterais. Somado-se a isso, a emergente resistência aos antibióticos pelas bactérias patogênicas leva a grandes esforços para descoberta de alternativas terapêuticas antimicrobianas que não induzam tal resistência (HAMBLIN e HASAN, 2004).

Neste contexto, as plantas medicinais têm se tornado uma importante opção terapêutica. Um dos fatores que contribui para a larga utilização de plantas para fins medicinais no Brasil é o grande número de espécies vegetais encontradas no país (NUNES *et al.*, 1999). Assim, tendo em vista a alta disponibilidade e o baixo custo, os produtos naturais contribuem para melhorar o acesso da população aos cuidados com a saúde. De acordo com a Organização Mundial da Saúde (OMS), cerca de 80-85% da população mundial de países em desenvolvimento, devido à pobreza e a falta de acesso à medicina moderna, dependem essencialmente das plantas para a saúde primária (PERAZZO *et al.*, 2004). Porém, poucas plantas têm sido cientificamente estudadas para uma avaliação segura de suas propriedades e eficácia, necessitando de estudos mais detalhados sobre seu uso terapêutico (NASCIMENTO; LOCATELLI; FREITAS, 2000). Dessa forma, a OMS, desde 1977, tem incentivado o estudo de plantas medicinais com o objetivo de avaliar cientificamente os benefícios da utilização de medicamentos fitoterápicos e de conhecer, ao mesmo tempo, os riscos de seu uso indevido (YUNES e CECHINEL FILHO, 2001).

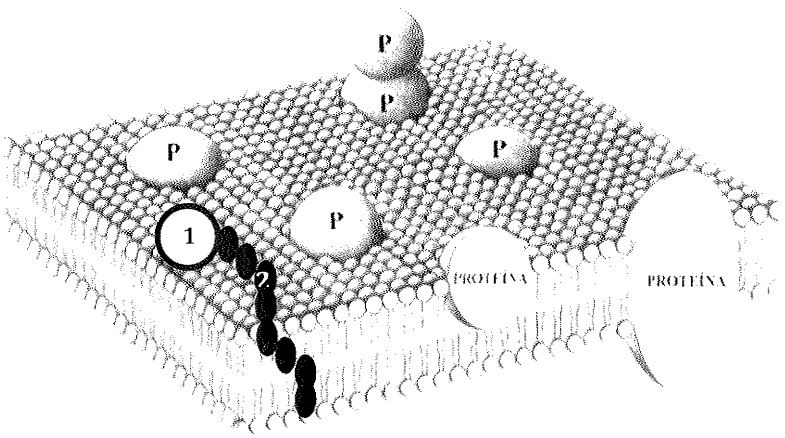
O líquido da castanha de caju (LCC) tem emergido como um composto promissor devido a sua variedade de propriedades biológicas, e muitas destas tem sido atribuídas ao ácido anacárdico, componente ativo extraído do LCC (TREVISAN *et al.*, 2006; DE LIMA *et al.*, 2008). Dentre suas atividades biológicas, tem sido relatado que o ácido anacárdico atua como anti-inflamatório e analgésico, antioxidante, antibacteriano e anti-tumoral (HIMEJIMA e KUBO, 1991; MUROI e KUBO, 1993; TREVISAN *et al.*, 2006; KUBO *et al.*, 2006; GREEN *et al.*, 2008; HEMSHEKHAR *et al.*, 2011; RIVERO-CRUZ *et al.*, 2011; ONASANWO *et al.*, 2012; AL-HAZZANI *et al.*, 2012; MAMIDYALA *et al.*, 2013).

Também tem-se observado que o ácido anacárdico possui uma potencial ação antibacteriana contra bactérias Gram-positivas, como o *S. mutans* (GREEN *et al.*, 2008), porém seu efeito em biofilmes não tem sido investigado. Sua atividade antimicrobiana tem sido relacionada em função da interação entre o comprimento da cadeia lateral hidrofóbica e o grupo hidroxila hidrofílico; a porção hidrofílica da molécula do ácido anacárdico liga-se a um hidrogênio intramolecular da membrana plasmática da célula-alvo, e, logo após, a porção

hidrofóbica da molécula é capaz de entrar na bicamada lipídica da membrana celular, rompendo a membrana plasmática (GREEN *et al.*, 2007) (Figura 1).

O ácido anacárdico apresenta-se como uma molécula anfipática, possuindo regiões hidrofílica e hidrofóbica, porém suas propriedades hidrofóbicas predominam na molécula, uma vez que a principal resposta para a atividade antibacteriana do ácido anacárdico depende da sua cadeia lateral hidrofóbica. Estudos têm relatado que a atividade antibacteriana do ácido anacárdico é proporcional a insaturação da sua cadeia lateral, ou seja, quanto maior o número de duplas ligações na cadeia lateral, maior efeito antibacteriano o composto exibirá (HIMEJIMA e KUBO, 1991; MUROI e KUBO, 1993; GREEN et al., 2008).

Figure 1. Esquema representativo do mecanismo de ação do ácido anacárdico.



Porção hidrofílica da molécula do ácido anacárdico (1) liga-se a um hidrogênio da membrana celular e, em seguida, a cadeia lateral hidrofóbica (2) penetra na bicamada lipídica da membrana celular, causando sua ruptura
Fonte: CITOLOGIA, 2014.

Neste estudo, uma mistura de ácidos anacárdicos foi isolada do LCC, sendo este extraído de castanhas de caju colhidas na estação experimental da Embrapa Agroindústria Tropical (Paraípaba, Ceará) durante a tempoarada de 2012. O extrato é composto por diferentes ácidos anacárdicos, que é mais eficiente do que os compostos isolados, e a caracterização destes foram previamente descritas por Trevisan *et al.* (2006). Por se tratar de um composto insolúvel em água, o extrato foi diluído em 5% de etanol e 1% de tween 20, obtendo-se uma emulsão de ácidos anacárdicos. Dessa forma, este estudo propôs-se a avaliar o efeito antibacteriano de uma nova emulsão composta por uma mistura de ácidos anacárdicos em culturas planctônicas e biofilmes de *S. Mutans*, sob diferentes formas de aplicação.

2 PROPOSIÇÃO

Esta tese de doutorado é apresentada em três capítulos, tendo como objetivos:

Capítulo 1: Investigar a atividade antibacteriana de uma emulsão de ácidos anacárdicos, extraídos do LCC, e de uma emulsão de um ácido anacárdico sintético, contra culturas planctônicas de *Streptococcus mutans*, bem como avaliar a citotoxicidade da emulsão natural *in vitro*.

Capítulo 2: Avaliar o efeito de diferentes concentrações de uma emulsão de ácidos anacárdicos extraídos do LCC contra biofilmes maduros de *S. mutans*.

Capítulo 3: Avaliar o efeito da aplicação única e aplicação diária de diferentes concentrações de uma emulsão de ácidos anacárdicos contra biofilmes de *S. mutans*, a fim de avaliar o efeito da emulsão de ácidos anacárdicos no biofilme maduro e no biofilme em formação, respectivamente.

3 CAPÍTULOS

Esta tese está baseada no artigo 46 do regimento Interno do Programa de Pós-graduação em Odontologia da Universidade Federal do Ceará, que regulamenta o formato alternativo para dissertações de Mestrado e teses de Doutorado, e permite a inserção de artigos científicos de autoria ou co-autoria do candidato. Dessa forma, esta tese é composta por três capítulos, contendo artigos a serem submetidos para publicação em revistas científicas, conforme descrito abaixo:

Capítulo 1

"*Effect of a natural and synthetic emulsion of anacardic acids on Streptococcus mutans.*" Denise L. Sousa, Ramille A. Lima, Meera D. Rathi, Nat Alia Tyhovych, Francisco Fábio O. Sousa, Lidiany K.A. Rodrigues, Malvin Janal, Simone Duarte. Este artigo será submetido à publicação no periódico "Food Chemistry".

Capítulo 2

"*Effect of anacardic acids emulsion on Streptococcus mutans biofilm.*" Denise L. Sousa, Ramille A. Lima, Meera D. Rathi, Francisco Fábio O. Sousa, Lidiany K.A. Rodrigues, Malvin Janal, Simone Duarte. Este artigo será submetido à publicação no periódico "Food Chemistry".

Capítulo 3

"*Evaluation of single and daily treatment of an anacardic acids emulsion against S. mutans biofilm.*" Denise L. Sousa, Ramille A. Lima, Meera D. Rathi, Francisco Fábio O. Sousa, Lidiany K.A. Rodrigues, Malvin Janal, Simone Duarte. Este artigo será submetido à publicação no periódico "PlosOne".

3.1 Capítulo 1

Effect of a natural and synthetic emulsion of anacardic acids on *Streptococcus mutans*

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Abstract

The aim of this study was to investigate the antibacterial activity of emulsions of natural and synthetic anacardic acids against planktonic cultures of *S. mutans* as well as its cytotoxic effect *in vitro*. The antibacterial activity of the emulsions was determined by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Cytotoxicity was evaluated using CellTiter-Blue cell viability. The natural emulsion dissolved in milli-Q water showed the strongest bactericidal activity against planktonic cultures of *S. mutans*, and both the MBC and MIC were 0.48 µg/ml. The MIC of the synthetic emulsion was 4.38 µg/ml, but its MBC wasn't determined (> 3,200 µg/ml). Two concentrations (662 µg/ml and 1,324 µg/ml) of natural emulsion were analyzed for cytotoxicity and both showed a moderate cytotoxic effect on fibroblasts (54% Ine 53%, respectively). We observed that the natural emulsion of anacardic acids exhibited a strong antibacterial effect against *S. mutans*, however its cytotoxic effects need to be further investigated.

Keywords: *Anacardium occidentale* Linn, *Streptococcus mutans*, Antibacterial Agents

1. Introduction

The cashew tree, *Anacardium occidentale* Linn, is a tropical tree originating in northeast Brazil. Its fruit consists of an outer shell (epicarp), a tight-fitting inner shell (endocarp), and a strongly vesicant cashew nut shell liquid (CNSL) (De Lima et al., 2008). CNSL contains active ingredients such as anacardic acid, cardanol and cardol (De Lima et al., 2008; Trevisan et al., 2006). It has many potential medicinal applications including analgesics and anti-inflammatories (Onasanwo, Fabiyi, Oluwole, & Olaleye, 2012; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011), and retains the capacity to act as an antioxidant (Trevisan et al., 2006; Kubo, Masuoka, Ha, & Tsujimoto, 2006; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011), an antibacterial agent (Muroi & Kubo, 1993; Rivero-Cruz, Esturau, Sanchez-Nieto, Romero, Castillo-Juarez, & Rivero-Cruz, 2011; Green, Tocoli, Lee, Nihei, & Kubo, 2008; Himejima & Kubo, 1991; Mamidyala, Ramu, Huang, Robertson, & Cooper, 2013; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011), an anti-tumor agent (Al-Hazzani, Periyasamy, Subash-Babu, & Alshatwi, 2012; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011) and a molluscicide agent (Sullivan, Richards, Lloyd, & Krishna, 1982; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011). The majority of CNSL properties are related to the presence of anacardic acid (Himejima & Kubo, 1991; Trevisan et al., 2006).

The biological activities of anacardic acid include its potent antibacterial activity against the Gram-positive bacteria, such as *Streptococcus mutans* (Green, Tocoli, Lee, Nihei, & Kubo, 2008). *S. mutans* is considered the primary agent associated with the initiation and development of caries lesions. It has a capacity to adhere to the enamel surface and to other bacterial species as well. *S. mutans* is also able to produce acid and survive in an acidic environment, which makes it an acidogenic and aciduric specie, respectively, and retains the ability to form a pathogenic biofilm (Krzysciak, Pluskwa, Jurczak, & Koscielniak, 2013).

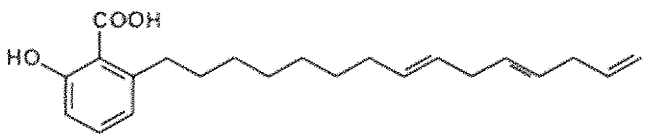
Anacardic acid's antibacterial activity against both Gram-positive and Gram-negative

bacteria is proportional to the degree of the side chain unsaturation and its antibacterial property increases as the side chain unsaturation increases (Kubo, Lee, & Kubo, 1999; Green, Tocoli, Lee, Nihei, & Kubo, 2008). However, studies have shown that anacardic acid is unstable for practical applications because of its side chain unsaturation and new research studies are being carried out to synthesize anacardic acid analogues possessing different side chains but preserving its antimicrobial effect (Green, Tocoli, Lee, Nihei, & Kubo, 2008; Kubo, Lee, & Kubo, 1999). The purpose of this study was to investigate the antibacterial activity of a natural emulsion of anacardic acids, isolated from CNSL, and a synthetic emulsion of anacardic acid against planktonic cultures of *S. mutans* as well as cytotoxic effect of natural emulsion *in vitro*. Our alternative hypothesis was that the natural emulsion, being composed of a mixture of anacardic acids, should be more efficient than the synthetic emulsion.

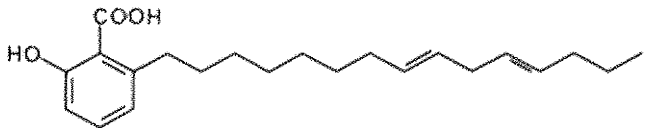
2. Materials and methods

2.1. Chemicals. The cashews (*A. occidentale* Linn) were harvested at the Embrapa Tropical Agroindustry Experimental Station, located at Paraibapa, Ceara, Brazil during the 2012 season. The cashew product extraction and isolation of anacardic acids from CNSL were previously reported in the literature (Trevisan et al., 2006). The natural extract is composed only by anacardic acids and its structures are shown in the Figure 1. The synthetic anacardic acid was purchased from Biovision, Inc. (San Francisco, USA) and its structure is shown in the Figure 2. The anacardic acids emulsions were obtained by dissolving them in 5% ethanol + 1% tween 20 (Sigma, St. Louis, USA). Milli-Q water and phosphate buffer saline (PBS) were used as vehicles and the pH was checked (Mettler Toleto, Columbus, OH, USA).

