LUCIANA ARANHA BERTO

ANTIMICROBIAL ACTIVITY OF PLANTS FROM BRAZILIAN CERRADO AGAINST *STREPTOCOCCUS MUTANS*

ATIVIDADE ANTIMICROBIANA DE EXTRATOS DE PLANTAS DO CERRADO BRASILEIRO CONTRA *STREPTOCOCCUS MUTANS*

Piracicaba

2014

Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

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Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in the area of Pharmacology, Anesthesiology and Therapeutics.

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, como parte dos requisitos exigidos para obtenção do título de Doutora em Odontologia, Área de Farmacologia, Anestesiologia e Terapêutica.

Orientador: Prof. Dr. Pedro Luiz Rosalen
Co-orientador: Prof. Dr. Francisco C. Groppo

Este exemplar corresponde à versão final da tese defendida por Luciana Aranha Berto e orientada pelo Prof. Dr. Pedro Luiz Rosalen.

Prof. Dr. Pedro Luiz Rosalen

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RESUMO

Apesar do constante desenvolvimento do conhecimento, prevenção e tratamento da cárie dental, esta doença continua tendo alta prevalência no Brasil e no mundo. Por este motivo, tem crescido o interesse por novos agentes farmacológicos que possam auxiliar no controle do biofilme dental, atuando contra o principal microrganismo associado ao desenvolvimento da cárie, o *Streptococcus mutans*. Desta forma, *o objetivo deste trabalho foi avaliar a atividade antimicrobiana do extrato de quatro plantas do cerrado brasileiro (e suas frações) contra S. mutans UA159*. Extratos hidroalcoólicos de *Lantana camara* (*Lc*)*, Copaifera langsdorffii* (*Cl*)*, Psidium guajava* (*Pg*) *e Cochlospermum regium* (*Cr*)*,* foram submetidos a testes de avaliação da atividade antimicrobiana para determinação das concentrações inibitória (CIM) e bactericida (CBM) mínima, inibição da aderência e queda de pH em solução. O extrato bruto das quatro plantas apresentaram potencial antimicrobiano e foram fracionados por gradiente de polaridade, sendo obtidas, para cada extrato, as frações hexânica (FHx), clorofórmica (FCh), acetato de etila (FAc) e aquosa (FAq). Estas foram submetidas aos experimentos já citados para determinação da(s) fração(ões) ativa(s) de cada extrato, selecionadas com base nos resultados dos testes de atividade antimicrobiana e no rendimento. Foram selecionadas, para a etapa subsequente de avaliação em biofilme, 6 frações ativas: *Lc*-FHx e *Lc*-FCl, ambas com CIM = 15,6 µg/ml, e rendimento 9,5 e 17,5%, respectivamente; *Cl*-FHx, que apresentou CIM = 15,6 µg/ml, atividade inibitória sobre a queda de pH em solução e rendimento igual a 21%; *Pg*-FHx, com CIM = 125 µg/ml, 93,4% de inibição da aderência na concentração de 62,5 µg/ml e rendimento igual a 2,0%; *Cr*-FHx, com CIM = 125 µg/ml, apresentou atividade inibitória sobre queda de pH do meio e rendimento igual a 1,0% e *Cr*-FAq, que obteve CIM elevado, entretanto, inibiu 86,7% de aderência bacteriana, na concentração de 62,5 µg/ml, sendo o rendimento desta fração 32%. As frações selecionadas foram submetidas a avaliações complementares, como: viabilidade bacteriana (*time kill*), inibição de formação e queda de pH em biofilme de *S. mutans*, utilizando discos de hidroxiapatita. Nos testes em biofilme*,* destacaram-se três frações: *Lc*-FHx, que em concentração 20xCIM, proporcionou redução na viabilidade do microrganismo e diminuição da formação do biofilme tratado diariamente

por 5 dias; *Lc*-FCh, que em concentração equivalente a 20xCIM, reduziu a formação de biofilme e *Cl*-FHx, que além de reduzir a formação de biofilme na concentração de 20xCIM, interferiu na viabilidade do microrganismo, nas duas concentrações testadas (10xCIM e 20xCIM). A composição química das frações ativas em biofilme foi analisada por CG-EM. As demais frações testadas nesta etapa não diferiram do controle negativo (veículo) nos testes aplicados. Nenhuma das frações avaliadas afetou a redução de pH do meio pelo biofilme. Em conclusão, as frações com polaridades baixa ou intermediária das espécies *Lantana camara* e *Copaifera langsdorffii* mostraram ter potencial para gerar novos compostos anti-cárie de origem natural, tendo apresentado atividade antimicrobiana sobre o biofilme formado por *S. mutans*.

Palavras-chave: *Streptococcus mutans*, biofilme oral, atividade antimicrobiana, *Lantana camara*, *Copaifera langsdorffii*, *Psidium guajava*, *Cochlospermum regium*.

ABSTRACT

Despite the continuous development of knowledge, prevention and treatment of dental caries, the disease continues to have a high prevalence in Brazil and worldwide. For this reason, there is been a strong interest for new pharmacological agents that can assist biofilm control, acting against the main microorganism associated with the development of caries, *Streptococcus mutans*. Thus, the aim of this study was to evaluate the antimicrobial activity of the extract of four plants from Brazilian cerrado (and its fractions) against *S. mutans* UA159. Hydroalcoholic extracts of *Lantana camara* (*Lc*), *Copaifera langsdorffii* (*Cl*), *Psidium guajava* (*Pg*) and *Cochlospermum regium* (*Cr*) were evaluated by means of antimicrobial tests for the determination of minimum inhibitory (MIC) and bactericidal (MBC) concentrations, inhibition of adhesion and pH-drop of *S. mutans*. The crude extract of four plants showed antimicrobial potential and were fractionated by polarity gradient, and 4 fractions were obtained for each extract: hexane (FHx), chloroform (FCh), ethyl acetate (FAc) and aqueous (FAq) fractions. These fractions were subjected to previously mentioned tests to determine the active fraction(s) of each extract. For the subsequent evaluation step in biofilm, 6 active fractions were selected: *Lc*-FHx and *Lc*- FCl, both with MIC = 15.6 µg/ml, and yield of 9.5% and 17.5%, respectively; *Cl*-FHx, which showed MIC = 15.6 µg/ml, an inhibitory activity on the pH drop in solution and yield of 21%; *Pg*-FHx with MIC = 125 µg/ml, 93.4% of inhibition of adhesion in the concentration of 62.5 µg/ml and yield of 2.0%; *Cr*-FHx, with MIC = 125 µg/ml, an inhibitory activity against pH drop and a yield of 1.0% and *Cr*- Aq, which showed a high MIC value, however, it inhibited 86.7% of bacterial adhesion at a concentration of 62.5 µg/ml, and had a yield of 32%. The selected fractions were subjected to additional tests as bacterial viability (time-kill), inhibition of formation and pH drop in biofilms of *S. mutans,* using hydroxyapatite disks. Three active fractions stood out in tests on biofilm: *Lc*-FHx which decreased the viability of the microorganism and biofilm formation in the concentration of 20xMIC, *Lc*- FCx, which reduced biofilm formation in the 20xMIC concentration, and *Cl* – FHx, which reduced biofilm formation in the 20xMIC concentration and interfered with the viability of the microorganism at the two tested concentrations (10xMIC and 20xMIC). The chemical

composition of active fractions was analyzed by GC-MS. The other fractions tested in biofilm did not differ from the negative control (vehicle). None of the evaluated fractions affected the pH drop by biofilm. In conclusion, *Lantana camara* and *Copaifera langsdorffii* fractions with low or intermediate polarities showed potential to generate new naturally occurring anticarie compounds and presented antimicrobial activity against biofilms formed by *S. mutans*.

Keywords: *Streptococcus mutans*, oral biofilm, antimicrobial activity, *Lantana camara*, *Copaifera langsdorffii*, *Psidium guajava*, *Cochlospermum regium*.

SUMÁRIO

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"Precisamos dar um sentido humano às nossas construções. E, quando o amor ao dinheiro, ao sucesso nos estiver deixando cegos, saibamos fazer pausas para olhar os lírios do campo e as aves do céu."

Érico Veríssimo

INTRODUÇÃO

Apesar do constante desenvolvimento do conhecimento, prevenção e tratamento da cárie dental, esta doença continua sendo a que possui maior prevalência dentro da Odontologia (Maia *et al.*, 2007) e representa um grande problema de saúde bucal, tanto no Brasil (Brasil, 2004; 2012) como na maior parte do mundo (Petersen *et al.*, 2005; More *et al.*, 2008; de Oliveira *et al.*, 2013).

