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LIDIA AUDREY ROCHA VALADAS MARQUES

DESENVOLVIMENTO, AVALIAÇÃO CLÍNICA E MICROBIOLÓGICA DE VERNIZ DENTÁRIO DE *COPAIFERA LANGSDORFFII* DESF

FORTALEZA

2019

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Tese apresentada ao Programa de Pós-graduação em Desenvolvimento e Inovação em Medicamentos da Universidade Federal do Ceará, como parte das exigências para a obtenção do título de Doutor em Desenvolvimento e Inovação Tecnológica em Medicamentos.

Orientadora: Profa Dra Mary Anne Medeiros Bandeira (Universidade Federal do Ceará).

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Esta Tese foi submetida como parte dos requisitos necessários à obtenção do GRAU DE DOUTOR em Desenvolvimento e Inovação Tecnológica em Medicamentos, outorgado pela Universidade Federal do Ceará, e encontra-se à disposição dos interessados na Biblioteca Setorial da referida Universidade.

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São Leopoldo Mandic

“A Deus e ao pai que a vida me deu, Djecílio Gonçalves Araújo (*in memoriam*), sem vocês eu não estaria hoje aqui realizando esse sonho de me tornar Doutora!”

.

“Sou voo longo a varar ventos,
Com brio e sofrer
Mas só faço a vida alçar
Fixada às asas do saber”
Alcinet Rocha

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RESUMO

Desenvolvimento, avaliação clínica e microbiológica de verniz dentário de *Copaifera langsdorffii* Desf. Lídia Audrey Rocha Valadas Marques. Orientadora: Profa. Dra. Mary Anne Medeiros Bandeira. Tese de Doutorado em Desenvolvimento e Inovação Tecnológica em Medicamentos. Universidade Federal do Ceará, 2019.

Introdução: O uso de produtos naturais com moléculas biologicamente ativas em diferentes tipos de formulações e formas de aplicações tem sido pesquisado como meio preventivo de doenças bucais. Estudos comprovam o potencial terapêutico da *Copaifera spp* ou copaíba em diversas funções, nas quais destacam-se as propriedades anti-inflamatória e antimicrobiana. Os vernizes dentários representam uma boa estratégia de prevenção, haja vista a grande aderência ao dente e liberação lenta, contínua e prolongada do princípio ativo. Objetivos: Realizar um estudo prospectivo de patentes de produtos de copaíba, desenvolver e avaliar a eficácia clínica e microbiológica de verniz dentário de *Copaifera langsdorffii* DESF (*C. langsdorffii*) na prevenção de lesões cariosas em crianças com alto risco de cárie dentária. Material e Métodos: Inicialmente realizou-se um estudo prospectivo de patentes de produtos dentários de copaíba em bancos de propriedade intelectual no Brasil e no mundo. No estudo piloto recrutou-se 24 crianças, de idade entre 36 e 71 meses, livres de cárie (ICDAS II= 0) e com bom estado sistêmico de saúde. As crianças foram divididas em 4 grupos para avaliação da dose-resposta na saliva após aplicação dos vernizes em diferentes concentrações (1%, 5%, 10% e 20%). Cada paciente recebeu a aplicação do verniz na face oclusal de todos os segundos molares decíduos e a saliva foi coletada antes e após a plicação do verniz para verificação da dose-resposta. A terceira etapa do estudo, trata-se de um estudo longitudinal, randomizado, controlado. Um total de 90 participantes, foram selecionados com os mesmos critérios de inclusão e exclusão do estudo piloto e foram randomizados em 3 grupos com 30 pacientes cada: O Grupo I – Verniz de *C. langsdorffii* 1%; Grupo II – Verniz de clorexidina 1%; Grupo III- Verniz de Flúor (5% NaF). Nessa etapa, cada paciente recebeu a aplicação do verniz na face oclusal de todos os segundos molares decíduos. O verniz foi aplicado 3 vezes para cada dente, no *baseline* (D0), após 3 meses (D90) e após 6 meses do início do tratamento (D180). Saliva foi coletada no *baseline* (D0), após 3 meses (D90), após 6 meses (D180) e, após 12 meses do início do tratamento (D360), para contagens de unidades formadoras de colônia (UFC) de *Streptococcus mutans* (*S.mutans*). As UFC foram contadas, sendo identificadas a partir das características morfológicas e os valores foram expressos em log10 (UFC/mL). A análise estatística foi realizada através do ANOVA e teste de Tukey. Resultados: No estudo prospectivo foram encontradas 9 patentes usando resina de óleo de resina de copaíba em produtos odontológicos. O Instituto Nacional da Propriedade Industrial (INPI-Brasil) apresentou o maior número de depósitos (5), seguido por Espacenet (2) e Free Patents (2). *C. Langsdorffii* foi destacado como a espécie mais amplamente utilizada nos produtos e depósitos de veículos nas formulações (3). No estudo piloto todas as concentrações apresentaram atividade antimicrobiana, sem diferença estatística entre as mesmas. Na análise comparativa do pré e pós tratamento, a concentração de 1% obteve melhor dose-resposta ($p=0,0026$). No estudo longitudinal os níveis de *S.mutans* (significância ± desvio padrão) foram os seguintes: Clorexidina D0: 0,58 ± 0,43; D90: 0,38 ± 0,23; D180:0,33 ± 0,14; D360: 0,55 ± 0,52), Flúor (D0:0,86 ± 0,37; D90:0,51 ± 0,33; D180:0,41 ± 0,24; D360:0,53 ± 0,44), Copaiba (D0:1,32 ± 0,61; D90:0,99 ± 0,57; D180:0,39 ± 0,22; D360:0,12 ± 0,19). Copaiba demonstrou redução significativa nos seguintes tempos: D360 versus D0 ($p < 0,0001$), D180 versus D0 ($p < 0,001$),

D360 versus D90 ($p < 0,001$), D180 versus D90 ($p < 0,001$), D360 versus D180 ($p < 0,05$). O verniz de clorexidina reduziu nos seguintes tempos: D180 versus D0 ($p < 0,05$). O grupo de Flúor reduziu *S.mutans* no D180 versus D0 ($p<0,001$). O grupo tratado com copaíba reduziu consistentemente as CFU em todo o período estudado. Na última coleta D360, a copaíba foi estatisticamente significante quando comparada ao grupo do flúor ($p <0,001$) e clorexidina ($p < 0,01$). Conclusão: Apesar da atividade biológica ser documentada há séculos, poucas foram as patentes para fins odontológicos encontradas com copaíba. A concentração de 1% obteve melhores respostas. Após três aplicações anuais, o verniz de copaíba demonstrou atividade antimicrobiana significante contra *S.mutans* por até 12 meses em crianças com alto risco de cárie. Futuros estudos são necessários para identificar efeitos anticárie para estabelecer o uso do verniz na prevenção da cárie.

Descritores: Odontopediatria; Cárie dentária; Produtos naturais; Saliva.

ABSTRACT

Development, clinical and microbiological evaluation of *Copaifera langsdorffii* Desf dental varnish. Lídia Audrey Rocha Valadas Marques. Orientadora: Profa. Dra. Mary Anne Medeiros Bandeira. Doctoral thesis in Development and Technological Innovation in Medicines. Federal University of Ceara, 2019.

Introduction: The use of biologically active natural products in different types of formulations and forms of applications has been researched to prevent oral diseases. Studies have demonstrated the therapeutic potential of *Copaifera langsdorffi* (copaiba) in several functions, especially the anti-inflammatory and antimicrobial properties. The varnishes represent a good prevention strategy, given the great adhesion to the tooth and slow, continuous and prolonged release of the active principle. Objective: The aim of the study was to do a prospective study of patents for copaiba products, develop and evaluate longitudinally the clinical and antimicrobial efficacy of a 1% *Copaifera langsdorffi* DESF (*C. langsdorffii*) dental varnish to prevent dental caries in high-risk children. Materials and Methods: Initially, a prospective study of copaiba dental product patents was carried out in intellectual property banks in Brazil and worldwide. In the pilot study, 24 children, aged between 36 and 71 months, caries-free (ICDAS II = 0) and with good systemic health were recruited. The children were divided into 4 groups for saliva dose-response evaluation after varnish application at different concentrations (1%, 5%, 10% and 20%). Each patient received the varnish application on the occlusal face of the all second deciduous molars and saliva was collected before and after the varnish application to verify the dose-response. The third stage of the study is a longitudinal, randomized, controlled study. A total of 90 participants were selected with the same inclusion and exclusion criteria from the pilot study and were randomized into 3 groups with 30 patients each: Group I - C. langsdorffii varnish 1%; Group II - 1% chlorhexidine varnish; Group III- Fluoride Varnish (5% NaF). At this stage, each patient received the varnish application on the occlusal face of the all deciduous second molars. Varnishes were applied to occlusal surfaces of second deciduous molars on the baseline (D0), 3 months after (D90), and 6 months after (D180). Saliva was collected before starting treatment (D0), 90 days after day 0 (D90), 6 months (D180) and 1 year after initiation of treatment (D360) to evaluate the *S.mutans* reduction. Microbiological analysis was performed in duplicates (1:10 and 1:100 mL dilutions). *S. mutans isolates* were identified by its characteristic colony morphology and the values were expressed as log10 (CFU/mL). Statistics were carried out by applying repeated measures analysis of variance, Tukey's multiple comparisons test, and paired t test. Results: In the prospective study, 9 patents were found using copaiba resin oil resin in dental products. The National Institute of Industrial Property (INPI-Brazil) had the largest number of deposits (5), followed by Espacenet (2) and Free Patents (2). C. Langsdorffii has been highlighted as the most widely used species in vehicle products and deposits in formulations (3). In the pilot study all concentrations showed antimicrobial activity, with no statistical difference between them. In the pre and post treatment comparative analysis, the 1% concentration obtained the best dose-response ($p = 0.0026$). In the longitudinal study we found the MS levels (mean± standard deviation) were as follows: Chlorhexine (D0: 0.58 ± 0.43; D90: 0.38 ± 0.23; D180:0.33 ± 0.14; D360: 0.55 ±0.52), Fluoride (D0:0.86 ± 0.37; D90:0.51 ± 0.33; D180:0.41 ± 0.24; D360:0.53 ± 0.44), Copaiba (D0:1.32 ± 0.61; D90:0.99 ± 0.57; D180:0.39 ± 0.22; D360:0.12 ± 0.19). Copaiba demonstrated significant *S.mutans* reduction: D360 versus D0 ($p < 0.0001$), D180 versus D0 ($p < 0.001$), D360versus D90 ($p < 0.001$), D180 versus D90 ($p < 0.001$), D360 versus D180 ($p < 0.05$). Chlorhexidine varnish significantly reduced *S.mutans*: D180 versus D0 ($p < 0.05$). Fluoride reduced *S.mutans* at D180

versus D0 ($p < 0.001$). Copaiba consistently reduced *S.mutans* throughout the 360-day period. At D360, copaiba produced lower *S.mutans* levels compared to fluoride ($p < 0.001$) and chlorhexidine ($p < 0.01$). Conclusions: Although biological activity has been documented for centuries, few patents for dental purposes have been found with copaiba. The 1% concentration obtained better responses. Three annual applications of the aqueous extract of Copaifera langsdorffii dental varnish showed substantial antimicrobial activity against S.mutans for up to 12 months in high-risk caries-free children. Further studies to identify the anticaries effect of this varnish are required to establish its use in caries prevention.

Key-words : Pediatric Dentistry; Dental Caries; Natural Products; Saliva.

LISTA DE ILUSTRAÇÕES

ILUSTRAÇÕES REFERENTES AO CAPÍTULO 1

ILUSTRAÇÕES REFERENTES AO CAPÍTULO 2

ILUSTRAÇÕES REFERENTES AO CAPÍTULO 3

LISTA DE TABELAS

TABELAS REFERENTES AO CAPÍTULO 2

TABELAS REFERENTES AO CAPÍTULO 3

LISTA DE ABREVIATURAS E SIGLAS

AAPD	American Academy of Pediatric Dentistry
CFU	Colony forming unit
C. langsdorffii	*Copaifera langsdorffii* Desf
CPI	Cárie precoce de infância
EEC	Early Caries Childhood
ICDAS	International Caries Detection and Assessment System
INPI	National Institute of Industrial Property
IPC	IPC - International Patent Classification
MBC	Minimal Bactericidal Concentration
MIC	Minimal Inhibitory Concentration
MSB	*Mitis salivarius bacitracin*
S. mutans	*Streptococcus mutans*
UFC	Unidades Formadoras de Colônias
WIPO	World Intellectual Property Organization

SUMÁRIO

1. INTRODUÇÃO

Nos últimos anos, na busca de novas substâncias com potencial farmacológico e biocompatíveis, observa-se o aumento no número de estudos quanto ao uso de produtos naturais em Odontologia, desenvolvimento de materiais com propriedades biológicas ativas e alternativas para o manejo de doenças bucais, em especial, a cárie dentária. De acordo com Romero *et al*. (2009), o uso de produtos naturais com finalidade medicinais é uma prática comum no Brasil e no mundo há várias gerações, tanto para o tratamento quanto para prevenção de doenças. Sendo assim, plantas medicinais têm sido estudadas para avaliar a atividade antimicrobiana frente à *Streptococcus mutans (S.mutans) e* na prevenção de doenças bucais. (FREIRES *et al*., 2015)

A copaíba é uma planta comum na América Latina e África Ocidental, pertencente à família Fabaceae, subfamília *Caesalpinioideae*. Da árvore da copaíba é extraído o óleo resina, que é utilizado para fins medicinais desde o início da colonização do Brasil, encontrando-se entre um dos mais importantes produtos naturais comercializados para outros países. Óleo-resinas são provenientes de secreções abundantes que podem recolher-se exteriormente por simples intercepção dos canais resinosos. O óleo resina da copaíba é constituído por terpenos que são os responsáveis pela característica resinosa do mesmo. O óleo é resultado da desintoxicação do organismo vegetal, e que funciona como defesa da planta contra animais, fungos e bactérias. Os principais constituintes químicos do óleo resina de copaíba são: β-cariofileno, α-humuleno, cedreno, cadineno, bisaboleno, entre outros representados na figura 1. (ALENCAR, 1982; VASCONCELOS *et al*., 2008; PIERI *et al*., 2009)