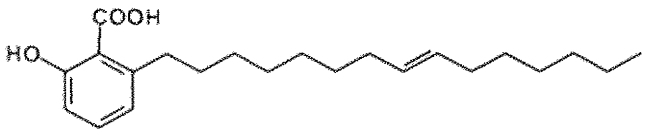
Anacardic acid -1 (I)



Anacardic acid-2 (II)



Anacardic acid-3 (III)



Anacardic acid-4 (IV)

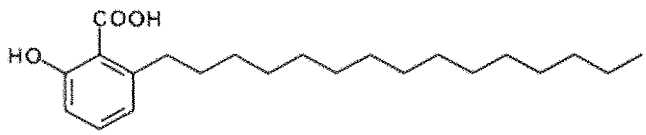


Figure 1. Structures of the anacardic acids from CNSL. (I) Anacardic acid – $C_{15:3}$, (II) Anacardic acid – $C_{15:2}$, (III) Anacardic acid – $C_{15:1}$, (IV) Anacardic acid – $C_{15:0}$. (Illustration from Trevisan *et al*, 2006).

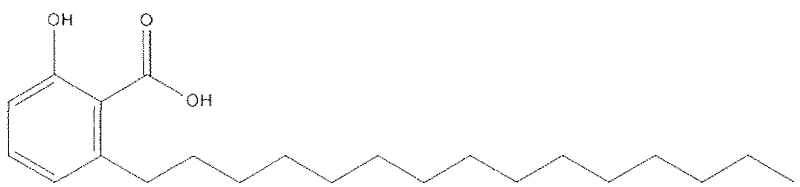


Figure 2. Structure of the synthetic anacardic acid purchased from Biovision, Inc. Anacardic acid – $C_{15:0}$ (Illustration from http://www.biovision.com/anacardic-acid-4549.html).

2.2. Microorganism and media. *Streptococcus mutans* UA159 (UAB577) was obtained from single colonies isolated on tryptic soy agar (TSA) plates, inoculated in tryptone yeast-extract broth (TYE) containing 1 % (w/v) glucose, and incubated for 18–24 h at 37°C in 5 % CO_2. The optical density was measured in a spectrophotometer (OD540) and the inoculum size was adjusted to 2.0×10^6 colony forming units (CFU)/ml.

2.3. Antibacterial activity. The antibacterial activity of the anacardic acids emulsions was determined by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in accordance with the CLSI guidelines (NCCLS, 1999; CLSI, 2006). For MIC determination, the broth microdilution method was used (NCCLS, 1999). In a sterile 96-well plate, it was performed serial dilution (1:3) of stock solution of the antimicrobial agent in TYE broth, and 5% of inoculum was added in each well. A 5% ethanol + 1% tween 20 solution and 0.12% chlorhexidine solution were used as negative and positive controls, respectively. The stock solution concentration used for the assay was 3,200 µg/ml and it was based on the highest concentration found in previous studies (Himejima & Kubo, 1991; Muroi, Nihei, Tsujimoto, & Kubo, 2004). The MIC value was detected by ELISA plate reader (SpectraMax M5, Molecular Devices, USA) at 540 nm after 24 hours of incubation at 37°C in 5 % CO_2. The MIC was defined as the lowest concentration of antimicrobial agent that completely inhibited growth of the organism in the wells (NCCLS, 1999). After the determination of the MIC, aliquot of 50 µl from well-plates showing no turbidity were plated onto a trypticase soy agar with 5% sheep blood (BD, USA) and incubated for 48 h at 37°C in 5 % CO_2. The MBC was defined as the minimal concentration of drug needed to kill most (≥99.9%) of the viable organisms after incubation (CLSI, 2006). Three replicates were performed.

2.4. Cell culture. NOR-10 (ATCC CCL-197) cell line was provided by the Department of Basic Science, New York University College of Dentistry, New York, New York. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serumk, 1% antimycotic solution and 0.1% gentamicin. The cells were incubated at 37°C in a humidified atmosphere of 5 % CO_2. The growth medium was replaced every 2 days. When cells had reached approximately 80% of confluency, the cells were washed with

phosphate-buffered saline (PBS) and trypsinized for 2 min at 37°C with 0.25% trypsin. Cells from passages two to four were used for the cytotoxicity assay and microscopy.

2.5. Cytotoxicity assay. The fibroblasts were grown in a flat-bottom plate (24-well culture plate, Corning, USA) and the cytotoxicity of the anacardic acids emulsion was analyzed using the CellTiter-Blue® Cell Viability Assay (Promega Corporation, Madison, WI, USA). The emulsion concentrations used for the cytotoxicity assay were approximately 1000x the MIC (C2) and half of this concentration (C1) of the anacardic acids emulsion that exhibited the highest MIC and highest pH. The experiment was randomly divided into three groups: one group was treated with C1, one group was treated with C2, and one group was not treated (control group). Briefly, fibroblast cells (5×10^3 cells/ ml) were planted in the culture cell plate overnight at 37°C. The cells were incubated with both concentrations of the anacardic acids emulsion for 1 h and after washed with PBS. CellTiter-Blue® reagent was added into each well. The plates were wrapped with aluminum foil and incubated for 4 h at 37°C in 5 % CO_2. After shaking for 10 s, the supernatant from the 24-well plate was transferred to a 96-well plate and the optical density (OD) in each well was read by an ELISA plate reader (SpectraMax M5, Molecular Devices, USA) at 560/590 nm. The cytotoxicity was calculated using the following equation:

Cytotoxicity (%) = [(OD control group – OD treated group)/ OD control group] x 100%

2.6. Fluorescence microscopy. Cells (5×10^3 cells/ml) were plated on culture slides and incubated overnight to allow adhesion. The medium was removed and replaced by anacardic acids emulsion (C1 and C2) for 1h. The cells were stained with Hoescht probe (Invitrogen, USA) following the manufacturer's instructions. Cells were treated with paraformaldehyde to fix the samples and changes in nucleus morphology were observed under a fluorescence microscope (Nikon Eclipse E600).

2.7. Statistical analyses. Prior to analysis, assumptions of equality of variances and normal distribution of errors were checked. The data were analyzed with a mixed model ANOVA with a fixed factor of group and a random intercept. When significant differences were detected, pairwise comparisons were made between all of the groups using t-tests based on the pooled standard error. IBM SPSS version 21 (IBM, Inc., Armonk, NY) was used to perform the analyses. The confidence interval was set at 95%.

3. Results

The MIC, MBC and pH of the natural and synthetic emulsions of anacardic acids using different vehicles for planktonic cultures of S. *mutans* are listed in Table 1. The natural emulsion of anacardic acids dissolved in milli-Q water showed the strongest bactericidal activity against planktonic cultures of *S. mutans*, and both the MBC and MIC were 0.48 µg/ml. The MIC of the natural emulsion of anacardic acids dissolved in PBS was 1.46 µg/ml and its MBC was 4.28 µg/ml. The MIC of both the synthetic emulsion of anacardic acid dissolved in milli-Q water and PBS was 4.38 µg/ml and the MBC wasn't found for both (>3,200 µg/ml). The pH of the natural and synthetic emulsions of anacardic acids dissolved in PBS (5.2 and 6.4, respectively) was greater than when dissolved in milli-Q water (3.4 and 3.7, respectively).

Table 1. MIC, MBC and pH of the natural emulsion of anacardic acids and synthetic emulsion of anacardic acid using Milli-Q water and PBS like vehicle against planktonic cultures of *S. mutans*.

	MIC (µg/ml)	**MBC (µg/ml)**	**pH**
Natural emulsion + Milli-Q water	0.48	0.48	3.4
Natural emulsion + PBS	1.46	4.38	5.2
Synthetic emulsion + Milli-Q water	4.38	> 3,200	3.7
Synthetic emulsion + PBS	4.38	> 3,200	6.4

In relation to the cytotoxicity, both concentrations (C1=662 µg/ml and C2=1,324

μg/ml) of the natural emulsion of anacardic acids indueed the death of eells and showed cytotoxic effect (Figure 3). The cytotoxicity levels were 54% (± 0.43) and 53% (± 0.45) after the treatment with C1 and C2, respectively. There was a significant reduction of cells after treatment with C1 and C2 when compared to the normal cells (control group) ($p < 0.05$). Figure 4 illustrates the cells' nuclei after the treatment with the natural emulsion of anacardic acids dissolved in PBS, showing the number of cells decreased significantly after the treatment.

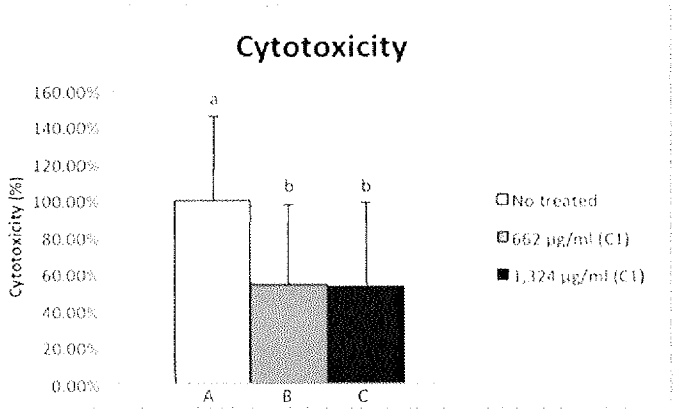


Figure 3. Mean of cytotoxicity of the cells treated with two concentrations of natural emulsion of anacardic acids. A = no treated cells (control group); B = cells treated with 662 μg/ml of anacardic acids emulsion; C = cells treated with 1,324 μg/ml of anacardic acids emulsion. Data represent the mean values and error bars represent standard deviations. Values marked by the different letters are significantly different from each other ($p < .05$).

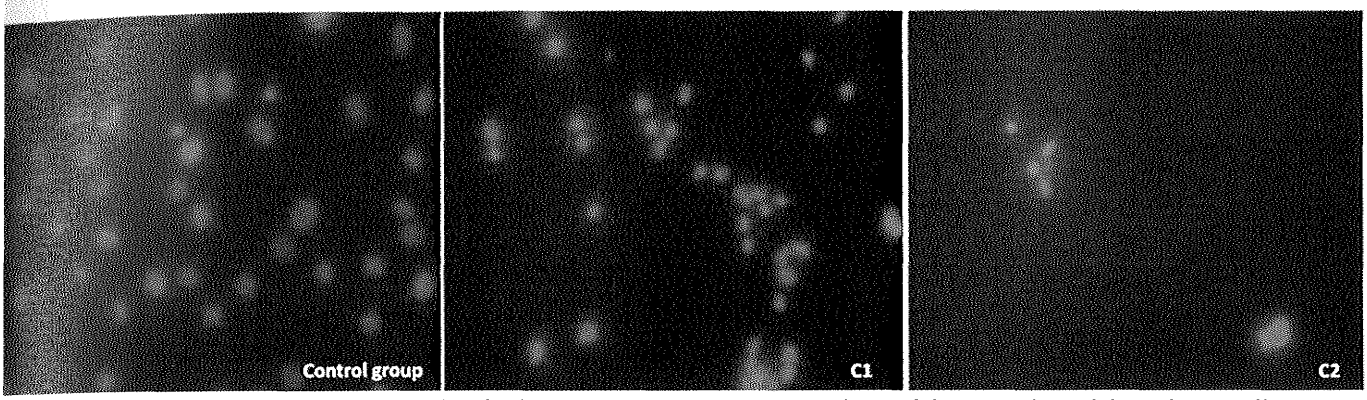


Figure 4. The fluorescence analysis of cells treated with two concentrations of the natural emulsion of anacardic acids. Control group = no treated cells; C1 = cells treated with 662 µg/ml of anacardic acids emulsion; C2 = cells treated with 1,324 µg/ml of anacardic acids emulsion. The nuclei are shown in blue. There was a reduction of the number of cells, and the cells also show a decrease in size of the nucleus in C1 and C2.

4. Discussion

In this study, we investigated the antibacterial activity of a natural emulsion of anacardic acids, isolated from CNSL, and a synthetic emulsion of anacardic acid against planktonic cultures of *S. mutans*. The cytotoxicity of the natural emulsion of anacardic acids was evaluated as well. The natural emulsion of anacardic acids is a mixture of different anacardic acids ($C_{15:3}$, $C_{15:2}$, $C_{15:1}$, $C_{15:0}$), and the synthetic emulsion is composed of just one kind of anacardic acid ($C_{15:0}$). Basically, the difference amongst these anacardic acids is the side chain unsaturation.

Studies (Himejima & Kubo, 1991; Muroi & Kubo, 1993; Green, Tocoli, Lee, Nihei, & Kubo, 2008) have related that the antimicrobial activity of anacardic acids have been proportional to the degree of the side chain unsaturation and can be summarized in the following decreasing order of antibacterial activity: $C_{15:3} > C_{15:2} > C_{15:1}$. In our study, the natural emulsion of anacardic acids is a mixture of different anacardic acids, which can explain the strong antibacterial activity of this emulsion. However, the synthetic emulsion is only composed of one type of anacardic acid ($C_{15:0}$). Anacardic acid ($C_{15:0}$) has previously been reported to show high selectivity toward Fe^{2+} and Cu^{2+}, and this finding implies that chelation might also play a role in the antimicrobial activity of anacardic acid by reducing

their bioavailability for bacteria (Fitzgerald, 1972; Nagabhushana, Shobha, & Ravindranath, 1995; Green, Tocoli, Lee, Nihei, & Kubo, 2007).