A cárie é uma doença multifatorial, cuja etiologia envolve a ação de microrganismos fortemente acidogênicos e acidúricos (Hamada & Slade, 1980), que interagem numa comunidade mista, permitindo a formação de um verdadeiro biofilme na superfície dental (Marsh, 2005; Shemesh *et al.*, 2007). O *Streptococcus mutans* é o microrganismo colonizador predominante da cavidade oral, além de ser considerado o mais importante agente etiológico associado à doença cárie. Na superfície dental, é uma das espécies que forma o biofilme e causa dissolução do esmalte devido à formação de produtos ácidos resultantes da metabolização de carboidratos fermentáveis da dieta (Marsh, 2005; Shemesh *et al.*, 2006; 2010; Selwitz *et al.*, 2007).

Por esta razão, o *S. mutans* tem sido intensamente estudado quanto aos seus fatores de virulência, dentre eles, a tolerância ao meio ácido favorecida pela atividade de deslocamento de prótons pela ATPase (Burne *et al.*, 1999; Quivey *et al.*, 2000) e os mecanismos de adesão da bactéria à superfície dental. Um importante fator de adesão deste microrganismo é a síntese de glucanos solúveis e insolúveis, por meio das enzimas glucosiltransferases - GTFs (Gibbons e Van Houte, 1975; Hamada & Slade, 1980; Marsh, 2005). Atualmente, três GTFs distintas, secretadas pelo *S. mutans*, estão bem caracterizadas molecular e bioquimicamente. São elas: GTF B - codificada pelo gene *gtfB*, que sintetiza glucanos insolúveis em água com ligações glicosídicas predominantes α (1→3), GTF C - codificada pelo gene *gtfC*, que sintetiza uma mistura de glucanos insolúveis e solúveis, com ligações glicosídicas predominantes α (1→6), e GTF D - codificada pelo gene *gtfD*, que sintetiza basicamente glucanos solúveis (Loesche, 1986; Hanada & Kuramitsu, 1989; Vacca-Smith & Bowen, 1998). Os glucanos, principalmente os insolúveis em água, têm

sido considerados os principais fatores de aderência e acúmulo de estreptococos cariogênicos sobre a superfície dental (Hamada & Slade, 1980; Rolla *et al.*, 1983; Tanzer *et al.*, 1985; Schilling & Bowen, 1992). Em acréscimo, tem sido demonstrado que estes glucanos aumentam a porosidade (Dibdin & Shellis, 1988; Van Houte, 1994; Bowen, 2002) bem como causam mudanças na composição inorgânica da matriz da placa (Cury *et al.*, 1997; 2000), tornando-a ainda mais cariogênica. Outros mecanismos também podem promover a colonização destes microrganismos por meio da formação de sítios de ligação para a bactéria, pela síntese de proteínas relacionadas à adesão (Shemesh *et al.*, 2007).

Além da adesão e da tolerância ao meio ácido, outros fatores de virulência do *S. mutans* já foram estudados. Desta forma, os mecanismos pelo qual o *S. mutans* se adere à superfície dental e demais fatores de virulência são alvos potenciais importantes para o controle do biofilme dental formado por este microrganismo (Shemesh *et al.*, 2007).

As estratégias mais comuns para prevenção ou controle da cárie dental têm sido a redução do biofilme bacteriano ou de patógenos específicos (Baehni & Takeuchi, 2003), especialmente por meio de agentes capazes de inibir estreptococos do grupo mutans e/ou seus fatores de virulência. Tem-se demonstrado que algumas substâncias de origem natural podem agir sobre os fatores de virulência dessas bactérias, apresentando potencial antimicrobiano e anti-cárie em testes in vitro e in vivo (Koo *et al.*, 2000; Duarte *et al.*, 2003).

Desta forma, extratos ou substâncias químicas isoladas de plantas utilizadas na medicina popular devem ser pesquisados como alternativas terapêuticas para o controle do biofilme dental patogênico (Tichy & Novak, 1998), representando uma imensa fonte para a descoberta de novas drogas, especialmente em países de grande biodiversidade, como Brasil (Braz-Filho, 1999). Inúmeras são as vantagens para o uso terapêutico desses extratos naturais, dentre elas o baixo custo e a grande disponibilidade para a população de baixa renda (Calixto, 2000).

Lantana camara, também conhecida como lantana ou cambará, *Copaifera langsdorffii*, popularmente chamada de copaíba, *Psidium guajava,* a goiaba e

Cochlospermum regium, o algodãozinho-do-cerrado, são espécies vegetais do cerrado brasileiro, um bioma rico e inexplorado, consideradas como plantas medicinais e utilizadas pela população local. Estudos já demonstraram ação antimicrobiana de extratos de destas plantas para alguns microrganismos patogênicos importantes.

O extrato de *Lantana camara* obteve significante atividade antimicrobiana contra 12 microrganismos, dentre eles *Streptococcus faecalis*, *Candida albicans* e *Staphylococcus aureus*, suportando a utilização popular desta planta como agente antimicrobiano para algumas doenças (Kumar *et al.*, 2006).

Compostos originados de *Copaifera langsdorffii* demonstraram atividade antimicrobiana contra microrganismos cariogênicos, como *Streptococcus salivarius*, *S. sobrinus*, *S. mutans*, *S. mitis*, *S. sanguinis* and *Lactobacillus casei* (Souza *et al*., 2011b) e microrganismos periodontopatogênicos, como *Porphyromonas gingivalis*, *Prevotella nigrescens*, *Fusobacterium nucleatum*, *Bacteroides fragilis*, *Actinomyces naeslundii*, *Bacteroides thetaiotaomicron*, e *Peptostreptococcus anaerobius* (Souza *et al*., 2011a).

O extrato de folhas de *Psidium guajava* também tem sido estudado com relação a suas propriedades antimicrobianas, sendo comprovada sua atividade contra *Bacillus cereus* (Biswas *et al.*, 2013), *Staphylococcus aureus* (Biswas *et al*., 2013; Sanches *et al.*, 2005; Vieira *et al*., 2001; Gnan e Damello, 1999) e *Yarrowia lipolytica*, um importante fungo patogênico (Sacchetti *et al*., 2005).

Gimenez *et al.* (2009) comprovaram a atividade do extrato alcoólico de *Cochlospermum regium* contra *Staphylococcus aureus*, *Streptococcus pyogenes* e *Klebsiella pneumoniae*. Uma fração originada da mesma planta também apresentou atividade contra *Staphylococcus aureus* e *Pseudomonas aeruginosa* (Solon *et al.*, 2009).

Desta forma, o presente estudo avaliou a atividade antimicrobiana de extratos hidroalcoólicos e frações de plantas do cerrado como *L. camara*, *C. langsdorffii, P. guajava* e *C. regium* contra *S. mutans*.

PROPOSIÇÃO

Estudar a atividade antimicrobiana de extratos e frações de plantas do cerrado brasileiro contra *Streptococcus mutans*.

Proposições Específicas:

1. Verificar a atividade antimicrobiana de extratos e frações de quatro plantas do cerrado brasileiro (*Lantana camara*, *Copaifera langsdorffii, Psidium guajava* e *Cochlospermum regium*) por meio de testes *in vitro* em células planctônicas de *S. mutans* UA159;

2. Identificar as frações ativas dos extratos vegetais que apresentarem atividade antimicrobiana ou que afetem os fatores de virulência (adesão e produção de ácido) contra *S. mutans* UA159 na forma planctônica;

3. Identificar as frações ativas com atividade antimicrobiana contra *S. mutans* UA159 em biofilme formado ou em formação, ou que afetem os fatores de virulência (adesão e produção de ácido) deste microrganismo estruturado em biofilme.

4. Demonstrar o perfil fitoquímico das frações ativas contra *S. mutans* em biofilme.

PREÂMBULO DO CAPÍTULO

Esta tese foi elaborada de acordo com a Informação CCPG/002/06, UNICAMP, de 13/09/2006 (Anexo I), que regulamenta o formato alternativo para dissertação e tese, permitindo a inserção de artigos científicos de autoria ou co-autoria do candidato.

Desta forma, a referida tese é composta por um capítulo contendo um artigo científico, intitulado "**Antimicrobial properties of plants from Brazilian Cerrado against *Streptococcus mutans***", que foi submetido para publicação na revista científica *Journal of Ethnopharmacology* (Anexo II).