Os canais óleo-resinosos localizam-se nas diferentes regiões e tecidos do vegetal, normalmente se encontram nas cascas, podendo serem encontrados também mais profundamente no lenho. Muitas vezes aparecem mais no inicio do desenvolvimento do vegetal, podendo perdurar por toda a vida a vida do mesmo. Assim, as incisões praticadas na planta podem retirar o conteúdo natural ou fisiológico desses canais secretores. (COSTA, 1994)

O óleo-resina de copaíba teve seu primeiro grande registro feito por Dom Frei Vicente do Salvador, pouco mais de um século da descoberta do Brasil. Em seu livro sobre a história do Brasil, dois capítulos foram exclusivamente para as plantas medicinais locais, onde destacou-se a árvora copaibera por ter um “óleo precioso” capaz de curar feridas. A árvore era chamada de kupa'iwa pelos índios, certamente seus verdadeiros descobridores, que significava que a árvore possuía algo em seu interior, no caso, o óleo. (TOBOUTI *et al*., 2017)

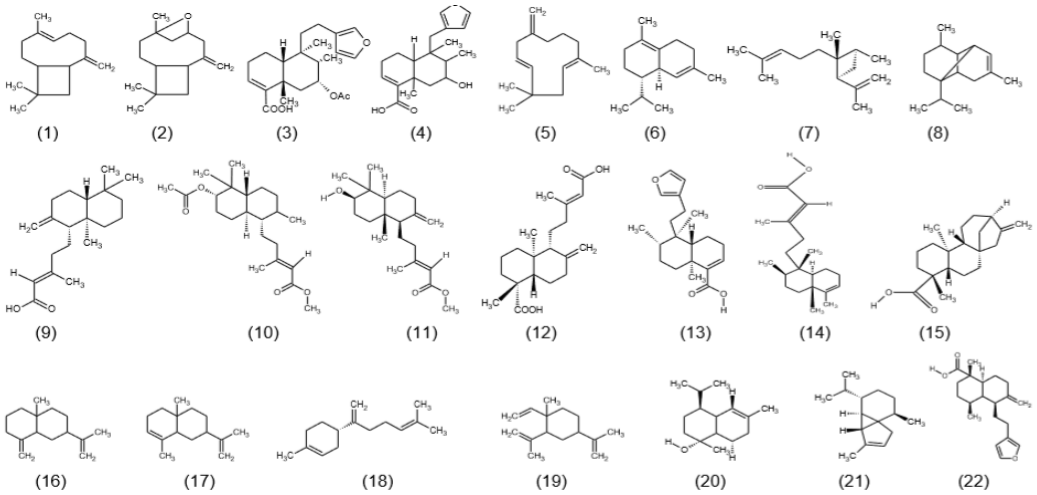


Figura 1: Principais constituintes químicos do óleo de copaíba.

Legenda: (1) - β-cariofileno, (2) - óxido cariofileno, (3) - ácido 7-acetoxi-hardwickiico, (4) - ácido 7-hidroxi-hardwickiico, (5) - α-humuleno, (6) - δ-cadieno, (7) - trans-α-bergamoteno, (8) - α-copaeno, (9) - ácido copálico, (10) - ácido 3-hidroxi-copálico, (11) - ácido 3-acetoxi-copálico, (12) - ácido ent-agático, (13) - ácido hardwickiico, (14) - ácido colavênico, (15) - ácido caurenóico, (16) - β-selineno, (17) - α-selineno, (18) - β-bisaboleno, (19) - β-elemeno, (20) - α-cadinol, (21) - α-cubebeno, (22) - ácido poliáltico.
Fonte: SILVA (2018).

A C*opaifera langsdorffii (C. langsdorffii)*, espécie escolhida nesse estudo, é nativa da floresta amazônica e comum na região do Mato Grosso, onde de acordo com um levantamento, é a espécie com maior importância na região de seu georreferenciamento. (BIESKI *et al*., 2015)

Figura 2: *Copaifera langsdorffii* Desf. (copaíba). Acervo de I.G.C. Bieski.

Fonte: BIESKI (2015).

Estudos comprovaram o potencial terapêutico da copaíba, onde destacam-se as propriedades anti-inflamatória, analgésica, cicatrizante, antisséptica, antitumoral, antibacteriana,

germicida e diurética. Existem relatos sobre outras propriedades, como ação antiviral, antidiarréica, antitetânica, antiulcerogênica e antiofídica, além de indicações para casos de hemorragias, dermatites, cefaleia, eczema e pneumonia. Na Odontologia, estudos demonstraram atividade antimicrobiana do óleo-resina de copaíba na forma de cimento odontológico, frente a *S.mutans*. (VEIGA JUNIOR; PINTO, 2002; VASCONCELOS *et al*., 2008; PIERI *et al*., 2009)

A cárie dentária é atualmente considerada uma disbiose que envolve interações entre a estrutura dentária, biofilme microbiano e exposição de açúcar, onde 35% dos casos não são tratados em todo o mundo. A cárie precoce de infância (CPI) se caracteriza a presença de cárie em crianças com até 6 anos de idade. (GIACAMAN, 2017; PITTS *et al*., 2017)

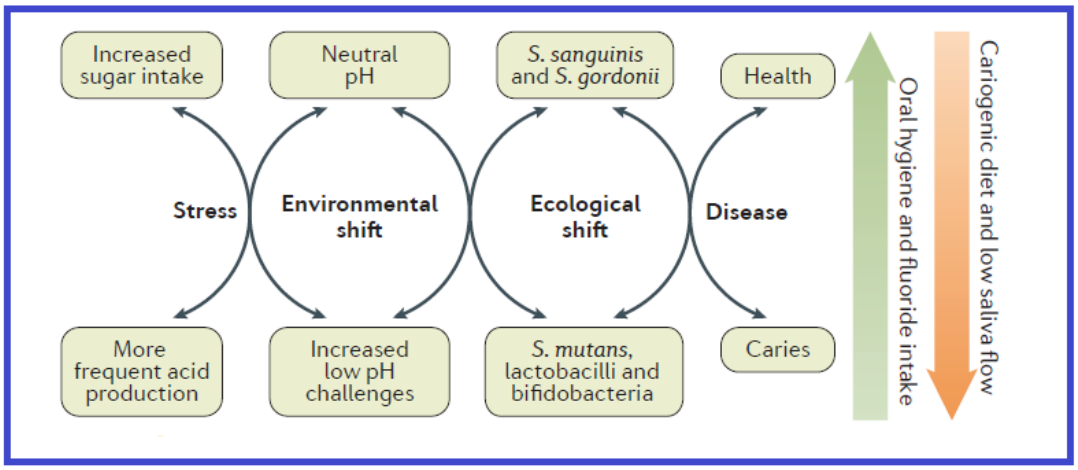


Figura 3: Hipótese da placa ecológica e etiologia da cárie dentária.
Fonte: PITTS *et al*., (2017).

Quando expostos a uma dieta rica em açúcar, comensais do grupo Streptococci metabolizam os carboidratos e produzem ácidos que vão desmineralizar a estrutura dentária. Entre os agentes que irão participar desse processo, os *S. mutans* destacam-se por terem o mecanismo de ação confirmado em relação à sacarose e à cárie dentária. Essa espécie é capaz de sobreviver, proliferar e produzir ácidos. Além disso, possuem glicosiltransferases, que são enzimas que permitem os mesmos formarem diversos polímeros extracelulares de açúcares e possuem adesinas permitindo, assim, as bactérias aderirem à estrutura dentária a à outras espécies do biofilme. (PITTS *et al*., 2017)

Apesar de não ser o agente etiológico, um aumento do número de *S.mutans* pode ser considerado um fator de risco para o início da lesão, o que torna esse grupo de bactérias, que são acidúricas e acidófilas, associadas ao desenvolvimento da lesão cariosa, sendo assim um

indicador de desequilíbrio da microbiota. Mesmo existindo mecanismos fisiológicos na boca, como a saliva, que vai auxiliar na remineralização, quando há um consumo de açúcar muito frequente na dieta, um desequilíbrio ecológico da microbiota bucal pode ocorrer. (GIACAMAN *et al*., 2010; ERIKSSON *et al*., 2017)

Os dentes mais acometidos pela cárie na dentição decídua são os segundos molares decíduos, devido a sua morfologia oclusal que favorece o acúmulo de placa bacteriana. Sabe-se, também, que prevenindo a cárie nos dentes decíduos, haverá prevenção na dentição permanente. No atual modelo crescente de Odontologia minimamente invasiva, defende-se que a cárie dentária pode ser controlada e prevenida de formas não-invasivas através de diversos produtos, nos quais podem-se citar os vernizes. (MANJI *et al*., 2018)

Como as crianças menores de seis anos ainda não possuem o hábito adequado de “cuspir”, as formulações mais adequadas para a prevenção da doença cárie nessa idade seriam os vernizes, ao invés de enxaguatório ou gel, e também devido a sua alta capacidade retentiva e de liberação lenta do princípio ativo. (DE LUCA *et al*., 2017)

Na prevenção da cárie dentária em saúde pública, devem ser implementadas abordagens e promoção de comportamento saudáveis, além de políticas como a fluoretação da água pública e estratégias em grupos de alto risco, com acesso restrito aos serviços odontológicos e fluoretos. (PITTS *et al*., 2017)

A incidência de cárie em crianças na faixa etária de 36 a 71 meses é bastante alta, uma alternativa interessante seria a utilização de vernizes com algum agente antimicrobiano que associado à escovação com dentifricio fluoretado, consequentemente, evitará o aparecimento das lesões de cárie. Vernizes com substâncias bioativas vêm sendo estudados em pesquisas *in vitro* como agentes antimicrobianos, o que se tornou um dos principais objetivos do presente trabalho. Em consequência do exposto, existe uma grande possibilidade de utilização nos pacientes infantis com a finalidade de controle da doença cárie através de uma possível atividade antimicrobiana desses vernizes à base do óleo-resina de copaíba.

2. OBJETIVO

2.1 OBJETIVO GERAL

Desenvolver e avaliar a eficácia clínica e microbiológica de um verniz incorporado a 1% de *Copaifera langsdorffii* Desf e comparar com diferentes vernizes dentários em crianças com alto risco de cárie residentes em um município sem fluoretação pública de água.

2.2 OBJETIVOS ESPECÍFICOS

- Realizar um estudo prospectivo baseado em patentes de produtos dentários com óleo de copaíba;
- Avaliar in vitro a atividade antimicrobiana do óleo-resina de *Copaifera langsdorffii* Desf em cepas de *S.mutans*.
- Avaliar a dose-resposta na saliva de crianças após a aplicação do verniz;
- Avaliar o aparecimento de lesões de cárie em segundos molares decíduos em crianças que receberam tratamento dentário com vernizes à base de copaíba, de clorexidina a 1% e o verniz fluoretado 5%;
- Avaliar a ação antimicrobiana dos diferentes vernizes comerciais *in vivo.*

CAPÍTULO 1

TITLE PAGE

This chapter refers to the article accepted on the journal Recents Patents on Biotechnology.

Products of dental use containing Copaiba oil: technology prospecting based on patents

Products of Dental Use Containing Copaiba Oil-Resin: Technological Prospecting Based on Patents

Abstract: *Background:* Copaiba oil-resin has been widely used and is especially found in neotropical regions, for which several pharmacological activities have been documented over the years. Prospective studies in intellectual property banks are important to increase competitiveness and thus generate new products in various research areas. *Objective:* A prospective study was carried out on patents of products containing copaiba oil-resin for dental use in intellectual property banks. *Methods:* The research was conducted with patent searches in six intellectual property banks of the world. Relevant information which describe the invention in the patent document were collected, processed and described. *Results:* The search found 9 patents using copaiba oil-resin in dental products. The National Institute of Industrial Property (INPI-Brazil) had the highest number of deposits (5), followed by Espacenet (2) and Free Patents (2). *C. Langsdorffii* was highlighted as the most widely used species in the products and deposits of vehicles in formulations (3). All the patents in the search are A61K code for medical, dental or hygienic purposes. *Conclusion:* Most of the found patents are related to the area of Microbiology, specifically with application in Cariology. Brazil is represented by the INPI and presented the highest number of patent applications when compared to other intellectual property banks.

Keywords: *Copaifera*; Intellectual property; Patents.

1. INTRODUCTION

Globalization and consequent intensification of market competition between companies requires that new products be launched consistently. This is no different in the area of dental materials, where technological innovation in companies has been a fundamental factor for maintaining and growing the industry [1,2].

The products of dental interest used in both the prevention and treatment of oral conditions undergo constant modifications to their compositions, in addition to being evaluated in advance by *in vitro* and *in vivo* studies until reaching the final consumer in seeking to benefit their therapeutic properties through the use of technological innovation. In pharmacotherapy, some studies comparing the viability of use between conventional pharmaceutical drugs and extracts of natural origin see some advantages in the latter, such as lower costs and reduced side effects, as well as safety in use [3]. Phytotherapeutic drugs already integrate the therapeutic system in developing countries by complementing or replacing conventional practices [4].

Due to the complex oral microbiota and its relation with pathogens in Dentistry, research concentrates its studies on the antimicrobial and/or anti-inflammatory effects of natural extracts such as *Aloe Vera* leaves, cashews (*Anacardium occidentale*) and guava (*Psidium guajava*) [5], neem leaves (*Azadirachta indica*), grape seeds (*Vtis vinifera L*.), propolis, and copaiba oil-resin (the focus of this study) [6-8].

Copaiba is a common plant in South and Central America, India and West Africa, belonging to the Fabaceae family and Caesalpinioideae subfamily. Since the beginning of colonization of Brazil, the copaiba tree has been a source for extracting resin oil used for medicinal purposes, being among one of the most important natural national products marketed to other countries. Among the main chemical constituents of the oil are β-caryophyllene, α-humulene, cedene, cadinene and bisabolene [9-11].

The Copaifera genus is present in South American countries such as Venezuela, Colombia, and especially in Brazil with the greatest species richness, where the trees can be found in the Southeast, Midwest and Amazon regions. Among the 72 cataloged species, 20 have already been found in Brazil, in which 16 are exclusive to the country. Among them are *Copaifera officinalis* L., *Copaifera guianensis* Desf., *Copaifera reticulata Ducke*, *Copaifera multijuga* Hayne, *Copaifera confertiflora* Bth., *Copaifera langsdorffi* Desf., *Copaifera coriacea* Mart. and *Copaifera cearensis* [12-15].