Another study (Muroi & Kubo, 1993) also indicated that the antibacterial activity of anacardic acids against *S. mutans* slightly decreased with the decreasing number of double bonds and this result indicates that the activity is affected not only by the lengths of the side chains, but also by the degree of unsaturation in the side chain. It is evident from these results that the mechanism of the bactericidal action of anacardic acids against *S.mutans* is due to a balance between the hydrophilic and hydrophobic parts of the molecule similar to their cytotoxic activity (Muroi & Kubo, 1993).

In relation to the antibacterial activity, our results showed that the natural emulsion of anacardic acids in Milli-Q water and PBS exhibited a strong antimicrobial activity in a low concentration (MIC of 0.48 µg/ml and 1.46 µg/ml, respectively) but the same result was not observed when using the synthetic emulsion in both Milli-Q water and PBS (MIC of 4.38 µg/ml for both). These results are in agreement with a previous study (Kubo, Nihei, & Tsujimoto, 2003) that investigated the antibacterial activity of anacardic acids against *S. mutans* and strains of methicillin-resistant *Staphylococcus aureus* (MRSA). These authors observed that among the compounds tested, anacardic acid-$C_{15:3}$, anacardic acid-$C_{12:0}$, and anacardic acid-$C_{10:0}$ retained the most potent antibacterial activity, each with an MBC of 6.25 µg/ml against MRSA, but no differences in their MICs and MBCs were noted, suggesting that their activity is bactericidal. On the other hand, anacardic acid-$C_{15:0}$ did not exhibit any activity against MRSA up to 800 µg/ml. Also another study (Himejima & Kubo, 1991) has demonstrated that cashew nut shell oil exhibited activity against Gram-positive bacteria and the most sensitive bacteria were S. *mutans* and *B. ammoniagenes* (MIC of 3.13 µg/ml).

The maximum antimicrobial activity of anacardic acid has been reported to be an interactive function of the hydrophobic alkyl (tail) chain length and the hydrophilic hydroxyl

group (head). Studies (Kubo, Muroi, & Kubo, 1995; Kubo, Nihei, & Tsujimoto, 2003; Green, Tocoli, Lee, Nihei, & Kubo, 2007; Green, Tocoli, Lee, Nihei, & Kubo, 2008) have proposed that the hydrophilic head moiety binds with an intermolecular hydrogen bond like a 'hook' attaching itself to a hydrophilic portion of the membrane, after which the hydrophobic tail portion of the molecule is then able to enter into the membrane lipid bilayer. As a result, disorder in the fluid bilayer of the membrane is created and this conclusion may be explained by the alcohols' non-ionic surfactant properties as well as by their non-specificity in the activity. According to these authors, it also appears that these biophysical processes are the major contributors to the antimicrobial activity of amphipathic alkanols.

In our study, we also evaluated the cytotoxicity of the natural emulsion of anacardic acids on fibroblast cells. The concentrations (C1=662 µg/ml and C2=1,324 µg/ml) used were based on the emulsion of anacardic acids that exhibited the highest MIC and highest pH. Our results show that C1 and C2 showed a moderate cytotoxic effect and reduced the cell numbers. An *in vitro* study (Kubo, Nitoda, Tocoli, & Green, 2011) showed that anacardic acids and cardols exhibited moderate cytotoxicity on melanoma cells. The same author observed that the cytotoxicity of anacardic acid ($C_{15:1}$) is dose-dependent and the concentration of 2.8 µg/ml lead to the loss of 50% of viable cells. Also, anacardic acids with varied chain lengths induced cytotoxicity towards several human cancer cell lines *in vitro* (Hemshekhar, Santhosh, Kemparaju, & Girish, 2011). Anacardic acids ($C_{12:0}$) and ($C_{15:1}$) exhibited nearly comparative cytotoxicity, suggesting the non-essentiality of unsaturation in the hydrophobic side chain. However, the molecular volume of the hydrophobic side chain is one of the main determinants of cytotoxicity (Hemshekhar, Santhosh, Kemparaju, & Girish, 2011).

Some studies using the same mixture of anacardic acids used in our study showed that the mixture of anacardic acids didn't exhibited cytotoxic effect in animal models (Carvalho et

al., 2011; Morais et al., 2010). Anacardic acid has become a compound of interest in recent years due to its anticancer, gastroprotection and antiinflamatory properties (Mota, Thomas, & Barbosa Filho, 1985; Morais et al., 2010; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011). The anticancer activity exhibited by anacardic acids could be due to its ability to act as a surfactant, although the possibility of chelating the essential metals can not be denied (Hemshekhar, Santhosh, Kemparaju, & Girish, 2011). Morais *et al.* (2010) showed that the pretreatment with the mixture of anacardic acids at doses of 10, 30 and 100 mg/kg markedly reduced the gastric damage induced by ethanol in a dose-related manner in mice and suggest that the gastroprotective role of anacardic acids can be mediated to a major extent by an antioxidant mechanism.

5. Conclusion

We observed that the natural emulsion of anacardic acids exhibited a strong antibacterial effect against planktonic cultures of *S. mutans*, and it can be considered a promising antimicrobial agent. We accepted the alternative hypothesis that the natural emulsion is more efficient than the synthetic emulsion. We suggest that further studies need to be conducted to evaluate the cytotoxicity of anacardic acids by employing other methodologies and concentrations.

6. Acknowledgment

We thank Dr. Nagila Ricardo, who provided us the anacardic acid. This research was supported by CAPES Foundation from whom the first and second authors received a scholarship in Brazil and during the PhD sandwich doctorate (Process 18494-12-9 and 7715-13-7, respectively).

7. Conflict of interest

All authors don't have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years

of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

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3.2 Capítulo 2

Effect of anacardic acids emulsion on *Streptococcus mutans* biofilm

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Abstract

The aim of this study was to evaluate the antimicrobial effect of an anacardic acids emulsion against *Streptococcus mutans* biofilm. The biofilms were formed on saliva-coated hydroxyapatite discs in batch culture during 5 days in the presence of 1% sucrose. At the end of the experimental period, single treatments with anacardic acids emulsion in two different concentrations (C1 = 132.4 μg/ml; C2 =1,324 μg/ml) were applied to biofilms. Biofilms were used for dry weight and colony-forming unit (CFU) analyses. Variable pressure scanning electron microscopy and confocal laser scanning microscopy were used to check biofilms morphology and viability, respectively. Significant decreases in the viability of biofilms were observed after treatment with anacardic acids emulsion. C2 presented a better antimicrobial effect than 0.12% chlorhexidine and C1 ($p < 0.05$). These results indicate that the anacardic acids emulsion has an antibacterial effect against *S. mutans* biofilm, statistically higher than chlorhexidine in the highest concentration tested, and therefore represents a promising antimicrobial agent.

Keywords: *Anacardium occidentale* Linn., biofilm, *Streptococcus mutans*.

Anacardic acid is an extract from the processing of cashew nut shell liquid (CNSL) and it has been recognized to have several biological activities (Sullivan, Richards, Lloyd, & Krishna, 1982; Himejima & Kubo, 1991; Muroi & Kubo, 1993; Trevisan et al., 2006; Kubo, Masuoka, Ha, & Tsujimoto, 2006; Green, Tocoli, Lee, Nihei, & Kubo, 2008; Rivero-Cruz, Esturau, Sanchez-Nieto, Romero, Castillo-Juarez, & Rivero-Cruz, 2011; Onasanwo, Fabiyi, Oluwole, & Olaleye, 2012; Al-Hazzani, Periyasamy, Subash-Babu, & Alshatwi, 2012; Mamidyala, Ramu, Huang, Robertson, & Cooper, 2013). The biological activities of anacardic acids have been reported as well, which include their potent antibacterial activity against *S. mutans* (Green, Tocoli, Lee, Nihei, & Kubo, 2008). Accordingly in our continuous efforts to investigate anacardic acids and oral biofilms (data not published yet), the aim of this investigation was to evaluate the effect of two different concentrations of an anacardic acids emulsion against mature *S. mutans* biofilm. Our null hypothesis was that there is no difference between the concentrations evaluated.

2. Materials and methods

2.1. Chemical. The cashews (*A. occidentale* Linn.) were harvested at the Embrapa Tropical Agroindustry Experimental Station, located at Paraibapa, Ceara, Brazil during the 2012 season. The cashew product extraction and isolation of anacardic acids from CNSL were previously reported in the literature (Trevisan et al., 2006). The anacardic acids extract was dissolved in 5% ethanol + 1% tween 20 (Sigma, St.Louis, MO), and milli-Q water was used as vehicle. The concentrations that were used were 132.4 μg/ml (C1) and 1,324 μg/ml (C2), according to the minimum inhibitory concentration and minimum bactericidal concentration found in the previous study (data not published yet).

2.2. Inoculum and biofilm model. *Streptococcus mutans* UA159 (UAB577) was obtained from single colonies isolated on agar plates, inoculated in tryptone yeast-extract broth containing 1% (w/v) glucose and incubated for 18–24 h at 37°C under microaerophilic

conditions (5% CO_2). Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs (HA) (surface area of 2.7 ± 0.2 cm^2; Clarkson Chromatography Products Inc., South Williamsport, Pa., USA) placed in batch cultures at 37°C in 5% CO_2 for 5 days. Hydroxyapatite disks were coated with filter-sterilized (0.22 μm; polyether sulfone low-protein-binding filter; Millipore Co., Bedford, Mass., USA) clarified human whole saliva for 1 h at 37°C; whole saliva was collected on ice from 1 donor who chewed paraffin film, and it was clarified by centrifugation (8,500 g, 4 ° C, 10 min) (Koo *et al.*, 2003). The biofilms were grown in tryptone yeast-extract broth containing 1% (w/v) sucrose and were kept undisturbed for 24 h to allow initial biofilm formation. The culture medium was replaced daily.

2.3. Treatment with anacardic acid on biofilm. At the end of the experimental period (fifth day), the biofilms were exposed to anacardic acids emulsion. The biofilms were dip-washed three times in 0.89% NaCl and immersed in C1 and C2 for 1 min. Subsequently, the biofilms were dip-washed in 0.89% NaCl again and collected. The 0.12% chlorhexidine and 5% ethanol +1% tween 20 solution were used as positive and negative controls, respectively.

2.4. Biofilm analysis. The biofilms were placed in 5 ml sterile saline solution, and the hydroxyapatite surfaces were gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication using three 15-s pulses at an output of 7 W (Fisher Scientific, Sonic Dismembrator model 100; USA). The homogenized suspension was used for dry weight and bacterial viability (colony forming units – CFU mg^{-1} of biofilm dry weight) as described in the previous study (Aires *et al.*, 2008). For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 ml biofilm suspension, and the resulting precipitate was centrifuged (10,000 g for 10 min at 4°C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed. And for bacterial viability, an aliquot (0.1 ml) of the homogenized suspension was serially diluted and plated on trypticase soy agar with 5% sheep blood (BD, USA). The plates

were incubated in 5% CO_2 at 37°C for 48 h, and then the number of CFU mg^{-1} of dry weight of biofilm was determined.

2.5. Variable pressure scanning electron microscopy (VPSEM). The HA discs were transferred to glass slides, with biofilms positioned upwards, and placed on the VPSEM [Zeiss EVO 50 (Carl Zeiss Microscopy, LLC, Thornwood, NY)] chamber. The images were captured at 100 Pa and 15.00 Kv and the working distance was 7.5 mm (Weber *et al.* 2013).

2.6. Confocal scanning laser microscope (CSLM). The organization of the live and dead bacteria on the biofilm surface was examined by CSLM. It was examined by using the Leica TCS SP5 microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a HCX APOL U-V-I 40X/0.8-numerical-aperture water immersion objective. The biofilms were stained with a live/dead BacLight bacterial viability kit (Molecular Probes. Invitrogen, Eugene, Oregon. USA). The stains were prepared in accordance with the manufacturer. The microplates were incubated at room temperature in the dark for 15 min and examined under CSLM (Paddock, 1999). The images were subsequently analysed using Fiji ImageJ (National Institutes of Health, Bethesda, MD, USA) to produce 3D projections.

2.7. Statistical analyses. Prior to analysis, assumptions of equality of variances and normal distribution of errors were checked. As the variances between groups were heterogeneous, data were rank transformed prior to analysis with a mixed model ANOVA with a fixed factor of group and a random intercept. When significant differences were detected, pairwise comparisons were made between all of the groups using t-tests based on the pooled standard error. IBM SPSS version 21 (IBM, Inc., Armonk, NY) was used to perform the analyses. The confidence interval was set at 95%.

3. Results

The bacterial viability (CFU mg^{-1} of biofilm dry weight) and dry weight (in mg) are showed in Figure 1. Significant decreases in the viability of *S. mutans* biofilms were observed

in relation to the negative control when biofilms were exposed to both concentrations of the anacardic acids emulsion ($p < 0.05$). There was a significant reduction of CFU when the biofilm was treated with the higher concentration (1,324 µg/ml) of the anacardic acids emulsion when compared to positive and negative controls ($p < 0.05$). There wasn't a significant difference between the lower concentration (132.4 µg/ml) and positive control ($p > 0.05$). In relation to the dry weight, there wasn't a significant difference amongst the two concentrations of the anacardic acids emulsions and both controls ($p > 0.05$).