CAPÍTULO 1

Antimicrobial properties of plants from Brazilian Cerrado against *Streptococcus mutans*

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Abstract

Ethnopharmacological relevance: Extracts from *Lantana camara* (lantana), *Copaifera langsdorffii* (copaiba), *Psidium guajava* (guava) e *Cochlospermum regium* (yellow cotton tree), plants from Brazilian Cerrado, an important and naturally rich biome, has been used in folk medicine by their antimicrobial and anti-inflammatory properties. *Objective:* The aim of this study was to evaluate the antimicrobial activity of extracts and fractions of mentioned plants from Brazilian Cerrado against *Streptococcus mutans*. *Material and methods:* The hidroethanolic extracts of the plants and their fractions (obtained by liquid-liquid partition of different polarities) were tested for their antimicrobial activity by preliminary evaluation - minimum inhibitory (MIC) and bactericidal (MBC) concentrations, pH drop in dense cell solution and adherence inhibition. After first tests, the fractions selected for their best antimicrobial activity were evaluated for their influence on *S. mutans* biofilm formed in hydroxyapatite disks by inhibition of formation, killing and pH drop assays. The chemical composition of the active fractions was assessed by GC-MS. *Results:* Hexanic and chloroform fractions from *L. camara* and hexanic fraction from *Copaifera langsdorffii* demonstrated strong antimicrobial activity, each one presenting MIC = 15.6 µg/mL and reduction on biofilm viability and formation. *Conclusion:* Apolar fractions of *Lantana camara* and *Copaifera langsdorffii* presented antimicrobial activity against *S. mutans* biofilm and can be useful as a source of natural anticarie compounds.

1. Introduction

Dental caries is one of the main common pathologies affecting humankind (More et al., 2008). This condition is biofilm-related infectious disease caused by a complex relation between microorganisms in the mouth, tooth surface and fermentable carbohydrates from diet (Loesch, 1986). *Streptococcus mutans* is considered the major etiologic agent of dental decay due to its ability to initiate the pathogenic biofilm formation, producing large amounts of extra and intra-cellular polysaccharides. In addition, this

microorganism is highly acidogenic and aciduric, promoting demineralization of dental enamel and decay (Galvão et al, 2012).

One of the most important tactic to combat caries disease consists on the control of the biofilm formed by *S. mutans*, targeting on its virulence factors such as acidogenicity and polysaccharide formation that promotes adherence of the microorganism to tooth surface (Koo and Jeon, 2009). In this context, natural products have been extensively studied, as an attempt to find effective anticarie agents (Jeon et al, 2011).

Medicinal plants have been used worldwide as traditional treatments for a variety of human diseases since ancient times. Derived from this knowledge, natural products have been the basis for the development of new lead compounds for pharmaceuticals (Palombo, 2011).

Lantana camara (lantana), *Copaifera langsdorffii* (copaiba), *Psidium guajava* (guava) and *Cochlospermum regium* (yellow cotton tree) are medicinal plants found in Brazilian Cerrado and used by local population as tradicional medicines innumerous infectious conditions and have been investigated for their antimicrobial activity (Kumar *et al.*, 2006; Gimenez *et al.*,2009, Souza *et al*., 2011a,b; Biswas *et al.*, 2013). Thus, the aim of this study was to evaluate the antimicrobial activity of extracts of mentioned plants from Brazilian Cerrado against *S. mutans.*

2. Material and Methods

2.1. Medicinal plants

Lantana camara, *Copaifera langsdorffii* and *Psidium guajava* were obtained from the Research Center for Chemistry, Biology and Agriculture (CPQBA), University of Campinas (UNICAMP), São Paulo, Brazil, and screened by germoplasm bank of the Collection of Medicinal and Aromatic Plants (CPMA) of the CPQBA/UNICAMP and identified by its curator Dr. Glyn M. Figueira. The plant *Cochlospermum regium* was

gently provided by Dr. Maria C. Vieira from the Federal University of Grande Dourados (UFGD), Mato Grosso do Sul, Brazil, and it was deposited in the herbarium of the UFGD, which has Dr. Zefa V. Pereira as the curator. Plants were collected from October 2011 to January 2013, in the morning. Detailed data from the plants are shown in Table 1.

Table 1. Medicinal plants used in the present study, with popular name, family, part used for the extract and registration number.

Medicinal plant	Popular name	Family	Part used	Herbarium/ registration number
Lantana camara	Lantana	Verbenaceae	leaf	CPQBA 1103
Copaifera langsdorffii	Copaiba	Caesalpinaceae	leaf	CPMA 2062
Psidium guajava	Guava	Myrtaceae	leaf	CPQBA 885
Cochlospermum regium	Yellow cotton tree	Cochlospermaceae	root	DDMS 4615

2.2. Preparation of plant extracts

For preparing extracts from *L. camara*, *C. langsdorffii*, *P. guajava* and *C. regium* the leaves or roots of the plants were washed with water, dried in an incubator at 40 °C, during 4 days, until they get brittle and then grounded into powder by a knife mill. Hidroalcoholic solution (ethanol 70%, v/v) was added to the powder to make a 20 % concentration and the mixture were homogenized (IKA Polytron/Ultra-turrax® T50 basic), during 1 minute, at 4000 rpm. Filtration was performed, new hidroalcoholic solution was added to the plant material and a second process in homegenized device was conducted. After new filtration, the solutions was homogenized and dried with a rotatory evaporator. The resultant material was the crude extract of the plants. Before antimicrobial tests, extracts were reconstituted with hidroethanolic solution 70% (v/v).

2.3. Fractionation of the extracts

The hidroethanolic extract of the plants was subjected to chemical liquid-liquid fractionation, based on a polarity gradient. The extract was solubilized in distilled water by an ultrasound bath and the fractionation was carried out using three organic solvents, in the proportion 2:1 (v/v), in the following order: hexane, chloroform and ethyl acetate, which originated the fractions FHx, FCh and FAc, respectively. The final residue obtained after

ethyl acetate fractionation was called aqueous fraction (FAq). The fractions were dried with a rotatory evaporator and the FAq was lyophilized. Each fraction was monitored by thin layer chromatography and developed under UV light (254 nm and 366 nm). Before being tested against *S. mutans*, the organic fractions were reconstituted with absolute ethanol and the FAq, with hidroethanolic solution 50% (v/v).

2.4. Analyses of the active fractions by GC-MS

The chemical composition of the fractions found to have activity against *S. mutans* biofilm was evaluated using a Hewlett-Packard 6890 gas chromatograph equipped with an HP-5975 mass selective detector and HP-5 capillary column (30m × 0.25mm × 0.25 μm). GC-MS was performed using split injection with the injector set at 250 °C, the MS detector set at 300 °C and the column set at 110 °C with a heating ramp of 5 °C/min and a final temperature of 280 °C, sustained for 26 min. Helium was used as the carrier gas at 1 mL/min. The GC-MS electron ionization system was set at 70 eV. A sample of each fraction was solubilized in methanol for the analysis. The quantitative analyses were performed using a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector under the same conditions previously described. Retention indices (RIs) were determined using injection of hydrocarbon standards and samples under the same conditions described above. The fractions components were identified by comparison with data described in the literature and the profiles in the NIST 05 mass spectral library.

2.5. Antimicrobial assays

The antimicrobial evaluation consisted in preliminary assays with the microorganism in suspension (MIC, MBC, inhibition of adherence and pH drop), followed by additional tests in biofilm (formation of biofilm, viability and pH drop). Only active fractions with promissory results in preliminary tests were selected for the experiments in biofilm. The criteria of selection were: MIC ≤ 500 μg/mL (Duarte et al., 2007), 80% of adherence inhibition in sub-MIC concentration and maintenance of the solution pH above 5.5.

2.5.1. Microorganisms

The present study used the microorganism *Streptococcus mutans* UA159, which was stored at -80 ºC in brain heart infusion (BHI - Difco®, Franklin Lakes, NJ, USA) broth containing 20 % glycerol.