Copaiba oil-resin is produced by the plant as a form of defense against animals, microorganisms and external damage, justifying its antimicrobial, anti-inflammatory, tissue neoformer and even analgesic properties [10,16]. *C. langsdorffii* oil has pharmacological activity against gram-positive and gram-negative bacteria, especially for topical use [17]. The antimicrobial activity may be related to the combination of sesquiterpenes and diterpenes, thus affecting the integrity of the bacterial cell wall. The oil has been scientifically proven activity against several pathogens, especially gram-positive bacteria such as *Staphylococcus* spp. and *Streptococcus* spp [18].

In dentistry, copaiba oil-resin is important due to its strong activity against oral bacteria and it can be used in appropriate formulations since the main oral diseases, caries and periodontal disease are strongly related to the dental biofilm [14].

Pieri et al. (2010) [19] evaluated the action of β-caryophyllene isolated from copaiba oil-resin on the adhesion of *Streptococcus mutans* bacterium, and found that it had better action than chlorhexidine. Pieri et al. (2016) [20] also evaluated the antimicrobial activity of β-caryophyllene isolated from copaiba oil-resin against dental plaque bacteria *in vitro*. The results demonstrated that β-caryophyllene prevented plaque-forming bacteria from proliferating. Other

studies have demonstrated antimicrobial activity of copaiba oil-resin in the form of dental cement against *S. mutans* [10, 11, 21].

The chemical composition of the copaiba extract varies according to the species, but it is basically composed by ß-caryophyllenes, the main bicyclic sesquiterpene found and which exhibits ample antifungal and antibacterial activity, with activity against all *Streptococcus* spp. strains being found in previous studies among them [22], as well as copalic acid, caurenoic acid, covalenic acid and chlorhequinic acid, with the latter being natural diterpenes [23]. This oil is already present in several pharmaceutical products of personal hygiene in Brazil, such as in shampoos, soaps, and lotions, among others. A well-defined standardized production of this extract may be an important strategy in manufacturing new oral hygiene items used to control caries disease and periodontal disease [24].

Studies involving natural products first address some issues such as: (1) fully elucidating their mechanisms of action; (2) the possibility of cytotoxicity in clinical use in humans; (3) isolating the active components of these extracts; and (4) modifying these isolated compounds for stronger inhibitory effects [25]. In the background, researchers simultaneously have the possibility to insert their invention in the market through patenting in intellectual property banks, which aims to guarantee security in profits and possible negotiations [1, 26]. However, the product needs to comply with characteristics such as: innovation, technology and the possibility of commercialization [2, 21, 27].

Studies on technological prospecting are important because patents deposited in a country are indicative of the technological and scientific scenario, as well as the market's interest in certain products. Thus, it can be predicated that patents have a very important role in the economic development of a country, where studies on patents demonstrate the current scenario by encouraging competitiveness and directing investments into research [26].

Due to the growing interest in the therapeutic activity of copaiba oil-resin and its intense commercialization, the present study aimed to carry out a prospective study based on the patent deposit of these products for dental purposes in intellectual property banks in Brazil and worldwide.

2. METHODS

2.1. ELABORATION OF THE PROSPECTIVE STUDY

A study of technological prospecting was carried out through the collection, treatment and analysis of information extracted from selected patent documents. The documentary research was conducted in December 2018. The searches were directed to patents deposited with formulations of copaiba oil-resin for dental use in databases of six reference intellectual property banks in the world:

- Instituto Nacional de Propriedade Industrial (INPI) - Brazil
- Unites States Patent and Trademark Office - United States
- Google Patents
- World Intellectual Property Organization (WIPO) - Europe
- Espacenet
- Free Patent On Line

2.2. SEARCH STRATEGY

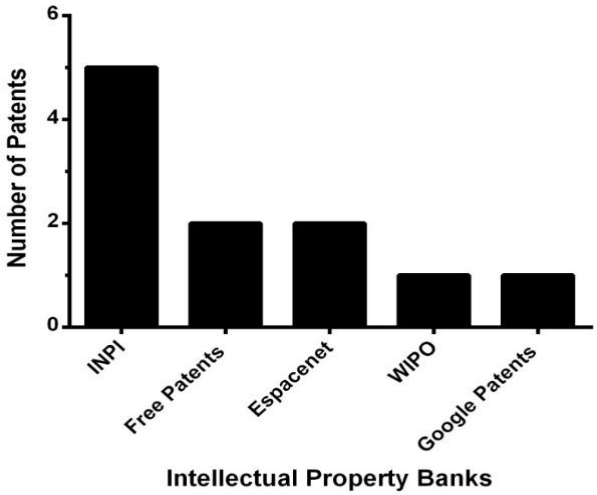
All patents that included the term 'copaiba' and/or 'copaifera' were considered for the search. The patent reports were selected after reading the title, abstract or report, and only dental patents were selected as inclusion criterion. Relevant information from the patent document was extracted and organized into tables and graphs were generated in the Graphpad Prism 6 program for

interpretation and to discuss the results found. The data represent the distribution of the number of patents by banks, the annual evolution of deposits, the species of copaiba used in patents and types of products for dental purposes according to IPC - International Patent Classification.

3. RESULTS

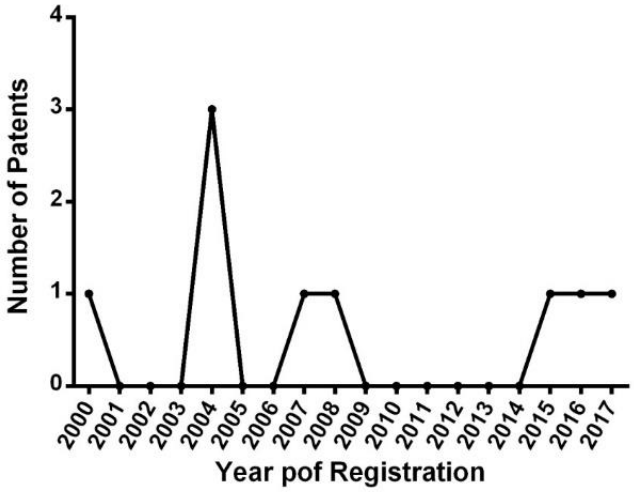
The initial search resulted in 11 patents found with the term ‘copaiba’ and/or ‘copaifera’ followed by title and abstract reading with the exclusion of 2 repeated results targeting dental applications with a total of 9 documents.

According to Graph 1, it is observed that the National Institute of Industrial Property (INPI) was the bank with the highest number of patent deposits of copaiba products (5), followed by Espacenet (2) and Free Patents (2), each with two patents. Other patent banks such as WIPO and Google Patents had only one reported patent.



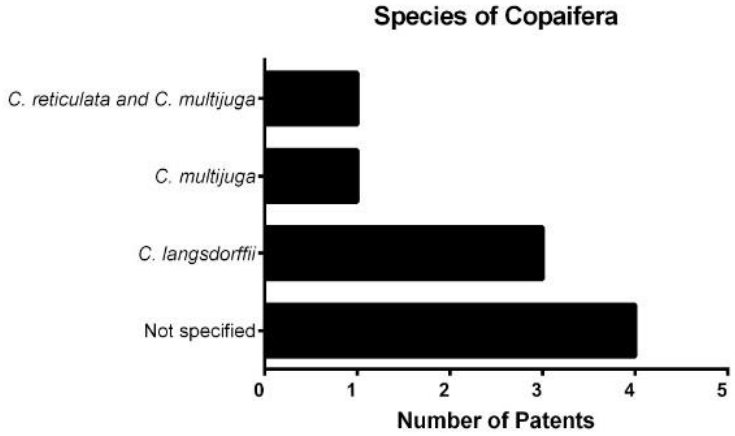
Graph 1. Number of patents found in intellectual property banks with the term ‘copaiba’ and/or ‘copaifera’.

Graph 2 shows the annual evolution in the number of patent deposits with copaiba resin oil, with the first registration occurring in 2000, and a greater number of the above mentioned intellectual property banks occurred in the 2003-2005 biennium.



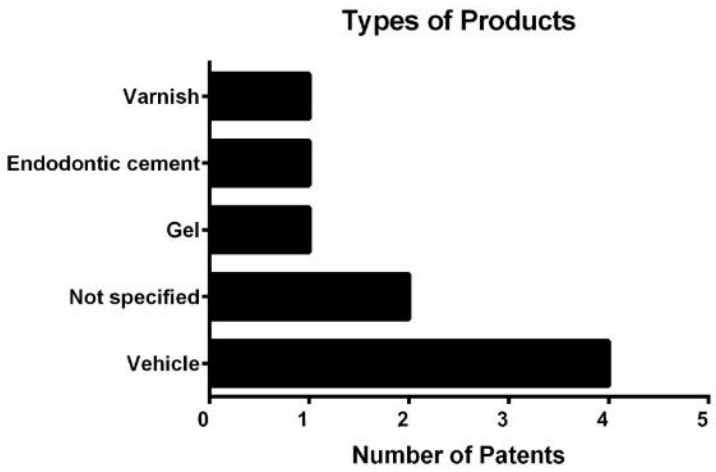
Graph 2. Distribution of the number of patents registered in intellectual property banks with the term ‘copaiba’ and/or ‘copaifera’ in the period 2000-2017.

It was verified that the majority of patents with the term ‘copaiba’ and/or ‘copaifera’ did not identify the species used. However, among patents that identified the *Copaifera* species, the highest number of patents registered was associated with *C. langsdorffi* species, followed by *C. multijuga* (Graph 3).



Graph 3. Main species of the *Copaifera* genus related to the deposited patents.

Graph 4 shows that the types of patented dental products with the term ‘copaiba’ and/or ‘copaifera’ were the largest in vehicle form (4). The remainder of the formulations are represented in the form of gels, varnishes, endodontic cement or were not specified.



Graph 4. Types of dental products deposited with the term ‘copaiba’ and/or ‘copaifera’.

4. DISCUSSION

In recent years, the search for new substances with pharmacological potential and biocompatibility highlights the growing number of studies regarding the use of natural products in Dentistry, with the development of materials with active and alternative biological properties for managing oral diseases, especially dental caries [28].

Copaiba oil-resin has been widely used and especially found in neotropical regions where bees of the *Apis mellifera* species are the main pollinating agents. There are records of copaiba oil-resin being used for almost 400 years, with several studies proving its innumerable biological activities, as it is effective against several microorganisms and commonly used in traditional medicine against various diseases [18,29]. In the present study, the *C. langsdorffii* species was the most found in the deposits.

The first patent with copaiba formulations for use in dentistry was deposited in 2000 at the Espacenet by inventors from Japan (JP2002114658 (A)[30], about an oral cavity composition that inhibits the activity of a protease produced by strictly Gram-negative anaerobic bacteria and increases antibacterial action on *Fusobacterium nucleatum*, can prevent periodontal diseases and bad breath, which comprises the combination of one or two or more kinds of plant such as copaiba, with one or two or more kinds of chemicals selected from the group consisting of 3-methyl-5-phenyl-1-pentanol, 11-dodecenic acid, 4-mthyl-4-decanolide, undecanoic acid and capric acid.

When analyzing patent deposits with copaiba in the dental area, Brazil is in 1st place with 5 patents deposited at the INPI with different formulations as vehicles (BR 1020170148009, BR 10 20150132310) [31,32], gel (PI 0404266-2)[33], endodontic cement (PI 0402262-9) [34] and varnish (BR1020160212626) [35], followed by Japan with a few deposits which provide a composition for oral cavity formulated with plant extracts, including copaiba, and a hydroxyapatite effectively promoting recalcification of tooth enamel in which tooth surface is decalcified, and positively suppressing dental caries (JP 2006062993) [36]

deposited at the Free Patents Online, Espacenet and WIPO; and also the previously mentioned JP2002114658 (A) [30] patent. Third, the United States with one patent deposited at the Free Patents Online and Google Patents that describes oral compositions having at least two botanical active ingredients derived from plants which provide particularly efficacious antimicrobial (antibacterial, antiviral, and/or antifungal), antioxidant, anti-inflammatory, anti-ageing, and/or healing properties to the oral compositions (US 20090087501A1) [37].

The deposit numbers among countries of intellectual property banks indicates that although there are worldwide deposits, Brazil has more interest in research and development of products with copaiba in the dental area. Thus, there is a situation in which Brazil, a developing country, features the highest number of products. However, there are few patents deposited. Several authors emphasize that the lack of interest of some researchers to patent their inventions gives priority to publish articles because of the unfamiliarity in the procedures for depositing patents [2].

Patents have had an international classification system since 1971 in order to standardize the searches which distributes them by codes, known as IPC - International Patent Classification, distributing the products in technological areas of classes A to H, where each class has its own division. This classification ends up being an efficient and easy access search tool, thus broadening dissemination of a product [38]. The years with the most deposits in the present study were between 2003 and 2005. After a time without deposits in the area there was growth from 2014 to 2017. In the search, the deposits were limited to a single type under the code A61K, which is related to products for medical, dental and hygienic purposes.

The development of dental materials with natural products is still quite limited, practically summarizing the topical formulations and without many clinical trials being published in the last decades. However, several studies have documented the pharmacological activity of natural products against dental biofilm, especially cariogenic [3, 39]. In this work, vehicles, gel and dental varnish formulations were found.

There are several reasons for the development of products with antimicrobial properties, among them increased microbial resistance, toxicity and high costs of various materials. The main antimicrobial agent in Dentistry is chlorhexidine, which causes tooth coloration when used for a prolonged period, changes in taste and it loses its pharmacological capacity with bacteria recolonization occurring such as *Streptococcus mutans* [35]. *C. langsdorffii* oil is effective against gram-positive and gram-negative bacteria, especially for topical use. Its effectiveness against the cariogenic bacteria is also emphasized [36].

Technological prospecting studies are considered a relatively recent activity and are currently being used to assist decision-making within a context of changes, especially with regard to the globalization of the economy and the acceleration of technological advances [2].

5. CONCLUSION

After an analysis of copaiba oil-resin product patents applied in Dentistry in intellectual property banks, it is concluded that the majority are related to the area of Microbiology, especially for application in Cariology.

Brazil is represented by the National Institute of Industrial Property (INPI) and obtained the highest number of patent applications when compared to other intellectual property banks.

Therefore, it is notable that interest in the protection of intellectual property in dental products with copaiba is still small, despite being a natural product widely used in research and in developing drugs.

CURRENT & FUTURE DEVELOPMENTS

Advances in research and development of medicines based on natural products have stood out in different areas (including Dentistry) in order to treat patients combined with the increase or the appearance of oral diseases. The complex chemical composition combined with the therapeutic properties of such substances stimulates the search for technological innovation of products of industrial interest. Among them, copaiba oil-resin arouses interest for its reported working biological properties.