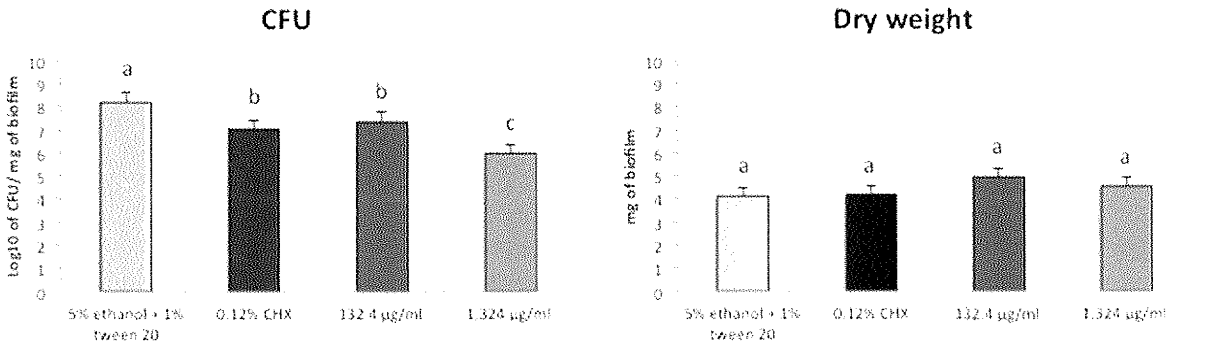

Figure 1. Effects of two concentrations of anacardic acids emulsion on the viability of *Streptococcus mutans* biofilm compared with negative (5% ethanol + 1% tween 20) and positive (0.12% chohrexidine) controls. Data represent the mean values and error bars represent standard deviations. Values marked by the different letters are significantly different from each other ($p < 0.05$).

Figure 2 illustrates the effects of the two concentrations of the anacardic acids emulsion on the morphology and structure of *S. mutans* biofilm. Regarding the morphological alterations, VPSEM didn't show any difference between the negative control and both concentrations (132.4 µg/ml and 1,324 µg/ml), and all the biofilms exhibited a similar structure.

Representative confocal laser scanning microscopy images of biofilms after treatment with both concentrations of anacardic acids emulsion are shown in Figure 3. Live (green) and

dead (red) bacteria can be observed on the surfaces of biofilms. Figure 3 (3A, 3B, 3C, 3D) and Figure 3 (4A, 4B, 4C, 4D) refer to biofilms treated with C1 (132.4 µg/ml) and C2 (1,324 µg/ml), respectively. A visual decrease in the proportion of live and dead bacteria was noticed in the biofilms treated with C1 when compared with positive and negative controls.

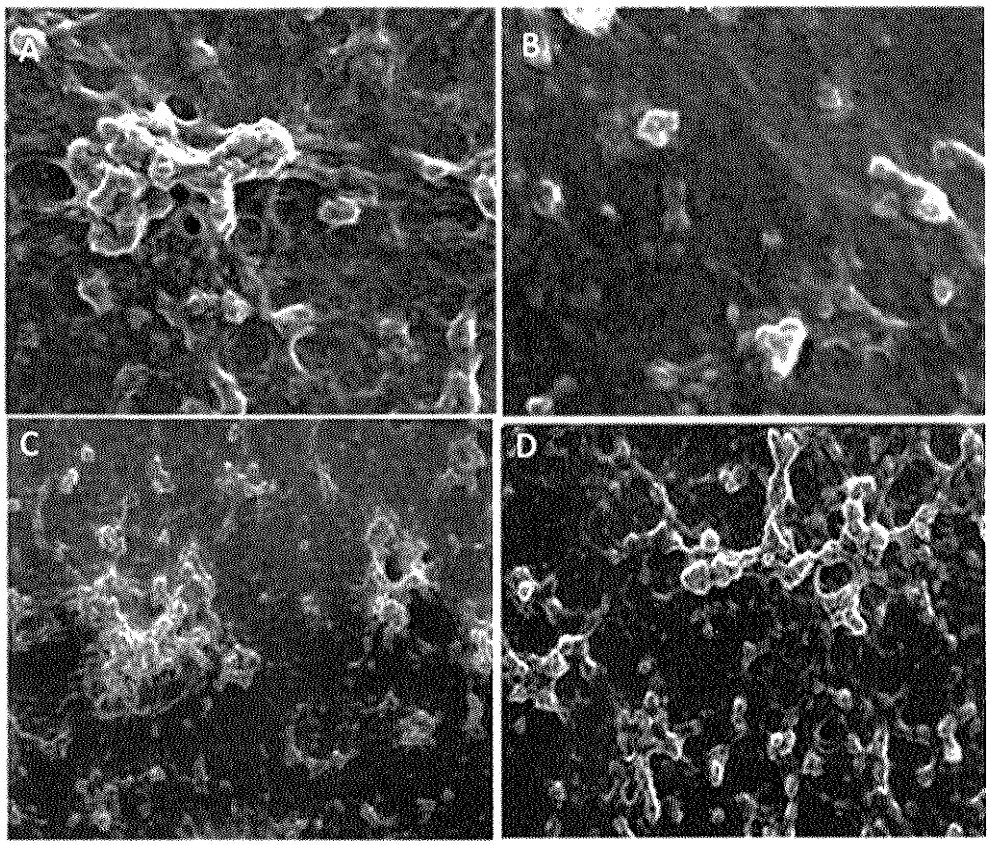

Figure 2. VPSEM images show the morphology and structure of *S. mutans* biofilm after the treatment with two concentrations of anacardic acids emulsion (Field width of 1.0 mm). A = Negative control (5% ethanol + 1% tween 20); B = Positive control (0.12% chlorhexidine); C = Biofilm after treatment with 132.4 µg/ml of anacardic acids emulsion; D = Biofilm after treatment with 1,324 µg/ml of anacardic acids emulsion.

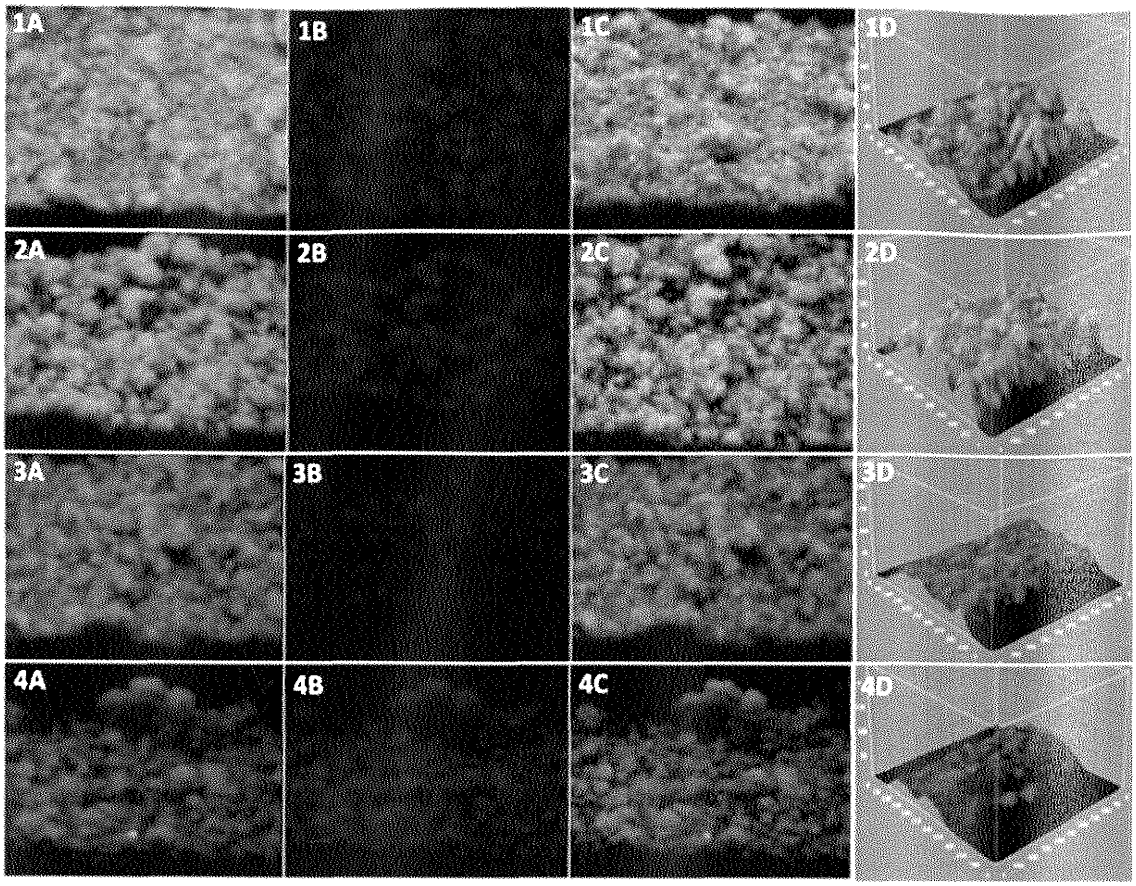

Figure 3. Mature biofilm of *S. mutans* after treatment with two concentrations of anacardic acids emulsion. (1) Distribution of bacteria on negative control biofilm (5% ethanol + 1% tween 20); (2) Distribution of bacteria on positive control biofilm (0.12% chlorhexidine); (3) Distribution of bacteria on biofilm treated with C1 (132.4 µg/ml); (4) Distribution of bacteria on biofilm treated with C2 (1,324 µg/ml). (A) Green, live bacteria. (B) Red, dead bacteria. (C) Overlap of green and red stacks. (D) Representative three-dimensional images of the structural organization of the biofilms: rendered images of the outer layers of biofilms.

4. Discussion

In this study, we aimed to evaluate the effect of two different concentrations of an anacardic acids emulsion against mature *S. mutans* biofilm. Some studies have related the antibacterial effect of anacardic acids in planktonic cultures of *S. mutans* but its effects on biofilm cultures have not been explored yet (Himejima & Kubo, 1991; Muroi & Kubo, 1993; Green, Tocoli, Lee, Nihei, & Kubo, 2008; Rivero-Cruz, Esturau, Sanchez-Nieto, Romero, Castillo-Juarez, & Rivero-Cruz, 2011). The anacardic acids emulsion used in this study was

isolated from CNSL containing an abundance of anacardic acids ($C_{15:3}$, $C_{15:2}$, $C_{15:1}$, $C_{15:0}$) and their structures were related previously (Trevisan *et al.*, 2006). In our investigation, we found that the anacardic acids emulsion has a strong antibacterial effect against *S. mutans* (data not published yet) and this is the first study evaluating the effect of this emulsion on biofilms.

We observed that the two concentrations (132.4 µg/ml and 1,324 µg/ml) tested in this study were able to decrease the viability of *S. mutans* in the biofilms. Studies have related that the antimicrobial activity of anacardic acids has been proportional to the degree of the side chain unsaturation and $C_{15:3}$ exhibited the highest antibacterial activity, followed by $C_{15:2}$ and $C_{15:1}$, respectively (Himejima & Kubo, 1991; Muroi & Kubo, 1993; Green, Tocoli, Lee, Nihei, & Kubo, 2008). Kubo *et al.* (1993) found that the antibacterial activity of anacardic acids against *S. mutans* decreases as the number of double bonds in the side chain decreases and that the bactericidal effect of anacardic acids on *S. mutans* is due to a balance between the hydrophilic and hydrophobic components of the molecule itself. Another study (Green, Tocoli, Lee, Nihei, & Kubo, 2007) found that anacardic acids' amphipathic property and alk(en)yl side chain, which is an important scaffold, allow it to act as a surface-active agent viz. surfactant and target the extracytoplasmic region of bacteria, thus not needing to enter the cell, and cause a biophysical disruption of the membrane of planktonic cultures of Gram-positive bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA).

In our study, although the anacardic acids emulsion was able to decrease the bacterial viability, it couldn't change the dry weight of biofilm. This result confirms the potent antimicrobial activity of the anacardic acids emulsion. However, the presence of extracellular polysaccharides (EPS) provide a barrier to the emulsion that typically has easy access to *S.mutans* in the planktonic state, which is shown in some studies (Himejima & Kubo, 1991; Muroi & Kubo, 1993; Green, Tocoli, Lee, Nihei, & Kubo, 2008; Rivero-Cruz, Esturau, Sanchez-Nieto, Romero, Castillo-Juarez, & Rivero-Cruz, 2011). Extracellular polysaccharides

(EPS) are the major reason the ineffectiveness of many antibiotics in eradicating biofilm bacteria. EPS also protect the integrity of various microniches within a biofilm from physical forces caused by the disruptive effects of surrounding liquid currents. This leads to a local buildup of chemicals in the immediate proximity of cells, with a significant effect on physiology and metabolism of bacteria. As a result, bacteria exposed to high concentrations of these molecules would have a different metabolic activity compared with those exposed to low concentration of signals (Alavi, 2012).

In agreement with what we observed in the dry weight analysis, VPSEM images showed that the structural components attributed to the EPS and the topographical morphology of biofilm after treatment with the anacardic acids emulsion were similar to the positive and negative controls. *S. mutans* biofilm topography displayed a largely unaffected extracellular matrix after treatment with C1 and C2, as compared to the negative control. This observation suggests that the anacardic acids emulsion does not seem to play a role in disrupting the mature biofilm. This may be due to the fact that when the biofilm is not exposed to the treatment, EPS continues to grow as a function of the biofilm's growth and structural organization, or also due the time and/or kind of application of the treatment.

On the other hand, CSLM images are in agreement with the CFU analysis. The images exhibited that after treatment with C1 and C2, there was indeed a reduction in live bacteria. This confirms that the anacardic acids emulsion acts as a bactericidal agent on *S. mutans* bacteria, as outlined in a number of other studies (Kubo & Muroi, 1993; Green, Tocoli, Lee, Nihei, & Kubo, 2008; Hemshekhar, Santhosh, Kemparaju, & Girish, 2011).