2.5.2. Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC and MBC tests were performed using the microtechnique described by da Cunha et al. (2013). Chlorhexidine 0.12% (m/v) (Sigma-Aldrich, St. Louis, MO, USA) was used as positive control and ethanol 3.5% or 5% (v/v) as negative control. Each well received 190 µL of BHI broth inoculated with *S. mutans* (final concentration of 1-2 x 10^5 CFU/mL) and 10 µL of the extract, fraction or controls. Tested concentrations of extracts and fractions varied from 3.9 to 1000 µg/mL and the final concentration of vehicle (ethanol) in each well was 3.5% and 5% when diluting extracts and fractions, respectively. The microplaque was incubated for 24 h at 37 °C in 5% CO_2. MIC was defined as the lowest concentration of extracts or fractions that inhibited microorganism growth indicated by 30 µL of resazurin 0.01% (Sigma-Aldrich, St. Louis, MO, USA). To determine MBC, an aliquot (10 µL) of each well with concentrations higher than MIC was subcultured on BHI agar and incubated for 48 h at the same conditions previously described. MBC was defined as the lowest concentration of the extracts or fractions that allowed no growth on the agar. The nature of antimicrobial effect of fractions was determined by the MBC/MIC ratio (CLSI, 2009). Thus, when MBC/MIC ratio for *S. mutans* was between 1 and 2, the fraction was considered bactericidal against this microorganism, and when the ratio was higher than 2, it was considered bacteriostatic.

2.5.3. Inhibition microorganism adherence

To determine the influence of the extracts or fractions on the microorganism adherence, the technique used by Castro et al. (2009) was performed. Non-treated concave bottom 96-well microplates were used to grow the microorganism in BHI broth with 1% sucrose (w/v) and the same inoculum and incubation conditions used in item 2.5.2 but

containing sub-MIC concentrations of extracts, fractions or control (3.5% or 5% ethanol, v/v). After incubation, the medium and non-adhering cells were removed, the wells were washed three times with sterile distilled water and adhering cells were stained with colorant crystal violet. The amount of adhered cells was quantified by measuring the absorbance at 575 nm.

2.5.4. Glycolytic pH drop in dense cell suspension

The effects of extracts or fractions on the acid production by *S. mutans* were assessed by pH drop test with dense cell suspensions (2 mg.cell-dry-weight/mL) as described by Murata et al. (2008). Cells of *S. mutans* from 18 h suspension cultures were harvested, washed once with salt solution (SS: 50mM KCl plus 1mM $MgCl_2$) and resuspended in the SS containing extracts or fractions in concentrations of 5xCIM and 10xCIM (based on literature and on solubility of the agent in the SS) or vehicle control (5% ethanol, v/v). The pH was adjusted to 7.2 with 0.1M KOH solution and glucose was added to achieve a concentration of 1% (w/v). The pH drop was evaluated with an pH glass electrode (Orion™, Thermo Fischer Scientific Inc, Waltham, MA, USA) attached to an pHmeter (Orion™ 290A+, Thermo Fischer Scientific Inc, Waltham, MA, USA) over a period of 90 min. Biocidal activity was observed by plating aliquots of cell suspension at each time point.

2.5.5. Activity of selected fractions on biofilm formation

Hydroxyapatite (HA) disks (surface area 1.47 cm^2; Clarkson Chromatography Products Inc., South Williamsport, PA, USA) were placed in a vertical position using a custom-made disk holder, in 24-well plates with ultrafiltered (10 kDa cutoff membrane; Prep/Scale; Millipore, MA) tryptone yeast extract (UFTYE, pH 7.0), with 1% (w/v) sucrose, containing *S. mutans* 2×10^6 CFU/mL and were incubated at 37 °C, 5% CO_2. After 19 h of undisturbed incubation to permit initial biofilm formation, biofilms were treated twice daily (at 10 am and 4 pm) until the 5th day of the experiment (115-hour-old biofilms) with selected fractions (concentrations of 10xCIM and 20xCIM) or control (5 % ethanol, v/v), diluted in adsorption buffer (AB; 50 mM KCl, 1 mM KPO_4, 1 mM $CaCl_2$, 0.1 mM

$MgCl_2$, pH 6.5). The UFTYE medium was replaced daily and a total of eight treatments were performed, each one with 1 min of exposition, according to da Cunha et al. (2013). The influence of selected fractions or control on biofilm formation was assessed by observing the bacterial viability (colony forming units - CFU/mL) after 5 days of treatments. At the end of the experimental period (115 h), biofilms were removed from UFTYE broth, washed once with AB and subjected to ultrasound bath and sonication (30 s pulse; output 7 W), to provide maximum recoverable viable counts. Cells suspensions were diluted, plated in BHI agar, and incubated for 48 h at 37 °C and 5% CO_2, for determination of UFC/mL.

2.5.6. Time-kill assay

To perform time-kill assay, biofilms of *S. mutans* were grown as described on item 2.5.5, with daily replacement of the medium, but with no treatments. Thus, 115 h-old undisturbed biofilms were exposed to selected fractions (concentrations of 10xCIM and 20xCIM), vehicle (5% ethanol, v/v) or positive control (chlorhexidine digluconate 0.12%, Sigma-Adrich, St. Louis, MO, USA), diluted in AB. After 1, 2 and 3 h of exposure, biofilms were removed from treatments, washed once in AB and subjected to the same process of sonication, dilution, plating and colony counting previously described (item 2.5.5). Killing curves were constructed by plotting log 10 CFU/mL versus time over 3 h (Duarte et al., 2006).

2.5.7. Glycolytic pH-drop in biofilm

Biofilms acid production during the exposure to selected fractions was evaluated using the technique described by Belli et al. (1991) with some modifications. The 115 h-old *S. mutans* biofilms grown in HA disks (without treatments, with daily replacement of the UFTYE) were washed once in SS and transferred to wells containing the selected fractions (concentrations of 10xCIM and 20xCIM) or vehicle (5% ethanol, v/v), diluted in AB. In the initial time point, the pH of these solutions were adjusted to 7.2 with

0.1 M KOH solution and glucose was added to a final concentration of 1% (w/v). The pH was then monitored every 15 min, during 90 min.

2.6. Statistical analysis

Triplicates from at least three separate experiments were conducted in each of the assays. An exploratory data analysis was performed to determine the most appropriate statistical test; the assumptions of equality of variances and normal distribution of errors were also checked. Data were analyzed using ANOVA and, when significant differences were detected, pairwise comparisons were made between all the groups using Tukey's method to adjust for multiple comparisons. The analysis was performed using the statistical program Biostat 5.0 (Mamirauá Institute, Manaus, Amazonas, Brazil). The level of significance was set at 5%.

3. Results and Discussion

Recently, medicinal plants have been widely investigated as a potential source of new and active therapeutic agents in an attempt to find alternatives to synthetic products for the treatment of numerous diseases (Gossell-Williams et al., 2006). In the present study, the antimicrobial properties of extracts from *L. camara* (*Lc*), *C. langsdorffii* (*Cl*), *P. guajava* (*Pg*) and *C. regium* (*Cr*) were tested against *S. mutans*, which is the major microorganism responsible for initiating oral pathogenic biofilm that causes dental caries (Loesch, 1986). The evaluation of crude extracts of *L. camara*, *C. langsdorffii*, *P. guajava* and *C. regium* against the *S. mutans* showed promissory results. All tested plants demonstrated activity on inhibition of microorganism growth (MIC and MBC tests) or at least on one of its virulence factors (inhibition of acidogenicity and/or adherence) at low concentration extract (Tables 2 and 3). Therefore, the four plant extracts were fractionated originating four fractions each (FHx, FCh, FAc and FAq) which were submitted to the same antimicrobial tests to determine the active fraction of each extract, which would be used in further biofilm assays. In addition, the yield of each fraction is in Table 2.

Lantana camara and *C. langsdorffii* extracts showed highest inhibitory activities, with low MIC values of 250 µg/mL and 125 µg/mL, respectively, and MBC of 500 µg/mL. *P. guajava* and *C. regium* showed very low inhibition of the microorganism growth, both with high MIC values of 1000 µg/mL and MBC of 4000 µg/mL (Table 2). *Lc*-FHx and *Lc*-FCh showed MIC of 15.6 µg/mL, while *Lc*-FAc and *Lc*-FAq showed higher values (62.5 and 1000 µg/mL, respectively), showing that active compounds are in the nonpolar fractions (FHx or FCl). For *C. langsdorffi,* FHx showed the best result, (MIC = 15.6 µg/mL), followed by FCh (MIC = 125 µg/mL), FAc (MIC = 500 µg/mL) e FAq (MIC = 1000 µg/mL). FHx of *P. guajava* and *C. regium* showed MIC value of 125 µg/mL, while FCh, FAc and FAq of both plants showed higher values, ranging from 500 to 1000 µg/mL. MBC values for the fractions ranged from 250 to 1000 µg/mL. The MBC/MIC ratio showed that all fractions demonstrated bacteriostatic effect against *S. mutans*, since ratio values are higher than 2 (CLSI, 2009; Galvão et al., 2012).