Although the number of patents such as oil for dental purposes is small, Dentistry topics present a variety of materials and formulations for clinical use in the market and its growth encourages the development of new products with the incorporation of copaiba resin oil. Brazil has shown to be a promising example in copaíba research, besides guaranteeing the protection of intellectual property based on scientific research. Future patents dealing with technological innovations are likely and the biological properties of copaiba should add to global demand, with costs that bring all these pharmaceutical technologies within global reach.

It is also expected that new formulations which incorporate copaiba oil-resin will be discovered in upcoming years and that these compounds could be used in preclinical and clinical trials.

LIST OF ABBREVIATIONS

INPI	=	Instituto Nacional de Propriedade Industrial
IPC	=	International Patent Classification
WIPO	=	World Intellectual Property Organization

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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CAPÍTULO 2

TITLE PAGE

This chapter refers to the article published on the Saudi Pharmaceutical Journal.

Dose-response evaluation of a copaiba-containing varnish against *Streptococcus mutans* in vivo

Dose-response evaluation of a copaiba-containing varnish against *Streptococcus mutans* in vivo

Abstract

Introduction: Dental caries is the most prevalent disease in humans and its incidence is particularly high during childhood. The use of medicinal plants is a common practice in Brazil. Objective: To evaluate the optimal antimicrobial concentration of *Copaifera langsdorffii* (copaiba) oil-resin, in the form of dental varnish, against *Streptococcus mutans* (*S. mutans*) in children. Methods: Twenty-four children, caries-free, aged until 6 years old, were selected to participate in this study. The varnish was applied to the occlusal surfaces of all deciduous molars. The antimicrobial activity was analyzed in saliva, whose collection was conducted in two phases: before applying the copaiba varnish and after use to verify the instantaneous effectiveness of *Copaifera langsdorffii* dental varnish in the reduction of *S. mutans*. The microbiological analysis was repeated twice, establishing dilutions of 1:10mL and 1:100mL. Results: Comparisons between different times within the same dilution were carried out by repeated measures analysis of variance (ANOVA) associated with Tukey's multiple comparisons test. Comparisons of conditions prior to and after treatment were performed using the t test for paired samples and it indicated that the 1% formulation promoted a more significant decrease in the number of *S. mutans* colonies (p=0,0026). Conclusion: Copaiba oil-resin, in the form of dental varnish, has antimicrobial activity against *S.mutans* in all the concentrations studied. Further studies to identify the long-term activity and anticaries effect of this varnish are required to establish its use in caries prevention.

Keywords: Dental caries; Saliva; *Streptococcus mutans*.

1.Introduction

Dental caries is currently considered a dysbiosis involving interactions between tooth structure, microbial biofilm and sugar exposure, where 35% of cases are not treated worldwide. The early childhood caries (ECC) is characterized by the presence of caries in children up to 6 years of age (Adams et al., 2017; Pitts et al., 2017).

When exposed to a high-sugar diet, diners in the streptococci group metabolize carbohydrates and produce acids that will demineralize the tooth structure. Among the agents that will participate in this process, *Streptococcus mutans* (*S. mutans*) is the primary pathogen, whose mechanism of virulence has been the most studied and known when compared to the other species (Pitts et al., 2017; Xiang et al., 2019).

Although it's not the etiological agent, an increase in the number of *S.mutans* can be considered a risk factor for the beginning of the lesion, which makes this group of bacteria, which are acidic and acidophilic, associated with the development of the carious lesion (Kaur et al., 2013; Eriksson et al., 2017).

There are physiological mechanisms in the mouth, such as saliva, which aid in remineralization, however when there is a very frequent consumption of sugar in the diet occurs the ecological imbalance of the oral microbiota. Then, the production of acids by acidogenic bacteria, such as *S. mutans*, is considered an important factor linked in the development of caries (Adams et al., 2017).

S. mutans begins colonization of the mouth early in life and is responsible for mature biofilm formation, resulting in early childhood caries (ECC). Habits such as unrestricted use of baby bottles and feeding (industrialized fruit juices, sweetened teas, fermented milk, milk with fermentable carbohydrates such as starches and sugar) while sleeping are strongly associated with the development of ECC (Ismail et al., 2008; Vasconcelos et al., 2008; Pitts et al., 2017).

In recent years, in the search for new substances with pharmacological and biocompatible potential, the number of studies on the use of natural products in dentistry and alternatives for the management of oral diseases, especially dental caries, has increased (Lobo et al., 2014; Freires et al., 2015).

According to Romero et al., the use of plants for medicinal purposes is a common practice in Brazil and has been passed from one generation to the next either for treatment or prevention of diseases. Medicinal plants have also been studied to evaluate their antimicrobial activity against *S. mutans*, as described in a study by Lobo et al. (2014).

Copaiba oil is widely used by the brazilian population in the treatment of microbial and inflammatory diseases and is commonly found in public markets, herb markets, and natural product stores. The copaiba tree belongs to the Leguminosae family, Caesalpinioideae subfamily, and *Copaifera* genus, and is commonly found in Latin America and West Africa. Species of this genus are popularly known as copaiba. This tree is found mainly in the southeast, midwest, and the Amazon regions. *Copaifera* comprises 72 species of which over 20 are found in Brazil. Chemically, the oil-resin of copaiba is a solution of diterpene acids in an essential oil containing sesquiterpenes, which are categorized into oxygenated and hydrocarbonated components (Paiva et al., 2002; Pieri et al., 2009; Romero et al., 2009).

Several species show demonstrated antimicrobial activity, especially C. multijuga, C. reticulata, C. langsdorffii, C. oblongifolia and C. officinalis (Diefenbach et al., 2017). Copaifera langsdorffii (C. langsdorffii), a species chosen in this study, is native to the Amazon rainforest and common in the Mato Grosso region, where according to a survey it's the species of greatest importance and use by the population in the region of its georeferencing (Bieski et al., 2015).

Paiva et al. (2002) reported antimicrobial, anti-inflammatory, and wound-healing activities in the diterpenoid fraction of copaiba oil-resin. Further, an in vitro study has investigated the antibacterial activity of a dental cement made with the copaiba oil resin and demonstrated the effectiveness of copaiba oil against *S. mutans* (Pieri et al., 2009).

Thus, the aim of this study was to evaluate, through microbiological testing, the optimal concentration of the copaiba oil-resin in the varnish form against *S.mutans* present in the saliva in vivo.

2.Materials and Methods

2.1 Extraction and chemical analysis of the oil-resin of copaiba

Samples of copaiba oil-resin, obtained from plant material of *Copaifera langsdorffii* Desf. (Fabaceae: Caesalpinioideae) deposited in the herbarium of Federal University of Mato Grosso- voucher Silva, R. R. et al. 1749, were received from the Federal University of Mato Grosso and originally obtained from Juruena Valle (Region: Midwest, Latitude: 10º 19 '05 "S, Longitude: 58º 21' 32" W, Height: 300m). Chemical constituents were identified by specialists at the Department of Chemistry, in the Federal University of Ceara (GC–MS, Shimadzu, model QP 5050, Japan). The main components of the *Copaifera langsdorffii* oil-resin used in the present study were: β-caryophyllene, α-humulene, cedrene, cadinene and bisabolene. The dental varnish were prepared at the School of Pharmacy of the same university, in random solutions with concentrations of 1%, 5%, 10%, and 20% of the oil-resin.

2.2 Study population

This study was approved by the Ethics Committee of the Federal University of Ceara (approval number 195.096). After signature of informed consent by parents or legal guardians, twenty-four 3–5 year-old children, from both genders, good general health and caries free, with ICDAS II (International Caries Detection and Assentment System) 0 and with high risk of caries, according to the criteria of the AAPD (American Academy of Pediatric Dentistry, 2014) were selected for the study. Children with a history of allergies or allergic diseases, e.g. asthma, urticaria, rhinitis, sinusitis, or intraoral soft tissue lesions were excluded from the study. None of the participants underwent antibiotic treatment up to 3 months prior to study initiation, nor during the course of this clinical trial.

2.3 Treatment application

Children were divided into four groups (6 children/group) with each group corresponding to one of the concentrations mentioned above. Initially, each patient chewed a piece a 3 × 3-cm plastic film (Parafilm®) for 60 s to stimulate

the production of saliva and release the bacteria from the dental biofilm. Saliva was collected using a plastic device and stored in sterile microcentrifuge tubes (Eppendorf®), which were stored in polystyrene box containing ice. To minimize the influence of the circadian rhythms on salivary flow, all samples were collected in the same session and conditions by the same operator between 9:00 and 11:00 AM.

Thereafter, the varnish was applied by the same operator to the deciduous molars, with relative insulation, of each patient after Robinson prophylaxis using brushes and pumice. After 10 seconds, a triple syringe was used to gently dry the varnish. The saliva of each subject was immediately collected and placed into sterile microcentrifuge tubes (Eppendorf®).

2.4 Microbiological analysis

Samples were transported to the laboratory for microbiological analysis in a hermetically sealed case containing ice, and analyzed no longer than 2 h after collection.

Saliva was homogenized on a tube shaker for 30 seconds. A volume of 0.1 mL of each sample was aseptically drawn and transferred into one sterile test tube containing 0.9 mL of saline. Procedure was repeated twice, establishing dilutions of 1:10 and 1:100. A corresponding volume of ten microliters of each dilution was plated onto *Mitis Salivarius-Bacitracin* (MSB) agar medium in triplicates. The plates were then incubated at 37ºC, during 48h, in jars under microaerofilic conditions. Bacterial counts were expressed as colony forming units (CFU)/mL of saliva and followed by phenotypical colony identification, as described elsewhere.

2.5 Statistical analysis

Quantitative variables, number of colony forming units (CFU) and relative reduction of CFU were initially analyzed by the Kolmogorov-Smirnov test to verify the normality of distribution. As such requirement was observed in all cases, then, for the descriptive statistics were calculated the average and standard deviation as well as parametric tests were employed for analytical statistics.

To compare four concentrations (1, 5, 10 and 20%) it was used analysis of variance (ANOVA) test associated with Tukey multiple comparison test to check for differences between the concentrations in pairs. Comparisons between pre- and post-treatment, considering a given concentration were made by t test for paired samples.

In all analyzes, we established the significance level of 0,05 (5%), and considered statistically significant P value less than 0.05. The GraphPad PRISM® software version 5.00 for Windows® (GraphPad Software, San Diego, California, USA, 2007) was used for both the achievement of statistical procedures as for the preparation of graphics.

Results were expressed as reduction in the number of *S. mutans* colony forming units (CFU) before and after varnish application treatment. Percentage reduction was calculated based on the formula above, where CFU (before) and CFU (after) correspond to the number of colony-forming units (CFU) obtained before and after treatment, respectively.:

$$\% \text{ Red CFU\%} = \frac{[\text{CFU(Before)} - \text{CFU(After)}]}{\text{CFU (Before)}} \times 100$$

3. Results

The patients reported no side effects after the application of different concentrations of varnish.

3.1 Comparison of different concentrations of copaiba

It became clear that the dental varnish copaiba in a concentration of 1% was more effective in reducing bacterial load of *S. mutans* in the samples analyzed at a dilution of 1:10 (Table) 01.

Table 1 - Number of colony forming units (CFU) of *Streptococcus mutans* in the saliva samples verified in 6 children with 1:10 dilution before and after the treatment with varnish containing the *Copaifera langsdorffii* oil-resin at concentrations 1, 5, 10 and 20%. In each concentration, comparisons between pre- and post-treatment were carried out by using the t test for paired samples.

Figure 1 - Effect of different concentrations of the *Copaifera langsdorffii* oil-resin applied in the form of varnish, in the relative reduction of the number of colony forming units (CFU) of *Streptococcus mutans*, expressed in percentage terms, measured in saliva samples, dilution 1:10.

In figure 1 it can be verified that at a dilution of 1:10 saliva, the antimicrobial dental varnish activity of copaiba oil-resin decreases with the increase in its concentration. The data are the average and standard deviation of measurements made in the saliva samples of 6 children. Comparisons between treatments were performed by using analysis of variance (ANOVA) test associated with Tukey multiple comparison test to check for differences between the concentrations two by two. No statistically significant differences were found between concentrations (ANOVA: F = 0,3694, P = 0,7760).

In table 2 by comparing the results of the pre- and post-treatment using a saliva sample at a dilution of 1:100, it is found that the concentration of 1% copaiba oil resin in the dental varnish is truly more effective in reducing the microbial load, in which the statistical difference presents a significant value (P = 0,0026).

Table 2 - Number of colony forming units (CFU) of *Streptococcus mutans* observed in the saliva samples of 6 children with dilution of 1:100 before and after the treatment with varnish containing *Copaifera langsdorffii* oil-resin on the concentrations 1, 5, 10 and 20%. In each concentration, comparisons between pre- and post-treatment were carried out by using the t test for paired samples.

Figure 2 - Effect of different concentrations of the *Copaifera langsdorffii* oil-resin applied in the form of varnish, the relative reduction in the number of colony forming units (CFU) of *Streptococcus mutans*, expressed in percentage terms, measured in saliva samples, dilution 1:100.

In examining figure 2, it is observed, in a similar way, a reduction of antimicrobial activity of dental varnish behavior with increasing concentration of copaiba oil resin incorporated into the formulation. The data correspond to the

average and standard deviation of measurements made in the saliva samples of 6 children. Comparisons between treatments were performed by using analysis of variance (ANOVA) test associated with Tukey multiple comparison test to check for differences between the concentrations two by two. No statistically significant differences were found between concentrations (ANOVA: F = 1,698, P = 0,2012).

When comparing the average reduction of UFC of *S. mutans* and standard deviations in the statistical analysis, it is observed that the dilution of saliva of 1:100 presents itself as better analysis option, since it has a higher statistical uniformity evidenced by the lower standard deviation.

Table 3 - Relative reduction in the number of colony forming units (CFU) of *Streptococcus mutans*, expressed in percentage terms, measured in saliva samples from 6 children, with dilution of 1:10 or 1:100, treated with varnish containing the *Copaifera langsdorffii* oil-resin at concentrations of 1, 5, 10 and 20%. In each concentration, comparisons between two dilutions were performed by using the t-test for paired samples.