5. Conclusion

We observed that the anacardic acids emulsion exhibited a strong antibacterial effect against *S. mutans* in the mature biofilm, and it can be considered a potent antimicrobial agent. The two concentrations analyzed were able to decrease the *S. mutans* viability in the biofilms.

6. Acknowledgment

We thank Dr. Nagila Ricardo, who provided us the anacardic acids, and Yan Deng for confocal scanning laser microscope images This research was supported by CAPES Foundation from whom the first and second authors received a scholarship in Brazil and during their PhD sandwich doctorate (Process 18494-12-9 and 7715-13-7, respectively).

7. Conflict of interest

All authors don't have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, our work.

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3.3 Capítulo 3

Evaluation of single and daily treatment of an anacardic acids emulsion against *S. mutans* biofilm

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Abstract

The objective of this study was to evaluate the effect of a single versus twice daily treatment of an anacardic acids emulsion against *Streptococcus mutans* biofilm. Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs for 5 days and treated with two concentrations of the anacardic acids emulsion (C1=662 μg/ml and C2=1,324 μg/ml). The discs were divided in four groups: one group was exposed to C1 and C2 twice daily for 1 min; one group was exposed just once on the fifth day; and the others two groups were negative (5% ethanol +1% tween 20) and positive (0.12% chlorhexidine) controls. Biofilms were analyzed for bacterial viability, dry-weight, and extracellular (EPS-insoluble and soluble) and intracellular (IPS) polysaccharides. Variable pressure scanning electron microscopy and confocal laser scanning microscopy were used to check morphology and viability, respectively. The C1 and C2 showed a significant reduction in the colony forming units (CFU) ($p<0.05$) but didn't change the amount of dry weight ($p>0.05$) in both biofilms. In relation to single treatment, C1 and C2 increased the total amount and content of EPS-insoluble, reduced the total amount and content of EPS-soluble when compared to the positive control, and didn't affect IPS levels ($p>0.05$). In relation to daily treatment, C1 and C2 didn't affect EPS-insoluble levels ($p>0.05$), increased the total amount of EPS-soluble when compared to both controls ($p<.05$), and decreased the total amount and content of IPS when compared to the negative control ($p< 0.05$). C1 and C2 didn't affect the morphology and structure after single treatment but they showed a different morphology when applied daily. Also C1 and C2 exhibited a decrease in live bacteria on both biofilms. The anacardic acids emulsion has a potent antibacterial effect and its action after the single treatment was more effective than the treatment daily on *S. mutans* biofilm.

Keywords: *Anacardium occidentale* Linn., biofilm, *Streptococcus mutans*.

Introduction

Dental caries is one of the most prevalent infectious diseases worldwide [1]. There are different species of bacteria that are instrumental in the development of dental caries, but *Streptococcus mutans* is one of the dominating species. *S. mutans* retains the ability to produce acidic metabolites (acidogenic and aciduric Gram-positive specie), to build up glycogen reserves, and to synthesize extracellular polysaccharides (EPS) [1, 2, 3]. Adherence to enamel surfaces as well as other bacterial cells is the main property of *S. mutans* that allows it to play a role in biofilm formation [4]. Extracellular polysaccharides contribute to the cariogenic potential of the biofilm by aiding in adherence of other bacteria and debris, hindering diffusion of acids by trapping them near the tooth surface, and allowing the biofilm to thicken and thus increase retention of acids [2, 5, 3].

Traditional medicinal plant extracts have been shown to inhibit the growth of oral pathogens, reduce the development of dental biofilms, and therefore suppress the development of dental caries [6]. Cashew nut shell liquid (CNSL) is contained in cashew nut (*Anacardium occidentale*) and it has a variety of functional properties as a molluscicide, insecticide, fungicide, and also as an antioxidant [7, 8 9]. The major components of CNSL include anacardic acids comprising 90% of it with trace amounts of cardanol and cardol [8]. These components have all been shown to have potential medicinal applications, although anacardic acid has been shown to provide better protection against various pathophysiological disorders as compared to cardanol and cardol [9, 10]. It has been particularly noted that anacardic acid has an antibacterial effect on *S. mutans* [11], but studies have only analyzed its antimicrobial effect on planktonic cultures [12, 13].

Therefore, the aim of this present study was to evaluate the effect of an anacardic acids emulsion against *Streptococcus mutans* biofilm after two treatments: 1) a single treatment, to evaluate the effect of the emulsion on the mature biofilm; and 2) a daily treatment, to evaluate the effect of the emulsion on the formation and composition of *S. mutans* biofilm. Our null hypothesis was that there wasn't difference between the single and daily treatment.

Materials and methods

Chemical. The cashews (*A. occidentale* Linn.) were harvested at the Embrapa Tropical Agroindustry Experimental Station, located at Paraibapa, Ceara, Brazil during the 2012 season. The cashew product extraction and isolation of anacardic acids from CNSL were previously reported in the literature [8]. The emulsion was obtained dissolving the anacardic acids extract in 5% ethanol + 1% tween 20 (Sigma, St. Louis, MO), and phosphate buffer saline (PBS) was used as vehicle. The concentrations used were 662 μg/ml (C1) and 1,324 μg/ml (C2) and according to previous study (data not published yet).

Inoculum and biofilm model. *Streptococcus mutans* UA159 (UAB577) was obtained from single colonies isolated on agar plates, inoculated in tryptone yeast-extract broth containing 1% (w/v) glucose and incubated for 18–24 h at 37°C under microaerophilic conditions (5% CO_2). Biofilms of *S. mutans* UA159 were formed on saliva-coated hydroxyapatite discs (HA) (surface area of 2.7 ± 0.2 cm^2; Clarkson Chromatography Products Inc., South Williamsport, Pa., USA) placed in batch cultures at 37°C in 5% CO_2 for 5 days. Hydroxyapatite disks were coated with filter-sterilized (0.22 μm; polyether sulfone low-protein-binding filter; Millipore Co., Bedford, Mass., USA) clarified human whole saliva for 1 h at 37°C; whole saliva was collected on ice from 1 donor who chewed paraffin film, and it was clarified by centrifugation (8,500 g, 4 ° C, 10 min) [14]. The biofilms were grown in tryptone yeast-extract broth containing 1% (w/v) sucrose and were kept undisturbed for 24 h to allow initial biofilm formation. The culture medium was replaced once daily in the morning.

Single and daily treatment with anacardic acids emulsion. To evaluate the effect of daily treatment with C1 and C2, the biofilms were exposed to C1 and C2 twice daily (10 a.m. and 4 p.m.) until the fifth day of the experimental period. The biofilms were dip-washed three times in 0.89% NaCl and immersed in anacardic acids emulsion for 1 min. After this, biofilms were dip-washed again and put back in the culture medium. To evaluate the effect of single treatment, the culture medium was replaced once daily and at the fifth day, the biofilms were exposed to C1 and C2 for 1 min. The 0.12% chlorhexidine and 5% ethanol +1% tween 20 solution were used as positive and negative controls, respectively.

Biofilm analysis. At the end of the experimental period, the biofilms were placed in 5 ml sterile saline solution, and the hydroxyapatite surfaces were gently scraped with a sterile spatula to harvest adherent cells. The removed biofilms were subjected to sonication using three 15-s pulses at an output of 7 W (Fisher Scientific, Sonic Dismembrator model 100; USA). The homogenized suspension was used for dry weight, bacterial viability (colony

forming units – CFU mg^{-1} of dry weight of the biofilm), and polysaccharide analyses (EPS-soluble, EPS-insoluble and intracellular iodophilic polysaccharides - IPS) as described in the previous study [15].

Dry weight. For the dry weight determination, three volumes of cold ethanol (-20°C) were added to 1 ml biofilm suspension, and the resulting precipitate was centrifuged (10,000 g for 10 min at 4°C). The supernatant was discarded, and the pellet was washed with cold ethanol, and then lyophilized and weighed [15].

Bacterial viability. An aliquot (0.1 mL) of the homogenized suspension was serially diluted and plated on trypticase soy agar with 5% sheep blood (BD, USA). The plates were incubated in 5% CO_2 at 37°C for 48 h, and then the number of CFU mg^{-1} of biofilm dry weight was determined [15].

Polysaccharide analyses. Soluble and insoluble extracellular polysaccharides (EPS-soluble and EPS-insoluble, respectively), and intracellular polysaccharides (IPS) were analyzed by colorimetric assay. The polysaccharide content was expressed per mg of dry weight. Briefly, an aliquot (3.9 ml) of the suspension was sonicated for 30-s pulses at an output of 7 W and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and the biofilm pellet was resuspended and washed in 5 ml of milli-Q water; this procedure was repeated three times. The supernatant was used for the EPS-soluble assay and biofilm pellet was used for the EPS-insoluble and IPS assays. All of the supernatants were pooled and three volumes of cold ethanol were added, and the resulting precipitate was collected by centrifugation and resuspended in 5 ml Milli-Q water; the total amount of carbohydrate was determined by the phenol–sulfuric acid method [16]. The EPS-insoluble was extracted using 1 N NaOH (1 mg biofilm dry weight/0.3 ml of 1 N NaOH) under agitation for 1 h 10 min at 37°C. The supernatant was collected by centrifugation, and the precipitate was resuspended again in 1 N NaOH; this procedure was repeated three times. The total amount of carbohydrate was determined by the phenol–sulfuric acid method [16]. The IPS were extracted with hot 5.3 M KOH (0.8 mg of biofilm dry weight/ml KOH) and 5.3 M HCl (0.8 mg of biofilm dry weight/ml HCl), and quantified using 0.2% I_2/2% KI solution and 1 M Phosphate Buffer (pH 7.0) [15,17].

Variable pressure scanning electron microscopy (VPSEM). The HA discs were transferred to glass slides, with biofilms positioned upwards, and placed on the VPSEM [Zeiss EVO 50

(Carl Zeiss Microscopy, LLC, Thornwood, NY)] chamber. The images were captured at 100 Pa and 15.00 Kv and the working distance was 7.5 mm [18].

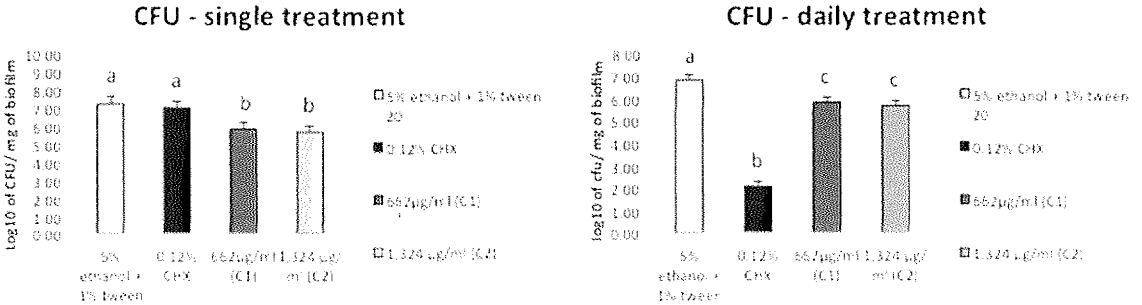
Confocal laser scanning microscope (CLSM). The organization of the live and dead bacteria on the biofilm surface was examined by CLSM. It was examined by using the Leica TCS SP5 microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) with a HCX APOL U-V-I 40X/0.8-numerical-aperture water immersion objective. The biofilms were stained with a live/dead BacLight bacterial viability kit (Molecular Probes. Invitrogen, Eugene, Oregon. USA). The stains were prepared in accordance with the manufacturer. The microplates were incubated at room temperature in the dark for 15 min and examined under a CLSM [19]. The images were subsequently analysed using Fiji ImageJ (National Institutes of Health, Bethesda, MD, USA) to produce 3D projections.

Statistical analyses. Prior to analysis, assumptions of equality of variances and normal distribution of errors were checked. As the variances between groups were heterogeneous, data were rank transformed prior to analysis with a mixed model ANOVA with a fixed factor of group and a random intercept. When significant differences were detected, pairwise comparisons were made between all of the groups using t-tests based on the pooled standard error. IBM SPSS version 21 (IBM, Inc., Armonk, NY) was used to perform the analyses. The confidence interval was set at 95%

Results

The bacteria viability and the dry weight of the biofilms treated with C1 (662 µg/ml) and C2 (1,324 µg/ml) of the anacardic acids emulsion are shown in Figure 1 and Figure 2, respectively. C1 and C2 showed a significant reduction in the CFU compared to the negative control after single and daily treatment ($p < 0.05$). After single treatment, C1 and C2 exhibited significantly less CFU than the positive control ($p < 0.05$). The amount of dry weight didn't change after treatment with C1 and C2 after both treatments when compared to the negative

control. There wasn't significant difference among C1 and C2 and the negative control



($p > 0.05$).

Figure 1. Bacteria Viability in *S. mutans* Biofilm after single and daily treatment with C1 (662 µg/ml) and C2 (1,324 µg/ml) of anacardic acids emulsion.
CFU of the *S. mutans* after treatment single and daily treatment with C1 and C2 of anacardic acids emulsuion compared with the positive and negative controls. Data represent the mean values and error bars represent standard deviations. Values marked by the same letters are not significantly different from each other ($p > 0.05$).