Table 2. Medicinal plants used in the study, their respective hexanic, chloroform, ethyl-acetate and aquous fractions with yield, MIC and MBC values, and MBC/MIC ratio.

Medicinal plant	MIC (CE) (µg/mL)	MBC (CE) (µg/mL)	Fraction (FR)	Yield (%)	MIC (FR) (µg/mL)	MBC (FR) (µg/mL)	MBC/MIC ratio[a]
L. câmara (lantana)	250	500	Hexanic*	9.5	15.62	500	32
			Chloroform*	17.5	15.62	500	32
			Ethyl-acetate	4	62.5	500	8
			Aquous	35	1000	-	-
C. langsdorffii (copaiba)	125	500	Hexanic*	21	15.62	250	16
			Chloroform	2.5	125	500	4
			Ethyl-acetate	27	500	>1000	-
			Aquous	37.5	1000	-	
P. guajava (guava)	1000	4000	Hexanic*	2	125	500	4
			Chloroform	6.5	500	>1000	-
			Ethyl-acetate	13	500	>1000	-
			Aquous	56.5	1000	-	-
C. regium (yellow cotton tree)	1000	4000	Hexanic*	1	125	500	4
			Chloroform	3.17	500	>1000	-
			Ethyl-acetate	28	1000	-	-
			Aquous*	32	1000	-	-

(*) Fractions selected for the biofilm assays. (CE) Crude Extract. ([a]) The fractions were considered bactericidal when the MBC/MIC ratio was between 1 and 2, and bacteriostatic if this ratio was higher than 2. (Galvão et al., 2012; CLSI, 2009). (-) Value not possible to determine.

The yield of fractions selected for the next step (tests on biofilm) was considered feasible for this *in vitro* model, ranging from 1% (*Cr*-FHx) to 56.5% (*Pg*-FAq), as showed in Table 2.

The implemented liquid-liquid fractionation process was considered efficient on separating active compounds in all evaluated extracts, since the MIC values of fractions were lower than the MIC of their original crude extract. This bioguided study is found to be successful, since active fractions present higher antimicrobial activity than their respective extract (Galvão et al., 2012).

Duarte et al. (2007) considered crude extracts with MIC values up to 500 µg/mL as strongly active and promissory for bioprospection of natural antimicrobial compounds. Thus, this study initially showed that *L. camara* and *C. langsdorffii* were promissory extracts, presenting MIC values 2 to 4 times inferior (250 µg/mL and 125 µg/mL, respectively) than the pre-established criteria (Duarte et al, 2007). Consequently, their fractions *Lc*-FHx, *Lc*-FCl e *Cl*-FHx showed even lower MIC values (15.6 µg/mL) indicating the strong activity of extracts compounds. In the other hand, according to the same criteria, the extracts from *P. guajava* e *C. regium* presented no antimicrobial potencial (MIC = 1000 µg/mL) to continue the investigation and would be discarded. However, their fractions *Pg*-FHx e *Cr*-FHx showed MIC values 8 times lower (125 µg/mL) than their original extract, and also affected virulence factors of *S. mutans* (acidogenicity and adherence), as will be shown forward (Table 3).

In the present study, *L. camara* showed strong antimicrobial activity against *S. mutans* and inhibition of adhesion at sub-MIC concentration (Tables 2 and 3). Kumar et al. (2006) also found that the extract of *L. camara* have significant activity against 12 microorganisms, including *Streptococcus faecalis*, *Staphylococcus aureus* and *Candida albicans*, supporting the use of this plant in folk medicine as an antimicrobial agent for some diseases.

Our study showed that the specie *C. langsdorffii* is also a promissory, with a low MIC value (15.6 µg/mL). However, a previous study observed no antimicrobial

activity of extracts from the bark of *C. langsdorffii* against *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus pyogenes* (Gonçalves et al. (2005). The lack of activity reported by the authors may be due to the absence of microorganisms sensibility to the tested extract or perhaps there is reduced active compounds concentration on the bark of *C. langsdorffii*. In our study the extract of the leaves of the plant (more sustainable source that the bark) were evaluated. Moreover, Souza et al. (2011b) tested the copalic acid isolated from the resin oil of *C. langsdorffii*, checking low MIC values for *S. mitis* (5 µg/mL), *S. mutans* (3 µg/mL) and *S. salivarius* (2 µg/mL). The lower MIC values obtained by the authors probably is due to use of an isolated compound. This fact ratifies the continuity of our study, aiming isolation and chemical identification of active substances from the evaluated extract (from leaf , not resin) of *C. langsdorffii*, based on evidences that the plant synthesizes secondary metabolic compounds active against *S. mutans*, which may also be present in the leaves of the specie.

Nader (2010) evaluated extracts of *C. regium* obtained from different parts of the plant root (bark, between the bark and core) after extraction using the solvents methanol, chloroform and hexane. The authors did not observed inhibition of *Staphylococcus aureus* growth with any of the extracts. In the present study, hydroethanolic extracts from *C. regium* showed moderate antimicrobial activity against *S. mutans* (MIC 1.000 µg/mL), also inhibiting the adherence of the microorganism at sub-MIC concentrations (500 µg/mL), an important fact to be considered in the search for new agents to control oral biofilm by decreasing cell adhesion and, consequently, microorganism virulence.

Gimenez et al. (2009) also tested the ethanolic extract of branches, leaves and roots of *C. regium* against some microorganisms and verified activity against *Staphylococcus aureus*, *Streptococcus pyogenes* and *Klebsiella pneumoniae* at concentration of 1.000 µg/mL. Like in the present study, the extract of *C. regium* showed moderate antimicrobial activity. However, the authors did not evaluated the activity on *S. mutans*.

Jebashree et al. (2011) evaluated the activity of ethyl acetate extract of *P. guajava* leaves against *S. mutans* obtaining MIC of 76 µg/mL. In our research, the extract of *P. guajava* showed moderate antimicrobial activity (1.000 µg/mL) however, the extract was obtained with hidroethanolic solution, allowing the extraction of different plant compounds what could explain the divergence of results.

Antimicrobial activity of methanolic and ethanolic extracts from *P. guajava* leaves was evaluated in a recent study (Biswas et al, 2013). The authors observed effective growth inhibition of *Bacillus cereus* and *Staphylococcus aureus* by both extracts tested, suggesting that guava possesses compounds containing antibacterial properties. This findings support our results which showed good activity of *Pg*-FHx against *S. mutans* in planktonic cells (MIC=125 µg/mL).

The inhibition of *S. mutans* adherence is an important activity to be investigated when searching for anticarie agents (Jeon et al., 2011). The binding and accumulation of microorganisms on teeth surface and to each other, mainly enabled by insoluble extracellular polysaccharides produced by the *S. mutans* glucosyltransferase enzymes, are important virulence factors of the oral biofilm (Schilling and Bowen, 1992; Koo et al., 2010).

The crude extract of *L. camara* inhibited 98.8 % of the adherence of *S. mutans* at the concentration of 125 µg/mL, while *P. guajava* and *C. regium* showed, respectively, 93 % and 83.7 % of inhibition at the concentration of 500 µg/mL. *C. langsdorffii* showed no effect on adherence of the microorganism (Table 3). As mentioned before, only two fractions showed activity on adherence properties of the microorganism: *Pg*-FHx, with 92.4 % on inhibition at the concentration of 62.5 µg/mL, and *Cr*-FAq, with 86.7 % of inhibition at the same concentration. The fractions of *L. camara* and *C. langsdorffii* presented no activity on the adherence on sub-MIC concentrations, what can infer that synergism of substances may be involved in the activity of crude extracts on adherence, which was annulled by the fractionation process in this two extracts.

Table 3. Results for inhibition of adherence and planktonic pH drop tests for extracts and fractions.

Medicinal plant	AIC (µg/mL)	Percentage of inhibition	Activity on pH drop	Fraction	AI C (µg/mL)	Percentage of inhibition	Activity on pH drop
L. camara (lantana)	125	98.8	(+)	Hexanic	*	-	(-)
				Chloroform	*	-	(-)
				Ethyl-acetate	*	-	(-)
				Aquous	*	-	(-)
C. langsdorffii (copaiba)	*	-	(+)	Hexanic	*	-	(+)
				Chloroform	*	-	(-)
				Ethyl-acetate	*	-	(-)
				Aquous	*	-	(-)
P. guajava (guava)	500	93	(+)	Hexanic	62.5	92.4	(-)
				Chloroform	*	-	(-)
				Ethyl-acetate	*	-	(-)
				Aquous	*	-	(-)
C. regium (yellow cotton tree)	500	83.7	(+)	Hexanic	*	-	(+)
				Chloroform	*	-	(-)
				Ethyl-acetate	*	-	(-)
				Aquous	62.5	86.7	(-)

*No inhibition was observed for the tested on sub-MIC concentrations.