4. Discussion

Resistance to synthetic antimicrobials and search for substances with pharmacological properties with lower adverse effects made increase the interest of the pharmaceutical industry for natural products. The development of dental materials with natural products is still quite limited, practically summarizing to topical formulations and without many clinical trials published in the last decades. However, several studies have documented the pharmacological activity of natural products against the dental biofilm, especially the cariogenic one (Marsh et al., 2000, Freires and Rosalen, 2016). In dental materials, phytomedicine has been used as antiinflammatory, antibiotic, analgesic, sedative agents in various types of formulations like toothpaste, mouthwash, endodontic solutions, among others.

In regards of dental caries, there are several risk indicators, those related to the disease directly, such as the exposure to sugar and consequent alteration of the microflora, as well as the modulating factors, such as socioeconomic aspects. Saliva is a biological material, being an excellent biomarker for systemic and oral diseases, especially in relation to dental caries, where approximately 10^8 microorganisms can be counted in 1 mL of saliva. Research associates counts of *S. mutans* in saliva to the experience of dental caries (Marsh et al., 2000, Kaur et al., 2013, AAPD, 2014).

Dental varnishes are pharmaceutical formulations for use in dentistry and generally have good acceptance by pediatric patients (Vasconcelos et al., 2008). They are composed of polymer matrices, excipients and the active principle. In the case of the present varnish the chosen matrix was insoluble, in this case ethylcellulose, used to modulate the release of the active principle and thus the substantivity was higher (De Luca et al., 2017). This type of formulation adheres to dental fissures and scars, gradually releasing the active principle, thus becoming a long-term therapeutic agent suitable for antimicrobial formulation (Franca et al., 2014).

The main function of varnishes is the prevention of dental caries because their properties contribute to the disruption of the dental biofilm, thereby decreasing the cariogenic microorganisms on the surface of teeth (Pessan et al., 2008).

We selected the varnish formulation rather than the mouthwash or gel formulations because children under 6 years of age do not have an adequate ability to eject saliva and also because varnishes promote a slow release of active ingredients. In addition to its easy clinical application, varnishes have good adhesive properties to teeth surfaces in children and show no contraindication for the age group studied (Pessan et al., 2008, De Luca et al., 2017).

There are several reasons for the development of new products with antimicrobial properties, since new microbial resistance, toxicity and high costs of dental materials are being marketed. The main antimicrobial agent in dentistry is chlorhexidine, which when used for a prolonged period causes tooth staining, mucosal irritation, alteration of the taste and loses its pharmacological capacity,

occurring the recolonization of bacteria as *S.mutans* (Vale et al., 2014). Current studies demonstrate weak evidence of the use of chlorhexidine for prevention of dental caries and reduction of *S. mutans* in children and adolescents (Walsh et al. 2015; Flamee et al., 2015).

Investments in patents are increasingly widespread, as the whole functioning of the capitalist system is related to innovation and scientific and technological advancement (Lima, 2006, Franca et al., 2014, Freires and Rosalen, 2016). This study is the first to use copaiba varnish, with patent number BR1020160212628 deposited on the National Institute of Industrial Property (INPI- Brazil).

S. mutans is closely related to the cariogenic biofilm due to the ability to synthesize glucans and fructan from sucrose using various glucosyltransferases (Gtfs) and a fructosyltransferase. Glucans provide specific binding sites for the colonization of *S. mutans* on the dental surface. Therefore *S.mutans* is considered a group of highly acidogenic and acid tolerant bacteria, virulence properties highly related to the capacity of dental demineralization and essential for the formation of bacterial biofilm (Klein et al., 2008). The effectiveness of copaiba oil against bacteria present in the oral environment is documented in the literature, not only in relation to *Streptococcus sp*., But also against *Enterococcus sp*., *Haemophilus sp*., *Aerococcus sp*., *Bacillus sp*., *Lactococcus sp*, among others (Klein et al., 2008, Pieri et al., 2016).

A study by Pieri et al (2016) has evaluated the inhibitory activity of copaiba oil-resin against the cariogenic microorganism *S. mutans* through a minimal inhibitory concentration test using the serial dilution technique in broth, and has reported inhibition of bacterial growth at all concentrations tested. Similarly, a study by Vasconcelos *et al.*(2008) reported the use of a formulation containing copaiba oleo resin, zinc oxide, and calcium hydroxide (cement).This cement showed antibacterial activity against *S.mutans* and *S. Sanguinis,* even in very small amounts at all dilutions analyzed.

Of all the copaiba oil-resin concentrations in dental varnish, the 1% showed the best antimicrobial activity, as evidenced by the relative decline of CFU of *S.mutans* in the samples studied.

The 1% formulation had stronger antimicrobial activity, probably because the copaiba active ingredients retained in the varnish matrix and is released locally. We also observed that higher concentrations of the copaiba ole resin lost the ability to retain its active ingredient, and in these situations, the active ingredient would be released so quickly that the varnish became unpleasant to patients and partially lost its antimicrobial activity. Therefore, during application, the lowest concentrations of the varnish would better retain the active ingredient, which was gradually released during contact with teeth and gums, there by promoting a higher antimicrobial activity.

Copaiba oil-resin has been tested for its antimicrobial activity against dental plaque-forming bacteria, with good *in vitro* and *in vivo* results, which gives credibility to the clinical use of the formulation (Paiva et al., 2002, Vasconcelos et al., 2008, Pieri et al., 2009, Romero et al., 2009). Therefore, this study represents an initial step towards future clinical trials, which can generate the development of a new product in the market, since the copaiba has a great economic importance worldwide. In addition, studies can be carried out with the association of fluoride in the varnish, since chemically it is a compatible association and thus the same also have the capacity of remineralization, since to be based only on bacterial profiles is somewhat limited in the case of dental caries.

5. CONCLUSIONS

The present study further supports the antimicrobial bactericidal effect of copaiba against *S. mutans*. The use of copaiba varnish could be a good prevention strategy for children aged between 3 and 5 years old and warrants the performance of additional randomized clinical trials to identify its antimicrobial efficacy and anticaries effect.

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Authorship

LARV, MFG, JMM, FOC and EMRN contributed in running the laboratory work and writing the manuscript; Prof. Dr. FVF contributed with data analyze. Profs Dr. MAMB and MMFF, performed extraction, isolation and characterization of isolated constituents. And participated on manuscript preparation and also contributed to critical reading of manuscript. Prof Dr SGFF performed the dental varnishes and also contributed to critical reading of manuscript. Prof Dr CSRF, CBMC and PLDL designed the study, manuscript preparation and also contributed to critical reading of manuscript.

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Figures and tables

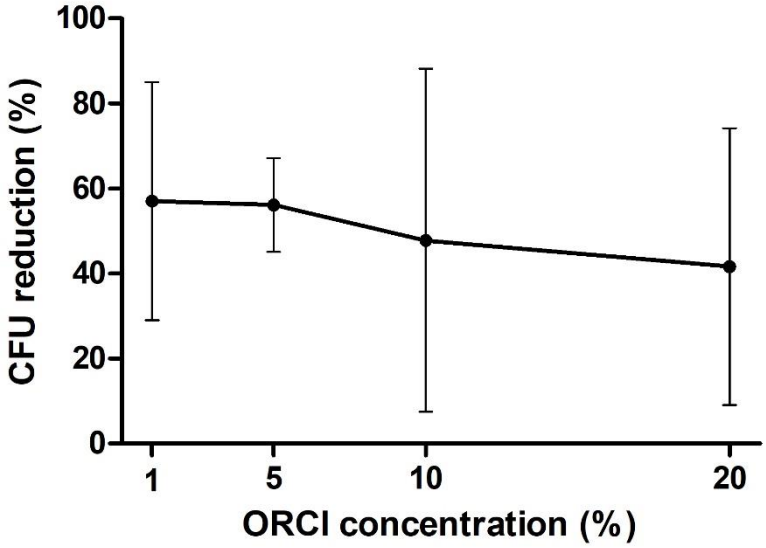

Figure 1 - Effect of different concentrations of the oil resin of *Copaifera langsdorffii* (ORCI) applied in the form of varnish, in the relative reduction of the number of colony forming units (CFU) of *Streptococcus mutans*, expressed in percentage terms, measured in saliva samples, dilution 1:10.

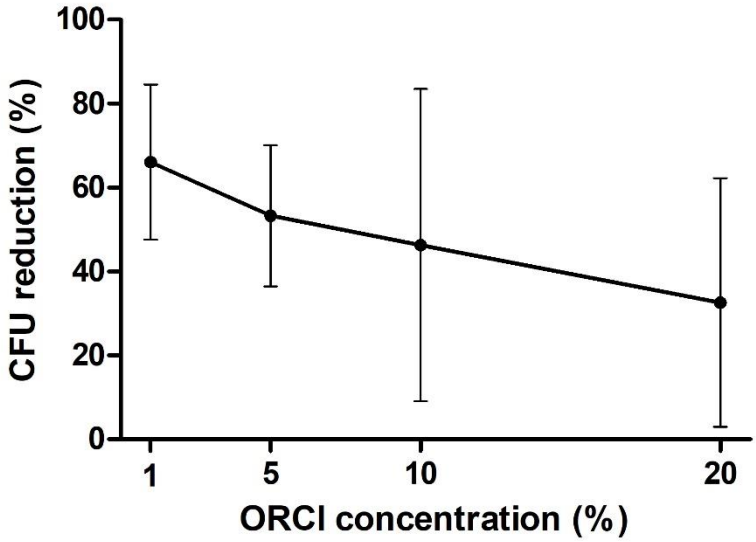

Figure 2 - Effect of different concentrations of the oil resin of *Copaifera langsdorffii* (ORCI) applied in the form of varnish, the relative reduction in the number of colony forming units (CFU) of *Streptococcus mutans*, expressed in percentage terms, measured in saliva samples, dilution 1:100.

Table 1 - Number of colony forming units (CFU) of *Streptococcus mutans* in the saliva samples verified in 6 children with 1:10 dilution before and after the treatment with varnish containing the oil resin at concentrations *Copaifera langsdorffii* 1, 5, 10 and 20%. In each concentration, comparisons between pre- and post-treatment were carried out by using the t test for paired samples.

Concentration (%)	Pre-treatment		Post-treatment		Significance (P value)
	Average	SD	Average	SD	
1 (n=6)	6972,00	4159,67	2571,83	1535,44	**0,0302***
5 (n=6)	5610,83	4047,03	2160,83	1425,83	**0,0275***
10 (n=6)	3479,60	4231,43	2426,40	3751,68	0,0928
20 (n=6)	6094,17	4448,37	3621,83	4295,63	0,0726

Table 2 - Number of colony forming units (CFU) of *Streptococcus mutans* observed in the saliva samples of 6 children with dilution of 1:100 before and after the treatment with varnish containing the oil resin of *Copaifera langsdorffii* on concentrations 1, 5, 10 and 20%. In each concentration, comparisons between pre- and post-treatment were carried out by using the t test for paired samples.

Concentration (%)	Pre-treatment		Post-treatment		Significance (P value)
	Average	SD	Average	SD	
1 (n=6)	33275,00	15146,79	11443,33	8760,58	**0,0026****
5 (n=6)	46388,33	37815,41	17496,67	11998,57	**0,0430***
10 (n=6)	11398,00	15693,41	8464,00	11950,68	0,1899
20 (n=6)	24608,33	16497,90	13218,33	7675,31	**0,0474***

Table 3 - Relative reduction in the number of colony forming units (CFU) of *Streptococcus mutans*, expressed in percentage terms, measured in saliva samples from 6 children, with dilution of 1:10 or 1:100, treated with varnish containing the *Copaifera langsdorffii* oil resin at concentrations of 1, 5, 10 and 20%. In each concentration, comparisons between two dilutions were performed by using the t-test for paired samples.

Concentration (%)	Dilution 1:10		Dilution 1:100		Significance (P value)
	Average	SD	Average	SD	
1 (n=6)	57,05	27,98	66,07	18,51	0,1924
5 (n=6)	56,10	11,01	53,30	16,86	0,6210
10 (n=6)	47,81	40,36	46,31	37,24	0,8934
20 (n=6)	41,62	32,59	32,57	29,64	0,4323

CAPÍTULO 3

TITLE PAGE

This chapter refers to the article submitted on the International Journal of Phytotherapy and Phytopharmacology (PHYTOmedicine).

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Clinical And Microbiological Evaluation Of *Copaifera Langsdorffii* Desf Dental Varnish In Children: A Randomized And Double-Blind Clinical Trial

Clinical And Microbiological Evaluation Of *Copaifera Langsdorffii* Desf Dental Varnish In Children: A Randomized And Double-Blind Clinical Trial

Abstract

Background: The use of biologically active natural products in different types of formulations and application forms have been researched to prevent oral diseases. Studies have demonstrated the therapeutic potential of *Copaifera langsdorffi* (copaiba) in several functions, especially its anti-inflammatory and antimicrobial properties. The varnishes represent a good prevention strategy given its great adhesion to the tooth and slow, continuous and prolonged release of the active principle. **Purpose**: The aim of this study was to longitudinally evaluate the antimicrobial efficacy of a 1% *Copaifera langsdorffi* (copaiba) dental varnish to prevent dental caries in high-risk children. **Methods**: This is a *longitudinal*, *randomized*, *controlled clinical trial.* Ninety (90) high-risk caries-free children (ICDAS II = 0) aged 36-71 months and in good health were recruited and randomly divided into three groups to receive treatment with varnishes containing: 1% copaiba, 1% chlorhexidine (positive control) or 5% fluoride (control). Varnishes were applied to occlusal surfaces of second deciduous molars at baseline (D0), 3 months after (D90), and 6 months after (D180). Saliva was collected before starting treatment (D0), 90 days after day 0 (D90), 6 months (D180) and 1 year after starting treatment (D360) to evaluate the *S. mutans* reduction. Microbiological analysis was performed in duplicates (1:10 and 1:100 mL dilutions). *S. mutans* isolates were identified by their characteristic colony morphology and the values were expressed as log10 (CFU/mL). Statistics were carried out by applying repeated measures analysis of variance, Tukey's multiple comparisons test, and paired t-test. **Results**: We found the *S. mutans* levels (mean± standard deviation) as follows: Chlorhexidine (D0: 0.58 ± 0.43; D90: 0.38 ± 0.23; D180:0.33 ± 0.14; D360: 0.55 ±0.52), Fluoride (D0:0.86 ± 0.37; D90:0.51 ± 0.33; D180:0.41 ± 0.24; D360:0.53 ± 0.44), and Copaiba (D0:1.32 ± 0.61; D90:0.99 ± 0.57; D180:0.39 ± 0.22; D360:0.12 ± 0.19). Copaiba demonstrated significant *S. mutans* reduction: D360 versus D0 ($p < 0.0001$), D180 versus D0 ($p < 0.001$), D360 versus D90 ($p < 0.001$), D180 versus D90 ($p < 0.001$), D360 versus D180 ($p < 0.05$). Chlorhexidine varnish significantly reduced *S. mutans at* D180 versus D0 ($p < 0.05$). Fluoride reduced *S. mutans* at D180 versus D0 ($p < 0.001$). Copaiba consistently reduced *S. mutans* throughout the 360-day period. At D360, copaiba produced lower *S. mutans* levels compared to fluoride ($p < 0.001$) and chlorhexidine ($p < 0.01$). **Conclusion**: Three annual applications of the Copaifera langsdorffii dental varnish showed substantial antimicrobial activity against *S. mutans* for up to 12 months in high-risk caries-free children. Further studies to identify the anticaries effect of this varnish are required to establish its use in caries prevention.