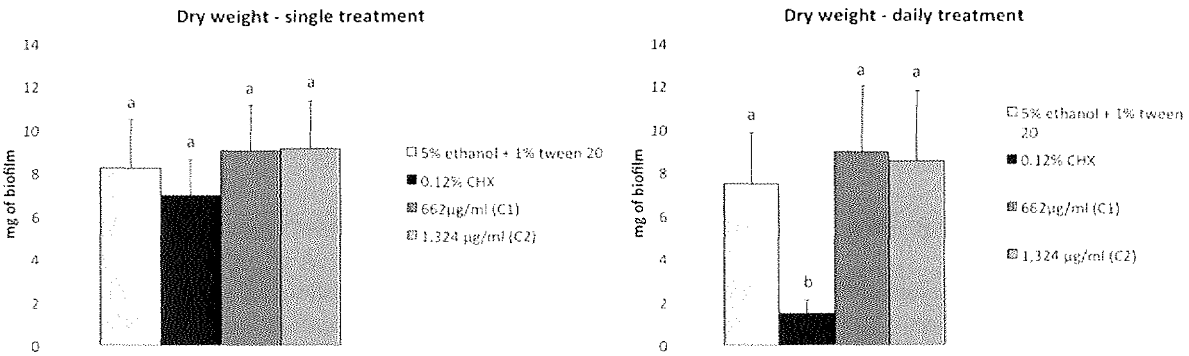


Figure 2. Dry Weight of *S. mutans* Biofilm after single and daily treatment with C1 (662 µg/ml) and C2 (1,324 µg/ml) of anacardic acids emulsion.
Mean of dry weight of the *S. mutans* biofilm after treatment single and daily treatment with C1 and C2 of anacardic acids emulsuion compared with the positive and negative controls. Data represent the mean values and error bars represent standard deviations. Values marked by the same letters are not significantly different from each other ($p > 0.05$).

The content (µg/mg of biofilm) of EPS (soluble and insoluble), and of intracellular polysaccharides (IPS) after single treatment with C1 and C2 of the anacardic acids emulsion are shown in Figure 3. The EPS-insoluble and IPS contents were statistically similar for all groups, showing that treatment with C1 and C2 didn't affect levels of IPS and EPS-insoluble ($p > 0.05$). In relation to EPS-soluble, C1 and C2 reduced the EPS-soluble when compared to the positive control. Particularly, this reduction was statistically significant after treatment with C1 ($p < 0.05$).

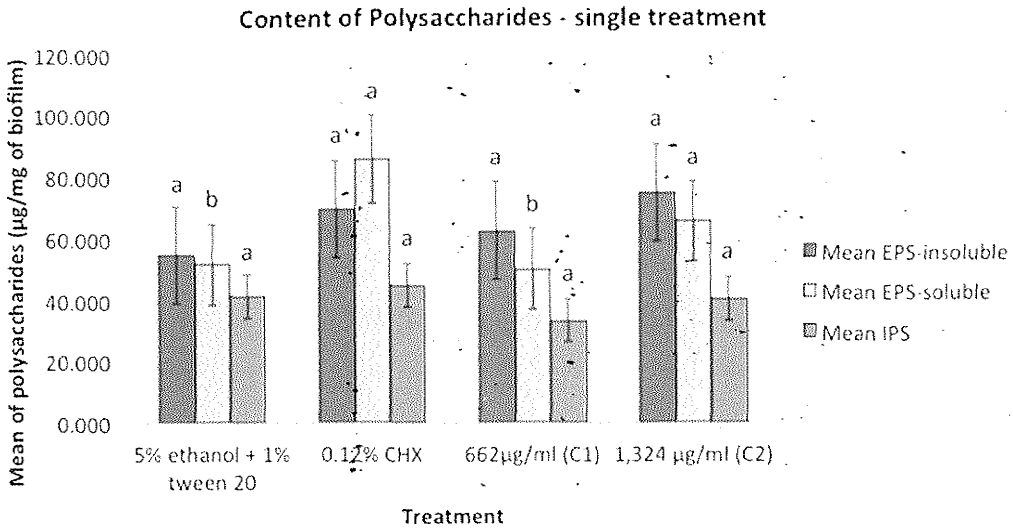


Figure 3. Amount and Content of Polysaccharides in *S. mutans* Biofilm After Single Treatment with C1 and C2 of the Anacardic Acids Emulsion.
The content (µg/mg of biofilm) of extracellular polysaccharides (EPS-insoluble and soluble, respectively), and of intracellular polysaccharides (IPS) in *S. mutans* mature biofilm after the single treatment with C1 and C2 of anacardic acids emulsion compared with the positive and negative controls. Data represent the mean values and error bars represent standard deviations. Values for each type of polysaccharide marked by the same letters are not significantly different from each other ($p > 0.05$).

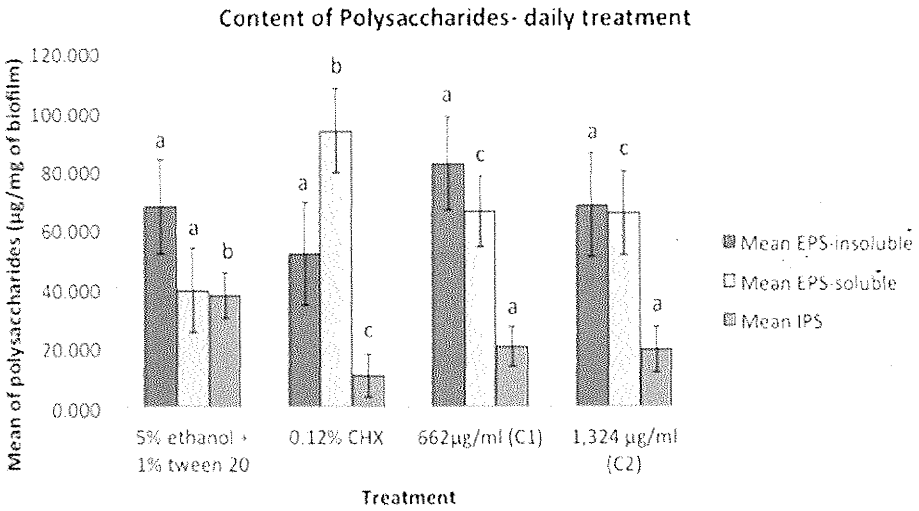


Figure 4. Amount and Content of Polysaccharides in *S. mutans* Biofilm After Daily Treatment with C1 and C2 of the Anacardic Acids Emulsion.
The content (µg/mg of biofilm) of extracellular polysaccharides (EPS-insoluble and soluble, respectively), and of intracellular polysaccharides (IPS) in *S. mutans* mature biofilm after the daily treatment with C1 and C2 of anacardic acids emulsion compared with the positive and negative controls. Data represent the mean values and error bars represent standard deviations. Values for each type of polysaccharide marked by the same letters are not significantly different from each other ($p \geq 0.05$).

Figure 4 shows the content (µg/mg of biofilm) of EPS-soluble, EPS-insoluble, and of intracellular polysaccharides (IPS) produced after the daily treatment with C1 and C2 of the

anacardic acids emulsion. The content of EPS-insoluble was similar for all groups, showing that treatment with C1 and C2 didn't affect EPS-insoluble levels ($p > 0.05$). In relation to EPS-soluble, C1 and C2 decreased the content of EPS-soluble in comparison with the positive control ($p < 0.05$) and increased in comparison with the negative control ($p < 0.05$). The IPS levels decreased after treatment with C1 and C2 when compared to the negative control ($p < 0.05$) and increased when compared to the positive control ($p < 0.05$).

Figure 5 and 6 show the VPSEM images after single and daily treatment with C1 and C2 of the anacardic acids emulsion, respectively. After single treatment, C1 and C2 didn't affect the morphology and structure of *S. mutans* biofilm when compared to the respective negative and positive controls. And after daily treatment, C1 and C2 showed a different morphology compared to the negative control and exhibited a higher production of polysaccharide.

Confocal laser scanning microscopy images of biofilm after single and daily treatment with C1 and C2 of the anacardic acids emulsion are shown in Figure 7 and 8, respectively. Live (green) and dead (red) bacteria can be observed on the surfaces of both biofilms. C1 and C2 exhibited a decrease in live bacteria after single and daily treatments.

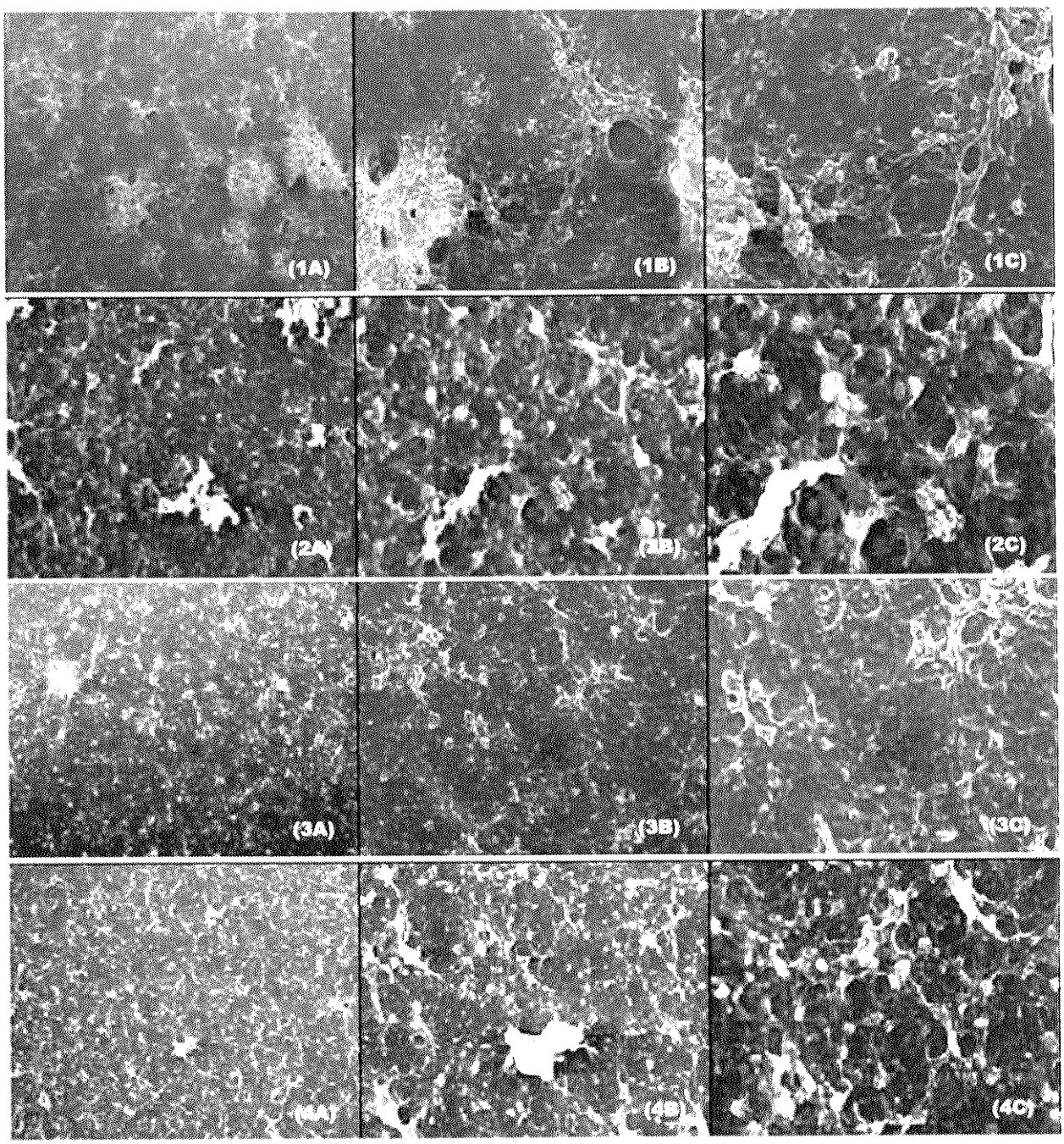


Figure 5. VPSEM images are showing the morphology and structure of *S. mutans* biofilm after the single treatment with anacardic acids emulsion.
(1) Negative control (5% ethanol + 1% tween 20); (2) Positive control (0.12% chlorhexidine); (3) 662 µg/ml of anacardic acids emulsion (C1); (4) 1,324 µg/ml of anacardic acids emulsion (C2). Field width of 3.0 mm (A), 1.0 mm (B), and 0.5 mm (C).