Another important virulence factor of *S. mutans* is its capacity of tolerating low enviromental pH and producing acid. Low pH leads to demineralization of the dental enamel and formation of carious lesions if the pH value falls below 5.5, which is the critical pH value for dissolution of dental hydroxyapatite (Loesche, 1986). The effects of extracts and fractions on the acidogenic and aciduric properties of *S. mutans* were examined by glycolytic pH-drop assay.

All the four crude extracts disrupted the acid production of the microorganism (Table 3), sustaining solution pH values above 5.5, however only the FHx of *C. langsdorffii* (Figure 1A) and *C. regium* (Figure 1B) preserved the property observed in the respective extracts (Table 3). The FHx of these two extracts sustained the solution pH above 5.5 during the first 45 min of experiment when tested in the concentration of 10xMIC and during the 15 first min, when tested in the concentration of 5xMIC (Figure 1). Once again, for *L. camara* and *P. guajava,* the fractionation may have influenced on the activity of the extracts, separating substances that could be acting synergistically.

Thus, the *Lc*-FHx, *Lc*-FCl, *Cl*-FHx, *Pg*-FHx, *Cr*-FHx and *Cr*-FAq reached the selected criteria for further analysis since they have showed the best activities against growth or/and one of the virulence factors of *S. mutans* (the selection criteria was described in item 2.5). So, those fractions were submitted to the tests on *S. mutans* biofilm structured on hydroxyapatite disc surface to mimic a more complex and resistant situation of this microorganism in the oral environment (Lemos et al., 2010).

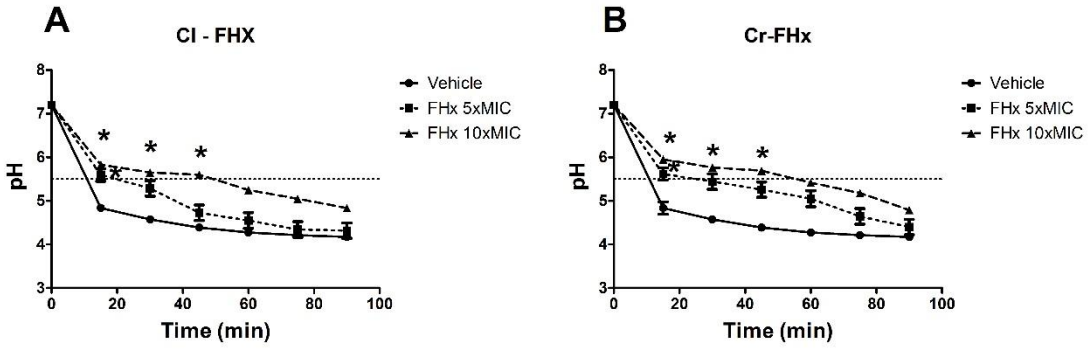

Figure 1. Glycolytic pH drop curves for hexanic fractions of *C. langsdorffii* (A) and *C. regium* (B), observed for 90 minutes. (*) pH above 5.5 and $p < 0.05$ when compared to vehicle (ANOVA-Tukey test).

Results for biofilm formation experiment are shown in Figure 2. The *Lc*-FHx, *Lc*-FCl and *Cl*-FHx showed a significant reduction on biofilm formation ($p<0.05$) at concentration of 20xMIC. When tested in the concentration of 10xMIC, no differences were observed between these fractions and the vehicle treatment ($p>0.05$). The *Pg*-FHx, *Cr*-FHx and *Cr*-FAq caused no reduction on the biofilm formation in the tested concentrations ($p>0.05$).

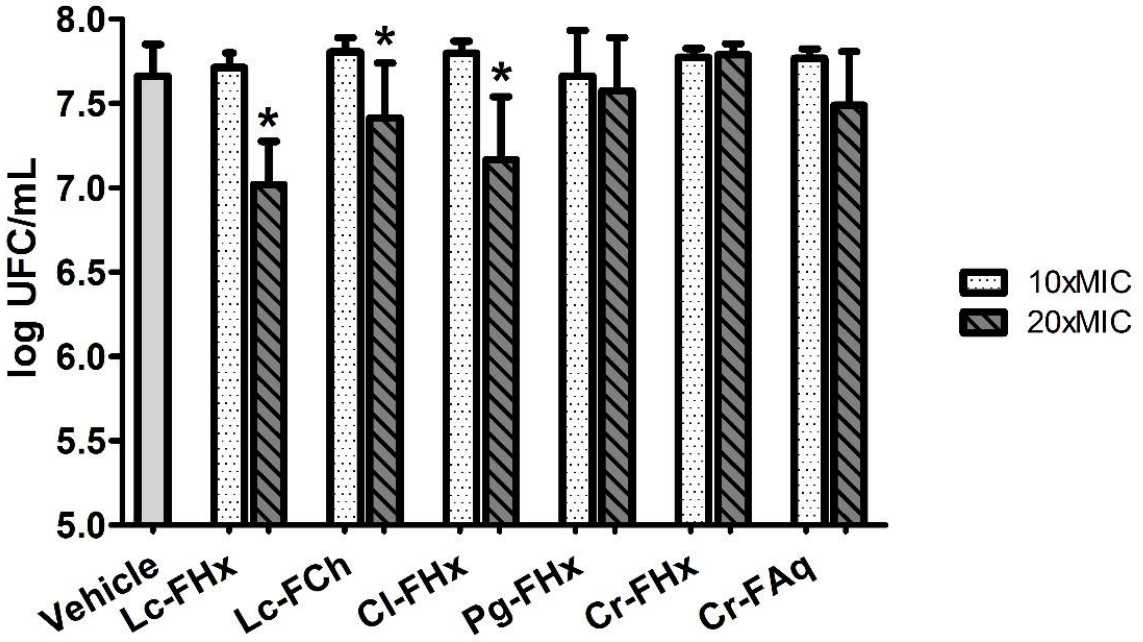

Figure 2. Effect of selected fractions *Lc*-FHx, *Lc*-FCh, *Cl*-FHx, *Pg*-FHx, *Cr*-FHx e *Cr*-FAq on *S. mutans* biofilm formation (logUFC/mL). (*) $p<0.05$ when compared to vehicle control (ANOVA/ Tukey).

Time-kill curves of *S. mutans* biofilm after the exposure to selected fractions can be observed in Figure 3. The *Lc*-FHx reduced microorganism viability after 2 and 3 h of contact ($p<0.05$), at concentration of 20xMIC (Figure 3A). The *Cl*-FHx also reduced the viability of *S. mutans* after 2 and 3 h of contact with the fraction at the concentration of 20xMIC ($p<0.05$) (Figure 3C). When tested in the concentration of 10xMIC, the reduction was observed only after 3 h of exposure ($p<0.05$). For the other evaluated fractions, no decline on the *S. mutans* viability on biofilm was observed for the two tested concentrations ($p>0.05$) (Figure 3 B, D, E and F).

The pH drop assay was performed on the *S. mutans* biofilm to evaluate the effect of selected fractions on its acid production. Nevertheless, none of the fractions caused alterations in the pH drop when compared to vehicle ($p>0.05$ – data not shown).

In the present study, the fractions that showed the best antimicrobial activity on preliminary assays with planktonic cells (MIC, MBC, inhibition of adherence and pH drop) were selected to be tested against *S. mutans* biofilm. In a bioprospection study, the results

of tests on planktonic cells have great importance on guiding the antimicrobial investigation, in addition to permitting a fast screening with a small amount of tested agents. However, these experiments are inefficient to reproduce physiological conditions of the microorganism in the oral cavity (Jeon et al., 2011), therefore, additional tests on *S. mutans* biofilm were conducted in the present study.