Keywords: Pediatric Dentistry; Dental Caries; Natural Products; Saliva**.**

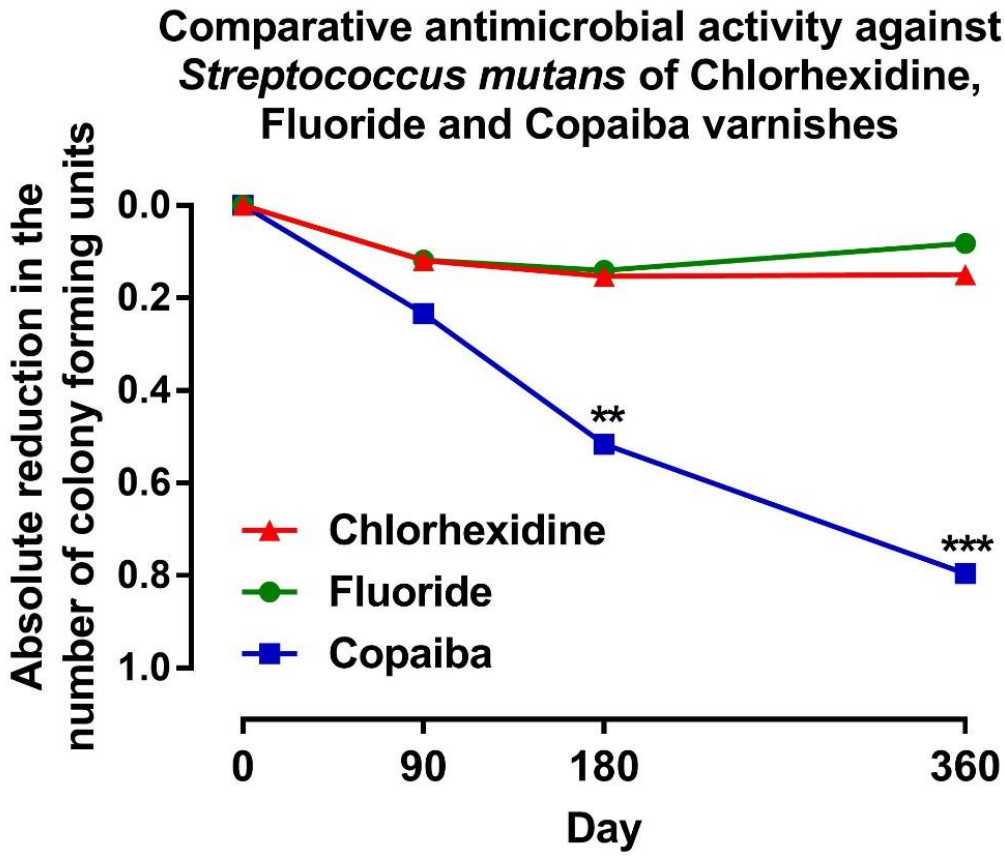

Figure 1: Graphical abstract of the manuscript.

INTRODUCTION

Medicinal plants have great biological and pharmacological diversity, being great targets in drug development (Vieira et al., 2018). There are more than 100 million bioactive molecules cataloged, and this number can be considered unlimited due to still unexplored possible chemical arrangements and resources (Freires and Rosalen, 2016).

Copaiba belongs to the Legminosae family, Caesalpinioideae subfamily and Copaifera genus, and the trees can reach up to 400 years of age. The oil is yellow-brown with several active components such as sesquiterpenes and diterpenes, which have anti-inflammatory, analgesic, antimicrobial and antitumor properties (Tobouti et al., 2017).

Copaiba resin oil has been widely used and especially found in neotropical regions where bees of the *Apis Mellifera* species are the main pollinating agents. There are records of copaiba oil use for almost 400 years, with several studies proving its innumerable biological activities, being effective against several microorganisms and commonly used in traditional medicine against various diseases (Romero et al., 2009; Tobouti et al., 2017).

The gender Copaifera is widely found in South and Central America, India and West Africa. The greatest species richness is found in Brazil, where trees can especially be found in the Southeast, Midwest and Amazon regions. Among the 72 cataloged species, 20 have already been found in Brazil, with 16 being exclusive to the country. Among them are *Copaifera officinalis* L., *Copaifera guianensis* Desf., *Copaifera reticulata* Ducke, *Copaifera multijuga* Hayne, *Copaifera confertiflora* Bth., *Copaifera langsdorffi* Desf., *Copaifera coriacea* Mart. and *Copaifera cearensis* (Tobouti et al., 2017, Diefenbach et al., 2018, Trindade et al., 2018).

Copaifera langsdorffii, the species chosen in this study, is native to the Amazon rainforest and common in the Mato Grosso region, where according to a survey it is the species with the greatest importance in the georeferencing region (Bieski et al., 2015).

Dental caries is the most prevalent disease in the world, and is called early childhood caries (ECC) when it occurs in children under 6 years of age (Parisotto et al., 2015, Pitts et al., 2017).

Even with the expansion of access to health services, early childhood caries (ECC) is still a public health problem (AAPD 2016), being the main cause of losing deciduous teeth early, negatively influencing speech, aesthetics, masticatory system and the dental arches (Parisotto et al., 2011).

The main products in preventing oral diseases are fluoride, chlorhexidine, triclosan, cetylpyridinium chloride and natural products, especially essential oils which have attracted attention due to antimicrobial activity. These have also been used as therapeutic alternatives against dental caries (Lobo et al., 2011, Lobo et al., 2014, Freires et al., 2015).

Buccal microbiota is quite diverse, where some species influence health, while others promote disease (Gomar-Vercher et al., 2004). Habits such as poor oral hygiene and poor diet may alter the equilibrium in the microbiota under healthy conditions, causing dysbiosis that will result in carious lesions (Pitts et al., 2017).

Dental biofilm is the main biological determinant in the development of dental caries (Sanz et al., 2017). Several microorganisms colonize dental biofilms, where *S. mutans* are strongly associated with dental caries, and are found in all niches such as saliva, tongue, oral mucosa and dental plaque (Manji et al., 2018).

After consumption of sucrose, *S.mutans* produce extracellular polysaccharides and are acidogenic and aciduric, being able to survive in adverse conditions. The oral cavity presents several genotypes of *Streptococcus mutans* (*S. mutans*) with different virulence capacities (Arthur et al., 2011; AAPD, 2016). Thus, although they are not the only ones involved in the process, they are a key contributor in forming dental biofilms (Klein et al., 2009).

The use of saliva as a biomarker has several advantages since it is a non-invasive, easy-to-collect medium and presents much information, even in a small amount of sample (Belstrøm et al., 2018). The salivary microbiota may help (for

example) in the diagnosis, prevention and control of caries, periodontal disease and gingivitis (Gomar-Vercher et al., 2014).

Healthy behavioral approaches and promotion should be implemented for the prevention of dental caries in public health, in addition to policies such as public water fluoridation and strategies in high-risk groups with restricted access to dental and fluoride services (Pitts et al., 2017).

The teeth most affected by caries in the deciduous dentition are the second deciduous molars due to their occlusal morphology which favors accumulating bacterial plaque. It is also known that preventing decay in primary teeth will prevent permanent dentition. In the current growing model of minimally invasive dentistry, it is argued that dental caries can be controlled and prevented in a non-invasive way through several products, among which varnishes can be mentioned (Manji et al., 2018).

As children under six years of age do not yet have the proper habit of “rinsing and spitting”, the most appropriate formulations for preventing caries disease at this age would be varnishes rather than a rinse aid or gel, and also because of their high retentive capacity and the slow release of the active principle (De Luca et al., 2017).

No reports have been found in the literature on the use of varnishes based on copaiba, thus the application of a patent of invention was deposited under protocol BR 1020160212628. The objective of this study was to evaluate the clinical and microbiological efficacy of *Copaifera langsdorffii* DESF C. langsdorffii) in children at high risk of caries.

MATERIAL AND METHODS

Copaiba oil resin

Samples of copaiba oil-resin obtained from *Copaifera langsdorffii* Desf. (Fabaceae: Caesalpinioideae) plant material deposited in the herbarium of the Federal University of Mato Grosso - voucher Silva, R. R. et al. 1749, were received from the Federal University of Mato Grosso and originally obtained from Juruena Valle (Region: Midwest, Latitude: 10º 19' 05" S, Longitude: 58º 21' 32" W, Height: 300m). Chemical constituents were identified by specialists at the

Department of Chemistry, in the Federal University of Ceara (GC–MS QP 5050 Shimadzu, Japan). The main components of the *Copaifera langsdorffii* oil-resin used in the present study were: β-caryophyllene, α-humulene, cedrene, cadinene and bisabolene.

In vitro Study

S. mutans ATCC UA159 strains were used in the present study to estimate the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC). The strain was activated by incubating it at 37ºC overnight in Brain Heart Infusion (BHI) culture medium in an anaerobic jar using the candle method for a period of 24 hours. An experimental study was carried out in which the potential antimicrobial activity of copaiba oil-resin on the standard *S. mutans* ATCC UA159 strain was investigated. Microbiological tests were carried out. All the tests were performed in triplicate and at two different times. The microdilution method was used in culture broth according to Standard M07-A10 of the Clinical and Laboratory Standard Institute (CLSI, 2015) to evaluate the antimicrobial activity. After this time, the cultures had their cell density adjusted in sterile 0.85% saline so as to achieve turbidity equivalent to the 0.5 tube of the McFarland scale (approximately 1.5 x 10^8 CFU/mL). The obtained suspension was diluted 100-fold in sterile BHI medium, resulting in a culture with approximately 10^6 CFU/mL. This suspension was used in the assay.

Clinical Study

This is a longitudinal, parallel, randomized, double-blind controlled clinical trial which adhered to the CONSORT checklist. The rules of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) were followed in order to improve the study methodology. The clinical phase occurred in the city of Aracati- CE, a city in which only 0.8% of the population has fluoridated water coverage.

Local and Population/Ethical Aspects

The study was carried out in the city of Aracati-CE. This study was approved by the Ethics Committee of the Federal University of Ceara (UFC), with number 195,096. The parents were invited to participate in the research and then informed so as to sign the clear and informed consent form (ICF). The sample selection (90 children) was carried out by means of a clinical examination of the patients in public schools and day care centers in the municipality, where children who were free from caries with 4 erupted primary second molars, aged between 36 and 71 months and of both genders were included. The high-risk caries classification was done according to the criteria of the American Academy of Pediatric Dentistry (AAPD), 2014; for example, consumption of sugar more than three times a day, lack of access to fluoridated water, presence of visible plaque, poor oral hygiene and absence of visits to a professional dentist. Exclusion criteria were: presence of any systemic disease or the application or use of any antibiotic or antimicrobial three months prior to starting the study.

Varnish Preparation

Copaiba oil was formulated as a varnish in the Pharmacotechnical laboratory of the Pharmacy course of the Faculty of Pharmacy, Dentistry and Nursing of the Federal University of Ceara in a standardized way in order to obtain similarity of color, odor, consistency and flavor.

A pilot study was initially performed to obtain the dose-response curve (Valadas et al., 2019). The concentration used in the main study was first checked as having the greatest relative reduction capacity of bacteria (%). All varnishes were stored in eppendorfs and encoded with letters of the alphabet.

Grouping and Application of Varnishes

The children were divided into 3 groups, with 30 participants each. Group I received application of 1% chlorhexidine varnish, 5% fluorine varnish group II, 1% Copaifera langsdorffii oil group (copaiba). Each patient initially chewed a piece of 3 × 3-cm plastic film (Parafilm®) for 60 s to stimulate the production of saliva and release the bacteria from the dental biofilm. Saliva was collected using a plastic device and stored in sterile microcentrifuge tubes (Eppendorf®), which

were stored in polystyrene box containing ice. To minimize the influence of the circadian rhythms on salivary flow, all samples were collected in the same session and conditions by the same operator between 9:00 and 11:00 AM. Each patient received an application of the varnish corresponding to their group in the four second deciduous molars. Prior to an application of varnishes, the teeth were professionally cleaned with a Robinson brush and pumice. The varnishes were applied with relative insulation onto the selected molars using a microbrush. After 10s, the varnish was subtly dried by air from a triple syringe. The cotton rolls were removed after 25 seconds to avoid saliva contamination. The varnish was applied 3 times for each tooth: at the baseline, after 90 days and 180 days of starting treatment. The presence or absence of caries was also recorded in the evaluated teeth as well as in the others during each evaluation and after the baseline. The saliva of each patient was collected at 4 moments: at the baseline, after 90 days, 180 days and 360 days of starting treatment.

Microbiological analysis

Samples were transported to the laboratory for microbiological analysis in a hermetically sealed case containing ice, and analyzed no longer than 2 h after collection.

Saliva was homogenized on a tube shaker for 30 seconds. A volume of 0.1 mL of each sample was aseptically drawn and transferred into one sterile test tube containing 0.9 mL of saline. The procedure was repeated twice, establishing dilutions of 1:10 and 1:100. A corresponding volume of ten microliters of each dilution was plated onto *Mitis Salivarius-Bacitracin* (MSB) agar medium in triplicates. The plates were then incubated at 37°C during 48 h in jars under microaerophilic conditions. Bacterial counts were expressed as colony forming units (CFU)/mL of saliva and followed by phenotypical colony identification, as described elsewhere.

Clinical Evaluation

The children were evaluated for the onset of carious lesions by the ICDAS II method in the same saliva collection periods.