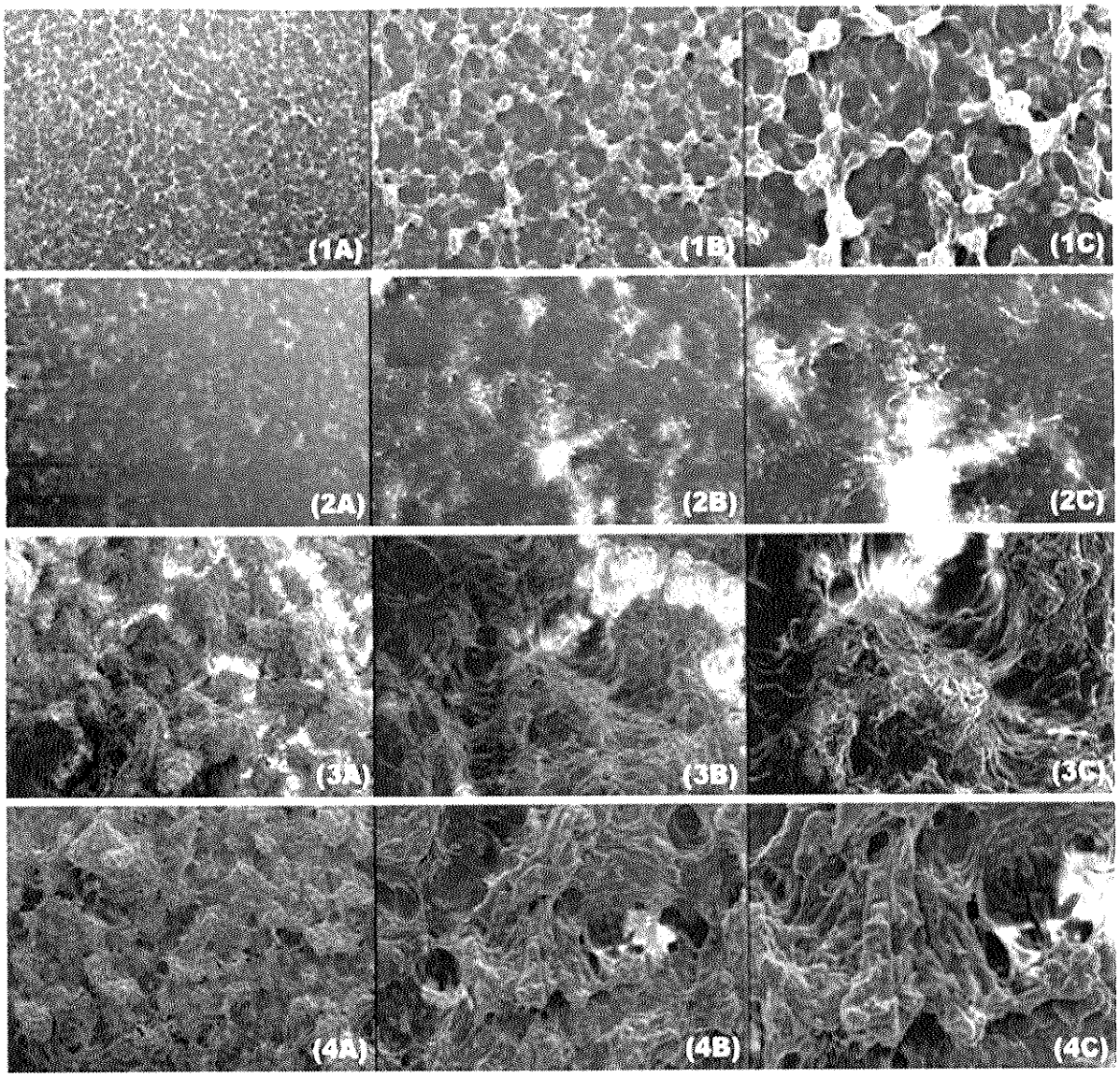


Figure 6. VPSEM images are showing the morphology and structure of *S. mutans* biofilm after the daily treatment with anacardic acids emulsion.
(1) Negative control (5% ethanol + 1% tween 20); (2) Positive control (0.12% chlorhexidine); (3) 662 µg/ml of anacardic acids emulsion (C1); (4) 1,324 µg/ml of anacardic acids emulsion (C2). Field width of 3.0 mm (A), 1.0 mm (B), and 0.5 mm (C).

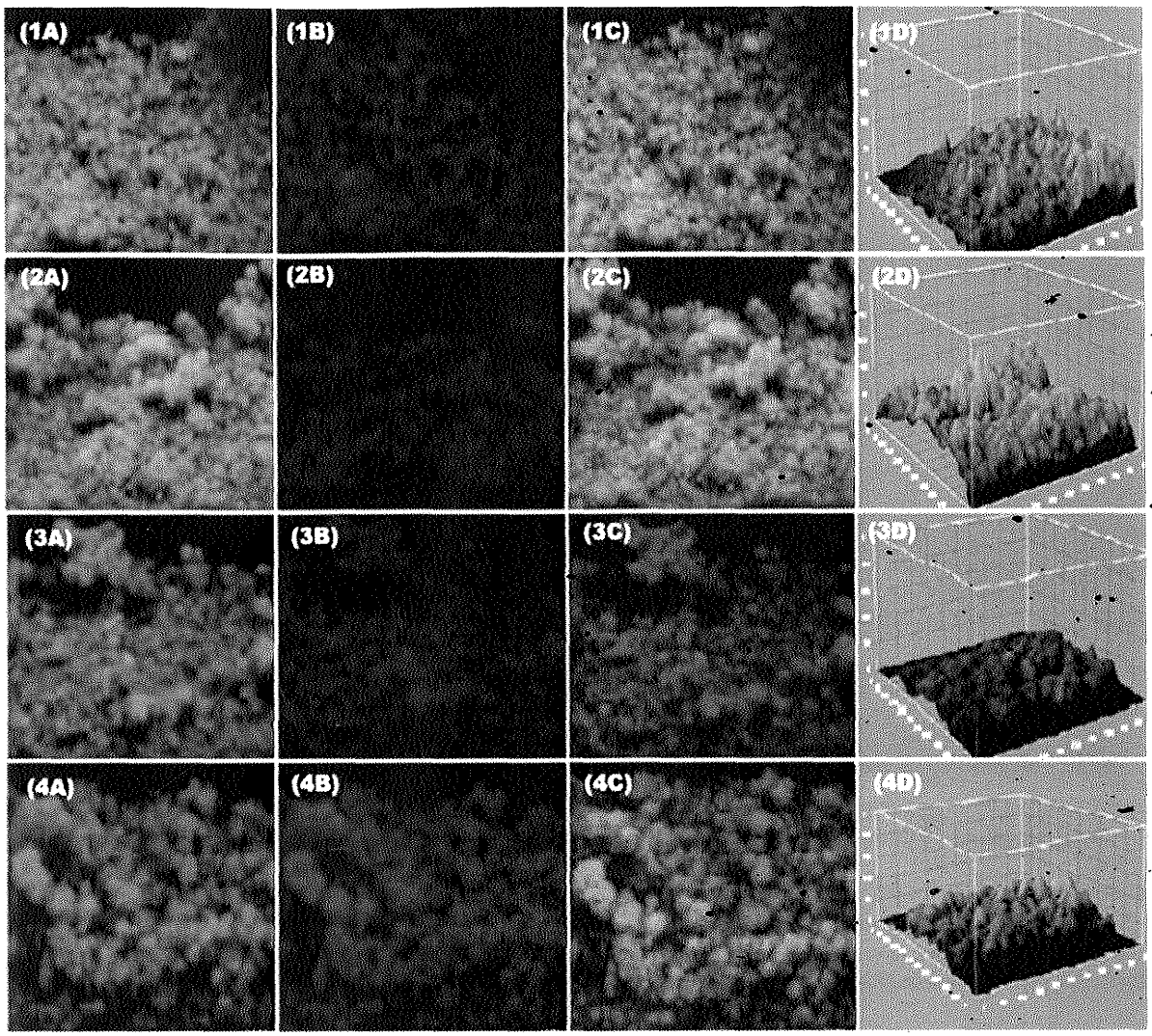


Figure 7. Confocal Laser Scanning Microscopy Images of *S. mutans* Biofilm after Single treatment with Anacardic Acids Emulsion.

(1) Distribution of bacteria on negative control biofilm (5% ethanol % 1% tween 20); (2) Distribution of bacteria on positive control biofilm (012% Chlorhexidine); (3) Distribution of bacteria on biofilm treated with C1 (662 µg/ml); (3) Distribution of bacteria on biofilm treated with C2 (1,324 µg/ml). (A) Green, live bacteria. (B) Red, dead bacteria. (C) Overlap of green and red stacks. (D) Representative three-dimensional images of the structural organization of the biofilms: rendered images of the outer layers of biofilms.

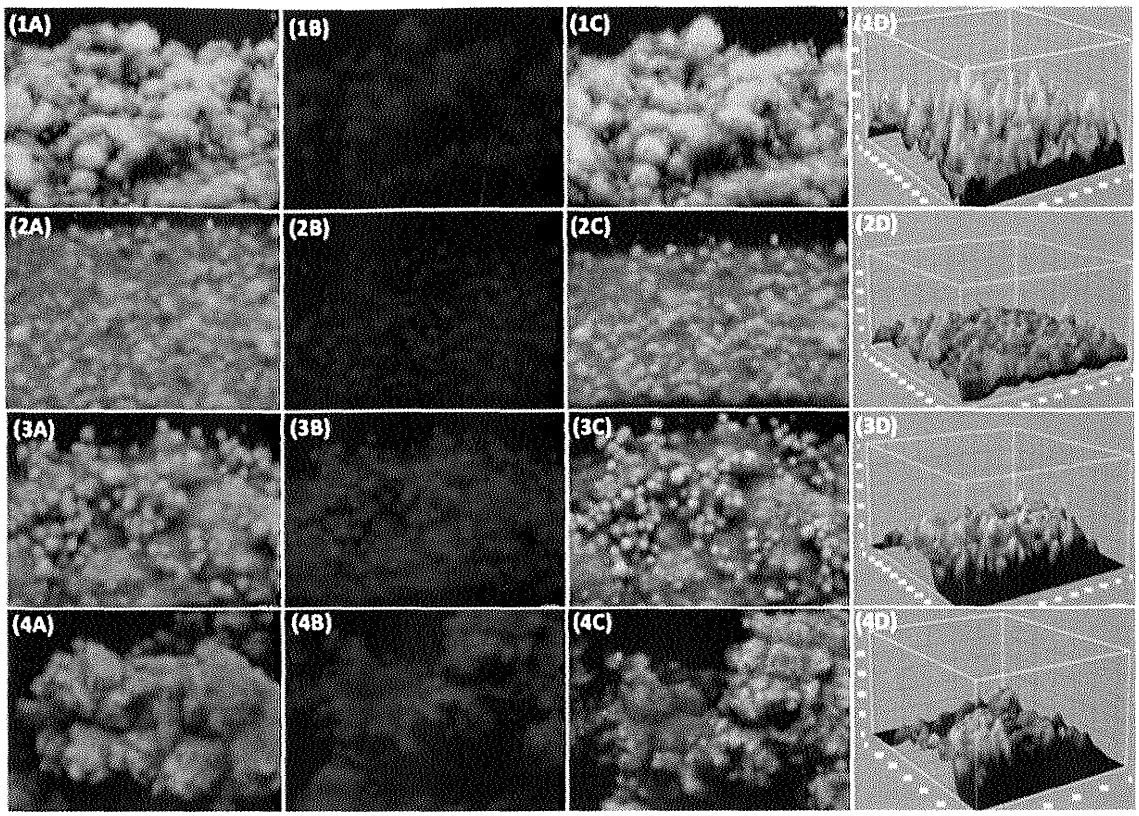


Figure 7. Confocal Laser Scanning Microscopy Images of *S. mutans* Biofilm after Daily treatment with Anacardic Acids Emulsion.
(1) Distribution of bacteria on negative control biofilm (5% ethanol % 1% tween 20); (2) Distribution of bacteria on positive control biofilm (012% Chlorhexidine); (3) Distribution of bacteria on biofilm treated with C1 (662 µg/ml); (3) Distribution of bacteria on biofilm treated with C2 (1,324 µg/ml). (A) Green, live bacteria. (B) Red, dead bacteria. (C) Overlap of green and red stacks. (D) Representative three-dimensional images of the structural organization of the biofilms: rendered images of the outer layers of biofilms.

Discussion

In this study, we aimed to evaluate the effect of a single and daily treatment of an anacardic acids emulsion against *Streptococcus mutans* biofilm. Although there are studies discussing the antimicrobial action of the anacardic acids [11,12,20], their effects on biofilm cultures have not yet been examined.

The results derived from our study showed that C1 (662 µg/ml) and C2 (1,324 µg/ml) significantly reduced the *S. mutans* viability as compared to the negative control after single and daily treatment. This indicates that anacardic acids emulsion does indeed act as an antibacterial agent. Anacardic acids have been proven to have a potent antibacterial effect against many species of bacteria, particularly against the Gram-positive species, as methicillin-resistant *Staphylococcus aureus* (MRSA), and the Gram-negative bacterium, *Helicobacter pylori*, which is known to cause acute gastritis [10,21]. However, all these studies evaluated the antibacterial effect of anacardic acid in planktonic cultures.

Anacardic acid's antibacterial activity is dependent upon the length and unsaturation of its alkyl side chain and with its antibacterial property increasing as the side chain length and unsaturation increases [20]. The emulsion used in this study is composed for different kinds of anacardic acids, which have different side chain unsaturation ($C_{15:3}$, $C_{15:2}$, $C_{15:1}$, $C_{15:0}$). Kubo et al. (1993) [22] found that the antibacterial activity of anacardic acids against *S. mutans* decreases as the number of double bonds in the side chain decreases and that the bactericidal effect of anacardic acids on *S. mutans* is due to a balance between the hydrophilic and hydrophobic components of the molecule itself. Another study [10] found that anacardic acids' amphipathic property and alk(en)yl side chain, which is an important scaffold, allow them to act as a surface-active agent viz. surfactant and target the extracytoplasmic region of bacteria, thus not needing to enter the cell, and cause a biophysical disruption of the membrane of planktonic cultures of Gram-positive bacteria, such as MRSA.

Our results also showed that the single and daily treatment of the biofilm with C1 and C2 had no effect on the dry weight of *S. mutans* biofilms in comparison to the negative control. The dry weight of *S. mutans* biofilm is comprised of various components. Oral biofilms contain 40% dry weight protein (mostly derived from saliva and bacteria), variable amounts of lipid, Ca, P, Mg, and F and glucan and fructan, which comprise 10-20% and 1-2% of the dry weight, respectively. The latter two components depend on the frequency of nutritional intake [23-31]. It is possible that the level of dry weight was not affected by the treatment because though C1 and C2 have a bactericidal role when applied to *S. mutans*, they do not have a lasting effect on the bacteria in the biofilm. Once the biofilms stopped being treated, some bacteria still continued to grow protein and polysaccharides when it was not exposed to the treatment, causing the levels of dry weight of the biofilms to stay consistent.