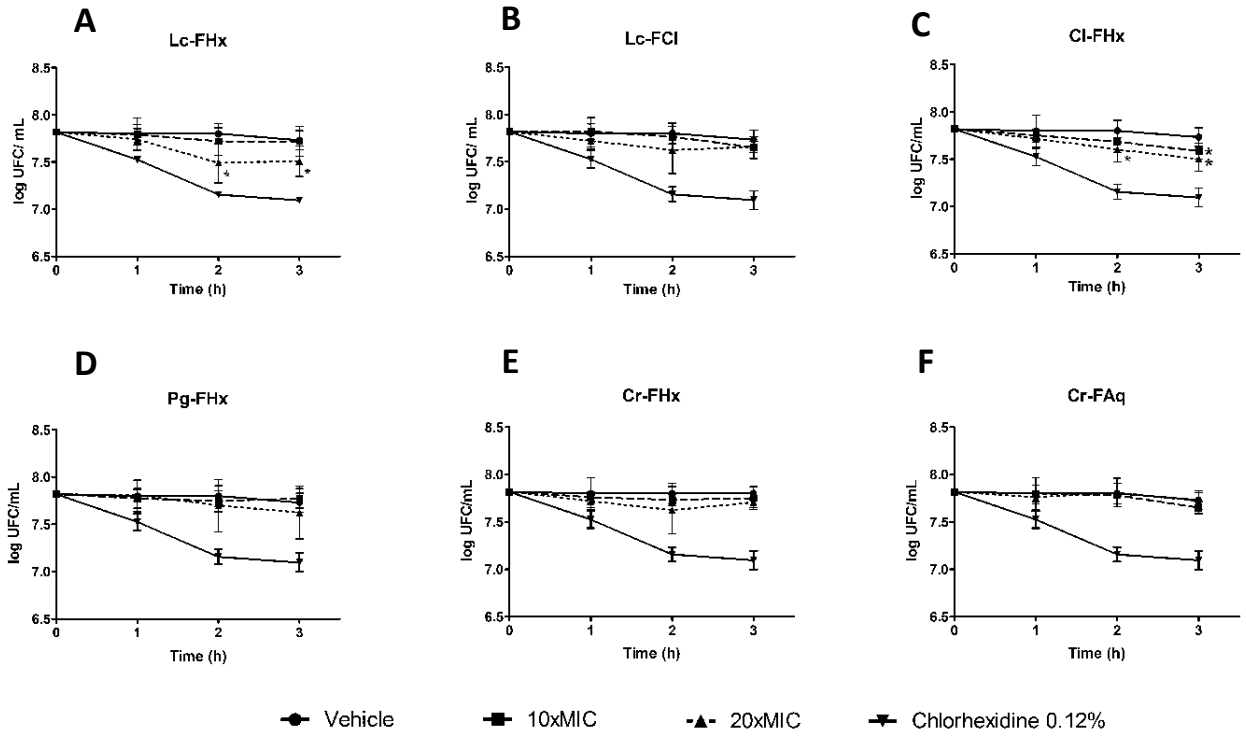

Figure 3. Time-kill curves of selected fractions (A) *Lc*-FHx, (B) *Lc*-FCh), (C) *Cl*-FHx), (D) *Pg*-FHx, (E) *Cr*-FHx e (F) *Cr*-FAq, on *S. mutans* biofilm (logUFC/mL). (*) $p<0.05$ when compared to vehicle control (ANOVA/ Tukey).

Lc-FHx, *Lc*-FCh and *Cl*-FHx affected *S. mutans* biofilm at the concentration of 20xMIC, confirming the inhibitory activity found in MIC and MBC tests (MIC=15.6 µg/mL). However, *Pg*-FHx and *Cr*-FHx, despite inhibiting the growth of microorganism in suspension (125 µg/mL), did not reduced viability or formation of biofilm. The same differences in results from planktonic cell and biofilm were observed for *Cl*-FHx and *Cr*-FHx, which reduced pH drop for the first experiment, however, did not affect acid production on biofilm.

It is well recognized that bacteria grown in suspension behave different from those embedded in biofilm (Lemos et al, 2010). Surface-bound bacteria exhibit a specific phenotype with molecular and physiological particularities, which confer protection and resistance of the microorganism to environment and to antimicrobial agents (Simões, 2011; da Cunha et al., 2013). As an example, antimicrobial concentrations required to inhibit bacterial biofilms can be up to 10-1000 times higher than those needed to affect the same microorganism grown planktonically (Simões, 2011). Therefore, the resistant structure of biofilm can explain the differences between our results for planktonic cells and biofilm model.

Figure 4 shows the chemical profile of the fractions that presented activity against *S. mutans* biofilm (*Lc*-FHx, *Lc*-FCh, and *Cl*-FHx). Most chemical compounds found in active fractions were identified by name or molecular mass (M) (Table 4). The unidentified substances (ni) are not main compounds since their relative percentage (rel %) ranged from 0.41% to 2.8 %. The three major compounds of *Lc*-FHx and *Lc*- FCh were the same (M = 468 / Rt 51.55 min and 51.50 min , M = 484 / Rt 56.05 min and 56.04 min , and M = 484 / Rt 59.19 min and 59.22 min, respectively) and may possibly be responsible for the pharmacological activities observed, although they were identified only by molecular mass and retention time with the methods used . Similarly, the two major compounds of *Cl*-FHx (M = 318 / Rt 26.51 min / 29.20 rel %, and M = 316 / Rt 29.88 min / 26.61 rel %), can be the active compounds.

Previous studies report the substances lantadene A (M = 552.8) and lantadene B (M = 552.8) as *L. camara* major and active compounds (toxic activity) (Tailor et al., 2013). However, in the present study, they were not identified in the active fractions (*Lc*- FHx and *Lc*-FCh).

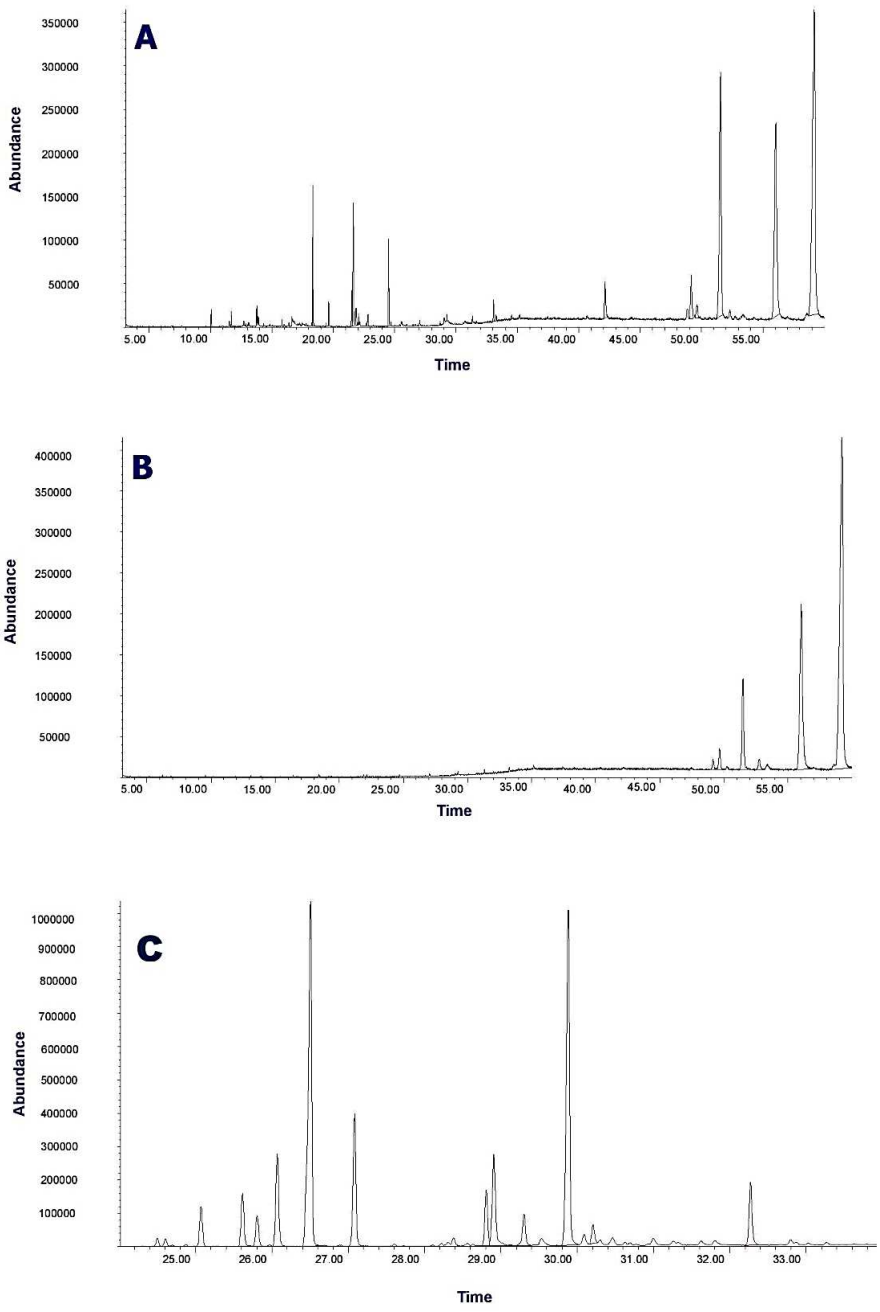

Figure 4. Chromatograms of fractions that presented activity against *S. mutans* biofilm, obtained through GC-MS. (A) *Lc*-FHx, (B) *Lc*-FCh, (C) *Cl*-FHx.