Statistical analysis

For the clinical trial, data on the number of CFU were initially transformed in order to homogenize the variances and make the distribution closer to normal. To do so, a logarithmic transformation was used according to the following equation:

Because there are values equal to zero, 1 to x has been added since the logarithmic function is only defined for $x > 0$.

The transformed values of the CFU number were initially analyzed by the Kolmogorov-Smirnov test to verify the normality of the distribution. Thus, mean and standard deviation were calculated for the descriptive statistics, as well as using parametric tests to analyze the data. Analysis of variance (ANOVA) was used to compare the three groups at each time (intergroup analysis), associated with Tukey's multiple comparison test to verify differences between the paired groups. Comparisons between the different times within each group (intragroup analysis) were performed by repeated measures analysis of variance (ANOVA), associated with the Tukey multiple comparisons test in order to verify differences between paired times. The level of significance was set at 0.05 (5%) in all analyzes, with a P-value less than 0.05 being considered as statistically significant. GraphPad Prism® software version 5.00 for Windows® (GraphPad Software, San Diego, California, USA, 2007) was used for both statistical procedures and graphing.

RESULTS

The in vitro tests showed one MIC of 2.0 µg/mL and MBC of 6.0 µg/mL), The average age of the participants were 4.9 years old.

First Dilution (1:10)

Table 1 shows the amount of *Streptococcus mutans*, expressed as the logarithm of the number of colony forming units (CFU) per mL of saliva, measured in saliva samples with dilution of 1:10 on days 0 (pretreatment), 90, 180 and 360 in patients treated with chlorhexidine, fluoride and copaiba varnishes on the first dilution. The data correspond to the mean and standard deviation of the logarithm

of the number of CFU verified in the saliva samples of the patients in each treatment group. At the end of treatment, the groups treated with copaiba varnish (p <0.0001) and fluoride (p <0.0001) had a statistical difference in relation to the start of treatment.

Table 1 – Amount of *Streptococcus mutans*, expressed as the logarithm of the number of colony forming units (CFU) per ml of saliva, measured in saliva samples with dilution of 1:10 on days 0 (pretreatment), 90, 180 and 360 in patients treated with chlorhexidine, fluoride and copaiba varnishes. The data correspond to the mean and standard deviation of the logarithm of the number of CFU verified in the saliva samples of the patients in each treatment group.

Day	Chlorhexidine	Fluoride	Copaiba	Significance (ANOVA)
	Mean ± SD	Mean ± SD	Mean ± SD	
0	0.58 ± 0.43	0.86 ± 0.37	1.32 ± 0.61[a,d]	**P<0.0001**
90	0.38 ± 0.23	0.51 ± 0.33[x]	0.99 ± 0.57[a,c,y]	**P<0.0001**
180	0.33 ± 0.14[y]	0.41 ± 0.24[x]	0.39 ± 0.22[x,z]	P=0.3580
360	0.55 ± 0.52	0.53 ± 0.44[x]	0.12 ± 0.19[b,d,x,z]	**P=0.0004**
Significance (repeated measures ANOVA)	**P=0.0107**	**P<0.0001**	**P<0.0001**	

SD: standard deviation; ANOVA: analysis of variance; The letters [a](P<0.001) and [b](P<0.01) denote statistically significant differences in relation to the chlorhexidine varnish on the same day, while the letters [c](P<0.001) and [d](P<0.01) indicate statistically significant differences in relation to the fluoride varnish on the same day (Tukey test); The letters [x](P<0.001) and [y](P<0.05) designate statistically significant differences in relation to day 0 in the same group, while the letter [z](P<0.001) denotes statistically significant difference in relation to day 90 in the same group (Tukey's test).

Second Dilution (1:100)

Table 2 shows the amount of *Streptococcus mutans*, expressed as the logarithm of the number of colony forming units (CFU) per ml of saliva, measured in saliva samples with dilution of 1:100 on days 0 (pretreatment), 90, 180 and 360 in patients treated with chlorhexidine, fluoride and copaiba varnishes, on the second dilution (1:100). The data correspond to the mean and standard deviation of the logarithm of the number of CFU verified in the saliva samples of the patients in each treatment group. At the end of treatment, the group treated with

chlorhexidine ($p < 0.05$) and copaiba ($p < 0.001$) varnishes had a statistical difference in relation to D0.

Table 2 – Amount of *Streptococcus mutans*, expressed as the logarithm of the number of colony forming units (CFU) per ml of saliva, measured in saliva samples with dilution of 1:100 on days 0 (pretreatment), 90, 180 and 360 in patients treated with chlorhexidine, fluoride and copaiba varnishes. The data correspond to the mean and standard deviation of the logarithm of the number of CFU verified in the saliva samples of the patients in each treatment group.

Day	Chlorhexidine	Fluoride	Copaiba	Significance (ANOVA)
	Mean ± SD	Mean ± SD	Mean ± SD	
0	0.45 ± 0.26	0.48 ± 0.20	0.89 ± 0.55[a.c]	**P<0.0001**
90	0.33 ± 0.13	0.36 ± 0.11	0.66 ± 0.44[a.c]	**P<0.0001**
180	0.30 ± 0.00[v]	0.34 ± 0.07[w]	0.38 ± 0.19[u.y]	P=0.0723
360	0.31 ± 0.18[w]	0.40 ± 0.28	0.10 ± 0.19[b.c.u.x.z]	**P<0.0001**
Significance (repeated measures ANOVA)	**P=0.0037**	**P=0.0417**	**P<0.0001**	

SD: standard deviation; ANOVA: analysis of variance; The letters [a](P<0.001) and [b](P<0.01) denote statistically significant differences in relation to the chlorhexidine varnish on the same day, while the letter [c](P<0.001) indicates statistically significant difference in relation to the fluoride varnish on the same day (Tukey test); The letters [u](P<0.001), [v](P<0.01) and [w](P<0.05) designate statistically significant differences in relation to day 0 in the same group, the letters [x](P<0.001) and [y](P<0.05) denote statistically significant differences in relation to day 90 in the same group, while the letter [z](P<0.05) indicates statistically significant difference in relation to day 180 in the same group (Tukey test).

Regarding the clinical data at the end of the treatment with the different varnishes (Table 3), the appearance of initial carious lesions in the group treated by the chlorhexidine varnish was observed.

Table 3 – Distribution of the lesions on the molars (Scores ICDAS II) of different groups treated with dental varnishes at the end of the clinical trial.

Tooth	Copaiba	Chlorhexidine	Fluoride
Score 0	0	0	0
Score 1	0	4	0
Score 2	0	1	0
Score 3	0	0	0
Score 4	0	0	0
Score 5	0	0	0
Score 6	0	0	0

DISCUSSION

C. langsdorffii oil is effective against gram-positive and gram-negative bacteria, especially for topical use. Its effectiveness against the cariogenic bacterial is also emphasized (Bieski et al., 2015). Saliva is a representative medium of the oral microbiota, which may reflect the changes in it, and was chosen because it is an accessible medium (Gomar-Vercher et al., 2014).

Among the risk markers for ECC are the *S. mutans* and *Lactobacillus* species, which are part of the oral microbiome (AAPD 2014). These may reflect different stages of the caries process and reveal changes in the oral microbiota (Gomar-Vercher et al., 2014). Although several species are involved in dental caries, *S. mutans* are still strongly associated with the disease; its high colonization in the oral cavity may be associated with the disease, since they are an indicator of microbial disequilibrium (Lobo et al., 2011; Lobo et al., 2014; Freires et al., 2017).

Streptococci, although not the only ones involved in dental caries, are one of the major colonizers of the oral cavity, initiating this colonization soon after tooth eruption (Manji et al., 2018).

Studies evaluating the use of chlorhexidine for a period of 6 months were insufficient to verify the effect on dental caries, as most do not show any effect on disease control (Jepsen et al., 2017). In the present study, the chlorhexidine varnish reduced the CFU for a period of six months ($p = 0.0107$), but in the last analysis (D360) it was observed that the CFU returned to the same levels of the initial period.

Vale et al. (2014) evaluated the time of recolonization of *S. mutans* after two consecutive days of treatment with 1% chlorhexidine gel. Saliva was collected before the study, and at days 1,7,14, 21 and 28, for evaluation of *S. mutans* levels. The levels decreased, but were not statistically significant.

The topical use of fluoride products in high concentrations (> 2,500 ppm) creates fluoride reservoirs, providing fluoride to the dental surface and promotes its penetration into the biofilm, being effective in reducing demineralization and increasing remineralization. Fluoride may present bactericidal activity (Pitts et al., 2017) with frequent professional applications. The group treated with fluoride

varnish in the present study showed a reduction of CFU throughout the study period ($p < 0.0001$).

The antimicrobial activity of copaiba oil may be related to the combination of sesquiterpenes and diterpenes, thus affecting the integrity of the bacterial cell wall. The oil has scientifically proven activity against several pathogens, especially gram-positive bacteria such as *Staphylococcus* spp. and *Streptococcus* spp. It is important to use a suitable methodology for the dilution of oil in research and validation by gas chromatography (Tobouti et al., 2017). In the present study, copaiba showed a significant reduction of CFU throughout all the period ($p < 0.0001$).

In the inter-group analysis in each period studied, the group treated with copaiba varnish was the only one to show statistically significant results for the two dilutions.

Copaiba oil has high activity against oral bacteria and can be used in appropriate formulations, since the main oral diseases, caries and periodontal disease are strongly related to the dental biofilm. However, in vitro and in vivo assays of these formulations must be well studied (Diefenbach et al., 2018).

According to Diefenbach et al. (2018), most of the studies which evaluate the antimicrobial activity of copaiba oil compare it with chlorhexidine, which is the positive control, where *S. mutans* are the most studied organisms, as well as the other studies with essential oils (Freires et al., 2015).

Pieri et al. (2010) evaluated the action of β-caryophyllene isolated from copaiba oil on the adhesion of *S. mutans* bacteria, in which it had better action than chlorhexidine. Pieri et al. (2016) evaluated the antimicrobial activity of β-caryophyllene isolated from copaiba oil against dental plaque bacteria in vitro. The results demonstrated that β-caryophyllene prevented plaque-forming bacteria from proliferating.

Dental varnishes stand out in preventing dental caries, and are widely accepted by pediatric patients, especially children under 6 years of age. Patients in this age group do not have adequate capacity to eject saliva, so the varnishes were the chosen formulation for the use of copaiba oil (Pessan et al., 2008; Vasconcelos et al., 2008). They are composed of polymer matrices, excipients

and active principle. In the case of the present varnish, the chosen matrix was insoluble (in this case ethylcellulose), used to modulate the release of the active principle and thus its substantivity was higher (Valadas et al., 2019; De Luca et al., 2017). This type of formulation adheres to dental scars and fissures, gradually releasing the active principle, disrupting the dental biofilm, becoming a long-term therapeutic agent which is suitable for antimicrobial formulation (Pessan et al., 2008, Franca et al., 2014).

Most of the randomized clinical trials with outcomes in dental caries are currently focused on the performance of restorative materials and with many biases in the sample. In the current phase of minimally invasive dentistry, studies with materials and preventive alternatives are important (Levey et al., 2017).

In the pilot study of copaiba dental varnish, all oil concentrations showed antimicrobial activity, however only 1% showed a reduction in *S. mutans* colony forming units (Valadas et al., 2019). It is believed that the greater complexity of the chemical constituents present in copaiba, a pharmaceutical form with lower concentration, presents a smaller interaction between the pharmaceutical excipients used in the formulation. In addition, the active ingredients of copaiba were probably retained in the varnish matrix and were released locally. It was also observed that higher concentrations of copaiba oil lost the ability to retain its active principle, and in these situations the active principle was released so quickly that the varnish partially lost its antimicrobial activity.

CONCLUSIONS

After three annual applications, copaiba varnish demonstrated significant antimicrobial activity against *S.mutans* for up to 12 months in children with high risk of caries. The fluoride and copaiba varnishes had good results regarding dental caries prevention. Future studies are needed to identify anticaries effects to establish the use of varnish in caries prevention.

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DISCUSSÃO

O desenvolvimento de materiais odontológicos com produtos naturais ainda é bastante limitado, praticamente resumido às formulações tópicas e sem muitos ensaios clínicos publicados nas últimas décadas. No entanto, vários estudos documentaram a atividade farmacológica de produtos naturais contra o biofilme dentário, especialmente o cariogênico. (VALADAS *et al*., 2019; FREIRES; ROSALEN, 2016)

Existem várias razões para o desenvolvimento de produtos com propriedades antimicrobianas, uma vez que cresce a cada dia a resistência microbianas, toxicidade e altos custos de diversos materiais. O principal agente antimicrobiano na Odontologia é a clorexidina que, quando utilizada por um período prolongado, causa coloração do dente, alteração do paladar e perde sua capacidade farmacológica, ocorrendo a recolonização de bactérias, como *S. mutans*. (VALE *et al*., 2014)

O óleo da *C. langsdorffii* é eficaz contra bactérias gram-positivas e gram-negativas, especialmente quanto ao uso tópico. Destaca-se também a eficácia do mesmo contra as cariogênicas. Não foram encontrados na literatura relatos da utilização de vernizes à base de copaíba, desse modo foi depositado o pedido de uma patente de invenção sob protocolo BR 1020160212628. (BIESKI *et al*., 2015)

No caso da cárie dentária, existem vários indicadores de risco, tanto diretamente relacionados à doença, como a exposição ao açúcar, e consequente alteração da microbiota, bem como os fatores moduladores, como aspectos socioeconômicos. A saliva é um material biológico, sendo um excelente biomarcador para doenças sistêmicas e orais, especialmente em relação à cárie dentária, onde aproximadamente 108 microrganismos podem ser contados em 1 mL de saliva. Pesquisas associadas à cárie utilizam contagens de *S. mutans* como biomarcadores de desequilíbrio da microbiota. (MARSH *et al*., 2000; KAUR *et al*., 2013; AAPD, 2014)

Os investimentos em patentes estão cada vez mais difundidos, pois todo o funcionamento do sistema capitalista está relacionado à inovação e ao avanço científico e tecnológico. Este estudo é o primeiro a utilizar verniz de copaíba sob

patente de número BR1020160212628. (VALADAS *et al*., 2017; FRANCA *et al*., 2014; FREIRES; ROSALEN, 2016)

Entre os marcadores de risco para a CPI destacam-se as espécies de *S.mutans* e *Lactobacillus,* que fazem parte do microbioma oral. (AAPD, 2016) Os mesmos podem refletir diferentes estágios do processo carioso e revelar alterações na microbiota oral (GOMAR-VERCHER *et al*., 2014). Apesar de diversas espécies estarem envolvidas na cárie dentária, os *S. mutans* ainda são fortemente associados à doença, onde uma alta colonização dos mesmos na cavidade oral pode estar associada à doença, por serem indicadores de desequílibrio da microbiota. (LOBO *et al*., 2011; LOBO *et al*., 2014; FREIRES *et al*., 2017)

Os *Streptococci,* apesar de não serem os únicos envolvidos na cárie dentária, são um dos maiores colonizadores da cavidade oral, iniciando essa colonização logo após a erupção. (MANJI et al., 2018)

Estudos que avaliaram o uso da clorexidina, por um período de 6 meses, foram insuficientes para constatar o efeito na cárie dentária, onde a maioria não demonstra efeito no controle da doença. (JEPSEN *et al*., 2017) No presente estudo, o verniz de clorexidina reduziu as UFC por um período de seis meses (p= 0,0107), na última análise (D360) observou-se que as UFC voltaram aos mesmos níveis do período inicial.