The content of EPS-insoluble didn't change after single and daily treatment with C1 and C2 of anacardic acids emulsion. Insoluble EPS are synthesized by glucosyltransferase (Gtf) enzymes, specifically GtfB and GtfC, which are encoded by the *gtfB* and *gtfC* genes [32]. Insoluble EPS play a role in contributing to the bulk and physical integrity and stability of the biofilm matrix and promotes tight adherence and coherence of bacterial cells bound to each other and to the apatitic surface, which ultimately lead to the formation of microcolonies of *S. mutans* in a matrix rich biofilm [32]. It is also involved in increasing the porosity of a dental biofilm matrix, allowing substrates to diffuse to the deepest part of the biofilm [5,29]. Insoluble EPS contain elevated amounts of α-1,3 linked glucose, which are critical for mediating bacterial adhesion and can make this polysaccharide more difficult to break down

[33,34,35]. Also, studies have shown that *S. mutans* growing in higher concentrations and frequencies of sucrose exposure form biofilms that are increasingly concentrated with EPS-insoluble [28,36,37,38]. This is consistent with our study where biofilms were grown in media that were supplemented with sucrose, which possibly serves as the reason why all groups in both treatments still formed similar levels of EPS-insoluble.

Furthermore, the IPS levels weren't affected after single treatment with C1 and C2 but these levels decreased compared to the negative control after treatment with C1 and C2 after daily treatment. In our study, it is possible that the bacteria that were treated with anacardic acids became weak and during the polysaccharide extraction process, the membranes of the weak cells were disrupted and IPS escaped from the inside of the cells, thus contributing to the overall decrease in IPS in daily treatment. Intracellular polysaccharides contribute to the incidence of dental caries by prolonging acid production and hence the period of lowered pH in dental plaque [39]. Upon depletion of nutrients in an oral biofilm, IPS also can be used as an endogenous source of carbohydrates for fermentation and persistence of the biofilm [40,41]. The *glgA* gene encodes glycogen synthase, which is an enzyme that is involved in synthesizing IPS and inactivation of this gene can result in the halt of the accumulation of IPS [42]. Considering that the levels of IPS did not change after single treatment and decreased after daily treatment, we can suggest that bacteria may use this polysaccharide as endogenous source of carbohydrates in the moments of nutrients depletion after the treatment.

Additionally, C1 and C2 reduced the levels of EPS-soluble compared to the positive control in the single treatment. However, the daily treatment with C1 and C2 increased the levels of EPS-soluble compared to both controls. Soluble EPS are synthesized by GtfD and act as primers for synthesis of EPS-insoluble by GtfB. They can also act as a reserve polysaccharide that can be metabolized by biofilm bacteria [32,35]. Therefore, it is possible that AA disrupts the metabolism of a mature biofilm.

Variable pressure scanning electron microscopy (VPSEM) is an accurate imaging technique that is used to observe the morphology of *S. mutans* biofilms with respect to topography and EPS preservation [18]. In our study, *S. mutans* biofilm topography displayed a largely unaffected extracellular matrix after treatment with C1 and C2 as compared to the positive and negative controls after the single treatment. This observation suggests that anacardic acids emulsion does not seem to play a role in targeting and hindering EPS mature. Also, our study displayed that the biofilms that were treated with C1 and C2 displayed a

different morphology and a higher production of polysaccharide in comparison to the negative control in the daily treatment.

Confocal lase scanning microscopy was used to observe the death of bacteria in the biofilms after exposure to both concentrations of anacardic acids emulsion. After both treatments, the images exhibited that C1 and C2 indeed a reduction in live bacteria. This suggests that anacardic acids emulsion act as a bactericidal agent on *S. mutans* bacteria, as outlined in a number of other studies [12,11,20].

In regards to the prevention of *S. mutans* biofilm formation, there are many questions that remain yet to be answered. The dosage at which anacardic acids emulsion is administered might have an effect on the inhibition of formation. In this present study, *S. mutans* biofilms were treated for one minute twice a day for five days at the specified concentrations of C1 and C2. Anacardic acids emulsion might be successful at preventing formation if a different regimen is adopted, with different concentrations or different frequencies of dosage. It would be interesting to observe the synergistic mechanism of anacardic acids emulsion with other natural compounds on inhibiting formation of *S. mutans* biofilms and thus preventing caries formation. Therefore, these are factors that we will take into account when continuing to investigate the role of anacardic acids emulsion.

Conclusion

We concluded that a single and daily treatment with anacardic acids emulsion against *Streptococcus mutans* biofilm reduced the bacterial viability in the biofilms. The single treatment didn't affect the matrix of EPS and the daily treatment increased it with C1 and C2. We rejected the null hypothesis. Considering this is the first study examining the effects of anacardic acid on *S. mutans* biofilm cultures, it will be necessary for further studies to be carried out to examine the specific mechanism.

Acknowledgment

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4 DISCUSSÃO GERAL

Estudos tem demonstrado que o ácido anacárdico é um promissor agente antimicrobiano devido a sua capacidade de reduzir o número de colônias bacterianas em culturas planctônicas de uma variedade de microrganismos, como, por exemplo, do *Streptococcus mutans* (RIVERO-CRUZ *et al.*, 2011; GREEN *et al.*, 2008; MUROI e KUBO, 1993; HIMEJIMA e KUBO, 1991). Entretanto, as bactérias responsáveis pelo desenvolvimento de lesões cariosas encontram-se reunidas dentro de uma complexa matriz de polissacarídeos, os biofilmes, o que as tornam mais resistentes aos agentes antimicrobianos (ARPANA e YADAV, 2008; COSTERTON; STEWART; GREENBERG, 1999).

Apesar da importância do biofilme na prevenção da doença cárie, bem como de outras doenças infecciosas, estudos avaliando a ação antibacteriana de ácidos anacárdicos limitam-se a culturas planctônicas, inexistindo na literatura estudos avaliando a ação destes em biofilmes. Nesse contexto, este estudo propôs-se a avaliar a ação de uma nova emulsão composta por uma mistura de diferentes ácidos anacárdicos, bem como avaliar o efeito desta emulsão em biofilmes de *Streptococcus mutans*.

O objetivo do **capítulo 1**, portanto, foi inicialmente avaliar a efetividade de uma emulsão natural de ácidos anacárdicos, extraídos do LCC, e de uma emulsão de um ácido anacárdico sintético, em culturas planctônicas de *Streptococcus mutans*. A finalidade deste estudo preliminar foi: 1) avaliar se as emulsões, natural e sintética, possuíam um efeito antibacteriano em culturas planctônicas de *S. mutans*; 2) investigar qual destas emulsões possuíam uma melhor atividade antibacteriana contra *S. mutans*; e 3) determinar quais seriam as concentrações inibitória mínima (CIM) e bactericida mínima (CBM), a fim de extrapolar esses resultados para culturas de *S. mutans* em biofilmes.

Estudos tem relatado que a atividade antibacteriana do ácido anacárdico é proporcional ao grau de insaturação da sua cadeia lateral (GREEN et al., 2008; HIMEJIMA e KUBO, 1991; MUROI e KUBO, 1993). Como a emulsão natural de ácidos anacárdicos é composta por uma mistura de quatro diferentes tipos de ácidos anacárdicos, que possuem cadeias laterais com diferentes graus de insaturação, esperava-se obter melhores resultados com esta emulsão do que com a emulsão sintética, que é composta por apenas um tipo de ácido anacárdico. Como se trata de um composto ácido, a água Milli-Q e o PBS foram utilizados como veículos das emulsões e os seus pH foram medidos.

Observou-se, portanto, que realmente a emulsão composta por diferentes ácidos anacárdicos possui uma melhor atividade antibacteriana contra *S. mutans*, tanto em água

quanto em PBS, do que a emulsão sintética. Além da sua potente ação antibacteriana, a emulsão natural ainda possui um baixo custo e uma maior acessibilidade, justificando uma maior investigação dessa emulsão, que foram conduzidas nos capítulos 2 e 3.

Como o objetivo inicial deste trabalho era utilizar esses resultados iniciais para estudos em biofilmes, e como se sabe que as bactérias organizadas em biofilmes apresentam um aumento expressivo na resistência aos antimicrobianos (COSTERTON; STEWART; GREENBERG, 1999), multiplicou-se o valor da CIM da emulsão de ácidos anacárdicos em PBS por 100 e 1000, dando origem ao estudo 2 (capítulo 2). Previamente, porém, investigou-se o possível efeito citotóxico da emulsão de ácidos anacárdicos em culturas celulares de fibroblastos. Para esta análise, utilizou-se a mais alta concentração que seria utilizada no modelo de biofilme (1000 vezes o valor da CIM da emulsão de ácidos anacárdicos) e metade deste valor.

Métodos de cultura celular para avaliar a toxicidade de materiais podem ser utilizados com sucesso, pois são reprodutíveis, rápidos, sensíveis e financeiramente acessíveis para a execução de estudos de biocompatibilidade *in vitro* (ROGERO *et al.*, 2003). Porém, cultura celulares primárias, como a utilizada no capítulo 1, são as que oferecem maior sensibilidade aos materiais testados, principalmente quando estes são tóxicos (STARK et al., 1986; VASINGTON *et al.*, 1967). Dessa forma, apesar das concentrações analisadas no capítulo 1 terem exibido uma moderada citotoxicidade, este resultado não significa que a emulsão de ácidos anacárdicos seja citotóxica, devendo outros métodos e outras concentrações serem investigados.

O **capítulo 2** objetivou avaliar o efeito de duas concentrações da emulsão de ácidos anacárdicos (C1 = 132,4 μg/ml; C2 =1.324 μg/ml) sobre biofilmes de *S. mutans*. Os resultados deste estudo demonstraram que a emulsão de ácidos anacárdicos reduziu a viabilidade de *S. mutans* em biofilmes maduros e a concentração mais elevada (1.324 μg/ml) mostrou-se mais efetiva do que o controle positivo (clorexidina a 0.12%). O peso seco do biofilme não alterou após o tratamento com ambas concentrações analisadas e isto pode ter ocorrido devido a um efeito bacteriostático da emulsão.

Devido aos bons resultados obtidos com o estudo do capítulo 2, partiu-se para a realização do **capítulo 3**. Neste estudo, utilizou-se a melhor concentração encontrada no estudo 2 e metade desta concentração (1.324 μg/ml e 662 μg/ml, respectivamente). Duas formas de tratamento do biofilme foram empregadas com a emulsão de ácidos anacárdicos: uma única aplicação, a fim de avaliar qual seria o efeito da emulsão no biofilme formado, e uma aplicação diária, com o objetivo de investigar se a emulsão seria capaz de interferir na

produção de polissacarídeos e consequentemente na formação do biofilme. Os resultados encontrados demonstraram mais uma vez o efeito antibacteriano da emulsão de ácidos anacárdicos ao reduzir a viabilidade bacteriana do *S. mutans* no biofilme após os dois tratamentos. Observou-se uma maior produção de polissacarídeo quando a emulsão de ácidos anacárdicos foi aplicada diariamente, que pode ter ocorrido porque o estresse provocado pelo tratamento levou as bactérias a produzirem mais polissacarídeo, mesmo sendo observado uma redução na viabilidade do *S. mutans*.

Parece ser clara, portanto, a atividade antibacteriana da emulsão de ácidos anacárdicos contra *S. mutans*. Porém, no que diz respeito a sua atuação em biofilmes maduros e na formação de biofilmes de *S. mutans*, ainda há muitas perguntas que necessitam ser respondidas. Novos estudos avaliando outras concentrações da emulsão, bem como diferentes tempos de aplicação do tratamento precisam ser conduzidos. Também seria interessante investigar o efeito sinérgico da emulsão de ácidos anacárdicos com outros compostos naturais e avaliar sua atuação na inibição biofilmes de *S. mutans*. Dessa forma, os estudos apresentados em três capítulos nesta tese são estudos iniciais e precursores de outros estudos, visando explorar mais esta emulsão de ácidos anacárdicos, que emerge como um forte antimicrobiano.

5 CONCLUSÃO GERAL

Com base nos resultados desta tese, pode-se concluir que:

I. A emulsão de ácidos anacárdicos obtida a partir do LCC exibiu um forte efeito antibacteriano em culturas planctônicas de *S. mutans*, podendo ser considerado um agente antimicrobiano promissor. Aceitou-se a hipótese alternativa de que a emulsão natural é mais eficiente do que a emulsão sintética. Novos estudos avaliando a citotoxicidade da emulsão de ácidos anacárdicos precisam ser conduzidos, empregando outras metodologias e utilizando outras concentrações.

II. A emulsão de ácidos anacárdicos apresentou um forte efeito antibacteriano em biofilmes maduros de *S. mutans*, reforçando seu promissor efeito antimicrobiano. As duas concentrações analisadas foram capazes de diminuir a viabilidade de *S. mutans* em biofilme. No entanto, rejeitou a hipótese nula, pois a maior concentração mostrou um efeito antibacteriano mais forte do que a menor concentração.

III. O tratamento único e diária com a emulsão de ácidos anacárdicos reduziu a viabilidade bacteriana de *Streptococcus mutans* em biofilmes maduros e em formação, respectivamente. A aplicação única da emulsão em biofilmes não afetou a matriz de polissacarídeos, e o tratamento diário aumentou a produção de polissacarídeos em ambas concentrações estudadas. Por essa razão, nós rejeitamos a hipótese nula. Considerando que este é o primeiro estudo a examinar os efeitos do ácido anacárdico em culturas de biofilme, propomos a realização de mais estudos, avaliando o mecanismo desta emulsão em biofimes.

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