The (-) -copalic acid (M = 304), found in the essential oil of *C. langsdorffii* (Souza et al., 2011b), was the only reported active compound against *S. mutans* identified for the this plant, though it was not identified in active fraction *Cl*-FHx in the present study.

Probably another chemical compound or synergistic combination of compounds such as those identified in Table 4 may be responsible for the antimicrobial activity against *S. mutans* observed in this study.

Table 4. Major compounds of *Lc*-FHx, *Lc*-FCh, *Cl*-FHx, active against *S. mutans* biofilm with their retention time (Rt) and relative percentage (Rel %).

	Lc-FHx		Lc-FCh		Cl-FHx	
Identification	**Rt (min)**	**Rel %**	**Rt (min)**	**Rel %**	**Rt (min)**	**Rel %**
2,4-bis (1,1-dimethylethyl) phenol	10,05	0,31	*	*	*	*
Hexadecane	11,70	0,33	*	*	*	*
n.i.	13,77	0,39	*	*	*	*
hexadecanoic acid methyl ester	18,33	3,00	*	*	*	*
hexadecanoic acid ethyl ester	19,62	0,48	*	*	*	*
methyl ester of 9,12-octadecadienoic acid	21,50	0,84	*	*	*	*
methyl 9,12,15-octadecatrienoic	21,63	3,65	*	*	*	*
n.i.	21,86	0,68	*	*	*	*
octadecanoic acid methyl ester	22,07	0,26	*	*	*	*
n.i.	22,82	0,41	*	*	*	*
butyl citrate	24,51	1,85	*	*	*	*
n.i.	33,05	0,49	*	*	*	*
beta-sitosterol	42,14	2,00	*	*	*	*
n.i.	49,17	2,79	*	*	*	*
n.i.	49,64	0,94	*	*	*	*
M = 468	51,55	20,86	51,50	9,50	*	*
M = 484	56,05	21,92	56,04	26,09	*	*
M = 484	59,19	38,80	59,22	60,13	*	*
n.i.	*	*	49,16	0,96	*	*
n.i.	*	*	49,67	2,06	*	*
n.i.	*	*	52,77	1,27	*	*
methyl isopimarato	*	*	*	*	25,07	2,82
M = 316	*	*	*	*	25,62	3,75
M = 318	*	*	*	*	25,81	2,21
M = 204	*	*	*	*	26,07	6,64
M = 318	*	*	*	*	26,51	29,20
M = 318	*	*	*	*	27,08	9,13
n.i.	*	*	*	*	28,81	2,80
n.i.	*	*	*	*	28,91	2,50
M = 316	*	*	*	*	29,30	2,45
M = 316	*	*	*	*	29,88	26,61
M = 316	*	*	*	*	30,21	1,33
M = 316	*	*	*	*	32,28	4,56

4. Conclusion

In conclusion, *Lantana camara* and *Copaifera langsdorffii,* particularly through their apolar fractions, presented antimicrobial activity against *S. mutans* biofilm and can be

valuable as sources of new natural anticarie compounds to control the oral biofilm. However, future studies should be conducted in preclinical and clinical models to validate the use of the extract or fraction of these plants in the development for therapeutic agents for dental caries.

Acknowledgments

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CONCLUSÃO

Em conclusão, as frações com polaridades baixa ou intermediária das espécies *Lantana camara* e *Copaifera langsdorffii* mostraram ter potencial para gerar novos compostos anti-cárie de origem natural, tendo apresentado atividade antimicrobiana sobre o biofilme formado por *S. mutans*. No entanto, estudos futuros devem ser conduzidos em modelos pré-clínicos e clínicos para validar a utilização do extrato ou fração dessas plantas na busca de agentes terapêuticos para a cárie dentária.

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ANEXO 1: Resolução da Comissão Central de Pós-Graduação da Unicamp que trata do formato alternativo para defesa da tese de doutorado.

INFORMAÇÃO CCPG/002/06

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG nº 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, sendo o projeto gráfico das capas definido pela PRPG.
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2º desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III, IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão retirados pelas Unidades em no máximo, cinco dias úteis para impressão preto e branco e 10 dias úteis para coloridas.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

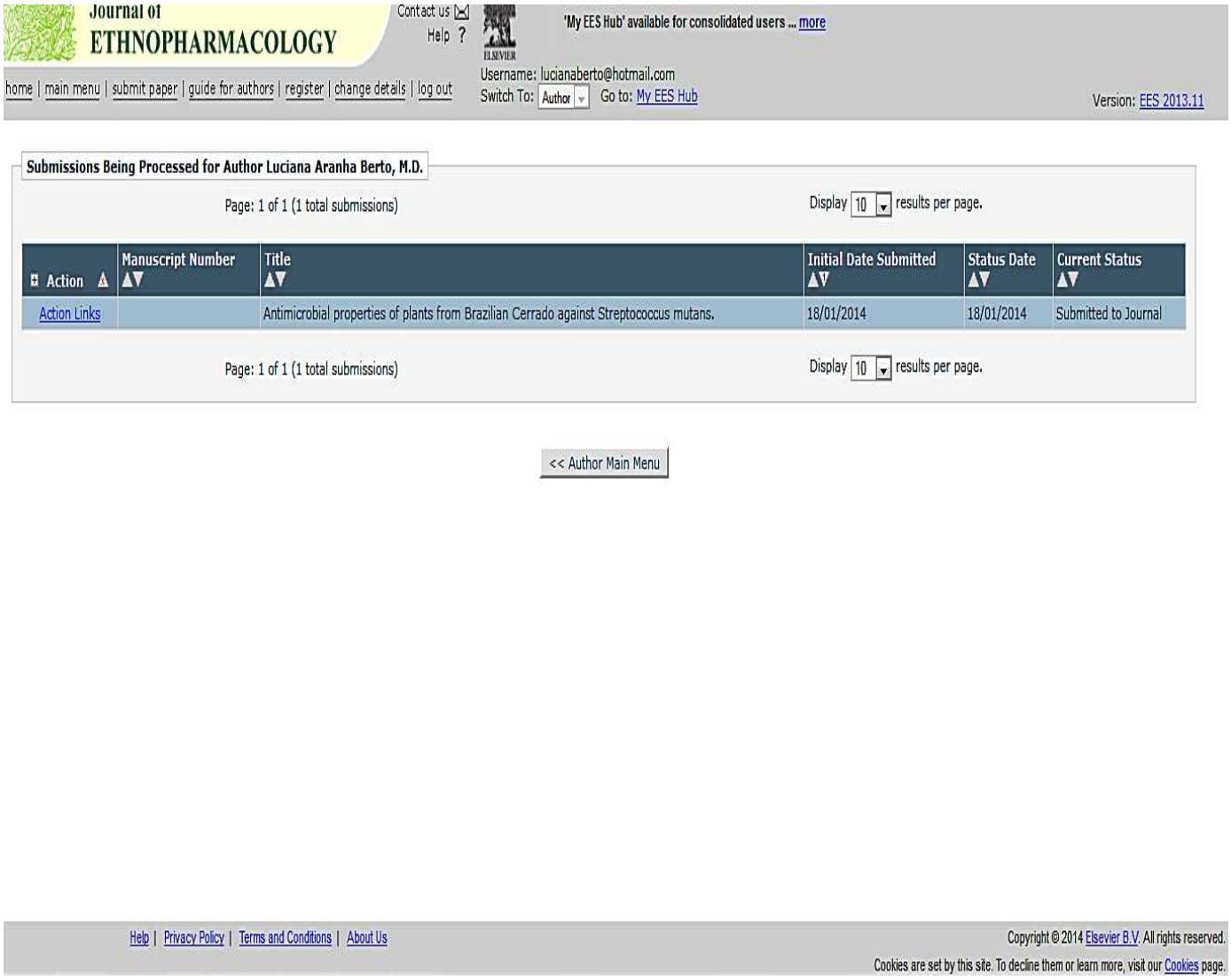
§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006



ANEXO 2: Comprovante de submissão do artigo científico ao periódico *Journal of Ethnopharmacology*.