Vale *et al*. (2014) avaliaram o tempo de recolonização dos *S.mutans* após tratamento de dois dias consecutivos com gel de clorexidina 1%. A saliva foi coletada antes do estudo, e nos dias 1,7,14, 21 e 28, para avaliação dos níveis de *S.mutans*. Os níveis diminuíram, mas não mostraram estatística significante.

O uso tópico de produtos fluoretados em altas concentrações (> 2.500 ppm) cria reservatórios de flúor, fornecendo flúor à superfície dentária e promove a penetração do mesmo no biofilme, sendo eficaz na diminuição da desmineralização e aumento da remineralização. Com frequentes aplicações profissionais, o mesmo pode apresentar atividade bactericida. (Pitts *et al*., 2017) No presente estudo, o grupo tratado com verniz fluoretado demonstrou redução por todo o período do estudo (p< 0,0001).

A atividade antimicrobiana do óleo de copaíba pode estar relacionada à combinação de sesquiterpenos e diterpenos, afetando, assim, a integridade da parede celular bacteriana. O óleo possui atividade cientificamente comprovada contra diversos patógenos, especialmente bactérias gram-positivas, como *Staphylococcus* spp. e *Streptococcus* spp. Em pesquisas com o óleo, é importante utilizar uma metodologia adequada para a diluição do mesmo e validação por cromatografia gasosa. (TOBOUTI *et al*., 2017) No presente estudo, a copaíba demonstrou redução significante por todo o período ($p< 0,0001$).

Na análise intergrupo, em cada período estudado, o grupo tratado com o verniz de copaíba foi o único a mostrar resultados estatisticamente significantes para as duas diluições.

O óleo de copaíba contém uma grande atividade contra bactérias orais podendo ser utilizado em formulações apropriadas, já que as principais doenças bucais, a cárie e a doença periodontal, são fortemente relacionadas ao biofilme dentário. Entretanto, estudos *in vitro* e *in vivo* dessas formulações devem ser bastante estudados. (DIEFENBACH *et al*., 2018)

De acordo com Diefenbach *et al*., (2018) a maioria dos estudos que avaliam a atividade antimicrobiana do óleo de copaíba o comparam com a clorexidina, sendo essa o controle positivo, onde os *S.mutans* são os organismos mais estudados, assim como os demais estudos com óleos essenciais. (FREIRES *et al*., 2015)

Pieri *et al*., (2010) avaliaram a ação do β-cariofileno isolado do óleo de copaíba na adesão da bactéria *S. mutans*, onde o mesmo teve melhor ação que a clorexidina. Pieri *et al*., (2016) avaliaram *in vitro* a atividade antimicrobiana do β-cariofileno isolado do óleo de copaíba contra bactérias da placa dentária. Os resultados demonstraram que o β-cariofileno impediu a proliferação das bactérias formadoras da placa.

Os vernizes dentários destacam-se na prevenção da cárie dentária, tendo bastante aceitação por paciente pediátricos, especialmente, menores de 6 anos de idade. Pacientes nessa faixa etária não possuem uma capacidade adequada para ejetar saliva, sendo assim, os vernizes foram a formulação escolhida para uso do óleo de copaíba. (PESSAN *et al*., 2008; VASCONCELOS *et al*., 2008).

Essas formulações odontológicas são compostas de matrizes poliméricas, excipientes e princípio ativo. No caso do verniz presente, a matriz escolhida foi insolúvel, neste caso a etilcelulose, utilizada para modular a liberação do princípio ativo e, assim, a substantividade foi maior (DE LUCA *et al*., 2017). Este tipo de formulação adere às cicatrizes e fissuras dentárias, liberando, de maneira gradual, o princípio ativo, rompendo o biofilme dentário, tornando-se um agente terapêutico de longo prazo adequado para formulação antimicrobiana (PESSAN *et al*., 2008; FRANCA *et al*., 2014).

Atualmente, a maioria dos ensaios clínicos randomizados em cárie dentária está voltada para o desempenho de materiais restauradores e com muitos vieses na amostra. Na atual fase da Odontologia minimamente invasiva, estudos com materiais e alternativas preventivas são importantes. (LEVEY et al., 2017)

No estudo piloto do verniz dentário de copaíba, todas as concentrações de óleo apresentaram atividade antimicrobiana, entretanto, a de 1% se destacou na redução das unidades formadoras de colônia de *S. mutans*. Acredita-se que o fato da maior complexidade dos constituintes químicos presentes na copaíba, uma forma farmacêutica com menor concentração apresenta uma interação menor entre os excipientes farmacêuticos utilizados na formulação. Além disso, provavelmente os ingredientes ativos de copaíba foram retidos na matriz do verniz e foram liberados localmente. Observou-se também que maiores concentrações do óleo-resina de copaíba perderam a capacidade de reter seu princípio ativo e, nessas situações, o princípio ativo foi liberado tão rapidamente que o verniz perdeu parcialmente sua atividade antimicrobiana.

CONSIDERAÇÕES FINAIS

Há séculos, as propriedades farmacológicas do óleo de copaíba são documentadas na literatura. O mesmo é um interessante produto a ser incorporado em materiais, inclusive os de uso odontológico, como os vernizes. Nos estudos realizados, confirmou-se a atividade antimicrobiana in vitro e eficácia clínica in vivo sobre *S.mutans*.

Após três aplicações anuais, o verniz de copaíba demonstrou efeito contra *S.mutans* por até 12 meses em crianças com alto risco de cárie. Os vernizes de flúor e copaíba tiveram bons resultados quanto à prevenção de cárie dentária. Futuros estudos são necessários para identificar efeitos anticárie para estabelecer o uso do verniz na prevenção da cárie.

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ANEXOS

ANEXO I-TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

“AVALIAÇÃO CLÍNICA E MICROBIOLÓGICA DO USO DE VERNIZES CONTENDO PRODUTOS NATURAIS PARA PREVENÇÃO DE CÁRIE EM CRIANÇAS”

Seu filho ou filha está sendo convidado a participar de um projeto de pesquisa. Sua participação é importante, porém, ele(a) não deve participar contra vontade própria ou contra a sua vontade. Leia com atenção as informações abaixo, sentindo-se livre para fazer qualquer pergunta que desejar, para que não haja dúvida alguma sobre os procedimentos a serem realizados.

a) O objetivo da pesquisa é testar o uso tópico (aplicado aos dentes), na forma de verniz, de um medicamento no tratamento da cárie

b) Durante o estudo você deverá fornecer informação sobre o estado geral de saúde do seu filho ou filha, bem como possíveis reações alérgicas que ele(a) já possa ter tido

c) A participação neste estudo consistirá de:

- Exame dentário de seu filho ou filha,
- Utilização de um medicamento sobre os dentes de seu filho ou filha (aplicação tópica) de um “remédio”, por 3 vezes: inicialmente, após 3 meses e após 6 meses, com freqüência de 1 (uma) vez ao dia.
- Coleta de saliva de seu filho ou filha por 4 vezes: inicialmente, após três meses, após 6 meses e após 1 ano. Para que seja feita a coleta seu filho ou filha terá que mastigar um pequeno pedaço de “chiclete” por um minuto, e a mesma se encontrará ligada a um pedaço de fio dental para evitar que o seu filho ou filha venha a engolir material. Depois de decorrido o tempo o mesmo será retirado da boca e colocado em um pequeno frasco.

d) A aplicação do “remédio”, a coleta de saliva e o exame dentário NÃO causarão DOR ao seu filho ou filha.

f) Seu filho ou filha **NÃO RECEBERÁ INJEÇÃO** de anestésico local.

g) Essa pesquisa oferece o raro risco de seu filho(a) ter alergia ao medicamento (remédio). Para diminuir as chances de alergias solicitamos informações sobre a saúde dele ou dela, estando preparados para suspender o tratamento, e usar remédios para alergia caso isso ocorra. Qualquer falta de informação de sua parte (responsável pela criança) a respeito das condições de saúde do seu filho (a) exclui totalmente a responsabilidade da equipe de pesquisa.

h) Após a aplicação do tratamento a saliva recolhida (conforme descrito acima) e analisada para descobrir se o remédio usado nos dentes de seu filho ou filha foi capaz de diminuir a número de bactérias da boca que causam cárie, podendo trazer como beneficio um tratamento para cárie no futuro.

i) A participação neste estudo lhe dá o direito de seu filho ou filha ser acompanhado (a) por um aluno estagiário para prevenção de novas cáries.

j) Você tem a liberdade de desistir ou interromper a participação do seu filho ou filha neste estudo no momento que desejar, sem necessidade de qualquer explicação.

k) Os resultados obtidos durante este estudo serão mantidos em sigilo. A da Faculdade de Farmácia, Odontologia e Enfermagem não o identificará por ocasião da exposição e/ou publicação dos mesmos (os dados serão publicados somente em revista científica e/ou congressos científicos não identificando o nome de seu filho ou filha).

l) O surgimento de resfriados ou viroses no dia da pesquisa, com conseqüente uso de medicações por período de tempo limitado, exclui seu filho ou filha do estudo.

m) Caso venham a surgir dúvidas ou perguntas, sinta-se livre para contactar a Dra. Patrícia Lobo (responsável pelo projeto) no telefone (85) 99845353.

Ao assinar este termo, você estará declarando que por meio de livre e espontânea vontade sua e de seu filho ou filha, ele(a) estará participando como voluntário do projeto de pesquisa citado acima, de responsabilidade da Dra Patrícia Leal Dantas Lobo da Faculdade de Farmácia, Odontologia e Enfermagem, da Universidade Federal do Ceará. Comitê de Ética em Pesquisa (COMEPE/UFC- TELEFONE (85) 33668344,

Fortaleza, ____de_____de 20___.

Nome da criança_____Data de nascimento_____

_____RG:_____

Assinatura do pai ou responsável

_____ _____

Assinatura da Testemunha1 *Assinatura da Testemunha2*

_____

Assinatura do responsável pelo projeto

ANEXO II- PARECER DO COMITÊ DE ÉTICA

UNIVERSIDADE FEDERAL DO CEARÁ/ PROPESQ

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO CLÍNICA E MICROBIOLÓGICA DO USO DE VERNIZES CONTENDO PRODUTOS NATURAIS PARA PREVENÇÃO DE CÁRIE EM CRIANÇAS
Pesquisador: Patrícia Leal Dantas Lobo
Área Temática:
Versão: 2
CAAE: 06987312.3.0000.5054
Instituição Proponente: Departamento de Clínica Odontológica
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 195.096
Data da Relatoria: 28/11/2012

Apresentação do Projeto:
O projeto em questão é um trabalho vinculado ao PIBIC/UFC e será realizado nas dependências da UFC/Campus Fortaleza com a participação de professores dos Cursos de Odontologia do Campus de Sobral e Fortaleza.
Neste trabalho, serão aplicados vários agentes antimicrobianos formulados como vernizes de aplicação tópica nos dentes de crianças na idade de 36 a 71 meses. Os agentes aplicados serão: verniz de fluor a 5%, verniz de clorexidina a 1%, verniz com extrato alcoolico de própolis verde a 15%, verniz de timol a 10% e carvacol a 10%, verniz com óleo resina de copaíba e verniz com extrato glico-alcóolico de calêndula. A concentração dos vernizes de copaíba e calêndula será determinada em estudo antes do início dos testes entre os vernizes, através da análise da tolerabilidade e curva dose-resposta das concentrações de 1%, 5%, 10% e 20%.
Na fase de estudo clínico, os vernizes serão aplicados no início, após 3 e 6 meses e serão realizadas análises microbiológicas da saliva destes pacientes, de forma a verificar alterações na contagem de S.mutans. Serão feitos exames clínicos para determinar a ocorrência de cárie dentária nestas crianças.

Objetivo da Pesquisa:

Endereço: Rua Cel. Nunes de Melo, 1127
Bairro: Rodolfo Teófilo **CEP:** 60.430-270
UF: CE **Município:** FORTALEZA
Telefone: (85)3366-8344 **Fax:** (85)3223-2903 **E-mail:** comepe@ufc.br

ANEXO III- COMPROVANTE DE ACEITE- ARTIGO DO CAPÍTULO I

Gmail **Lídia Valadas <lidiavaladas@gmail.com>**

SUBMISSION | BMS-BIOT-2019-7

Recent Patents on Biotechnology BIOT <biot@benthamscience.net> 09 de junho de 2019 15:59

Para: lidiavaladas@gmail.com

Dear Dr. Valadas,

I hope you are doing well. With reference to your manuscript entitled as "Products of dental use containing copaiba oil-resin: technology prospecting based on patents" to Recent Patents on Biotechnology, I would like to inform you that the manuscript has now been accepted by the experts in the field for publication in Recent Patents on Biotechnology.

Sincerely,

Editorial Office
Recent Patents on Biotechnology
Bentham Science Publishers
biot@benthamscience.net

ANEXO IV - COMPROVANTE DE PUBLICAÇÃO- ARTIGO DO CAPÍTULO II

Saudi Pharmaceutical Journal 27 (2019) 363–367

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Original article

Dose-response evaluation of a copaiba-containing varnish against streptococcus mutans in vivo

Lídia Audrey Rocha Valadas[a,*], Mariana Fernandes Gurgel[b], Joelma Martins Mororó[b], Said Gonçalves da Cruz Fonseca[a], Cristiane Sá Roriz Fonteles[c], Cibele Barreto Mano de Carvalho[d], Francisco Vagnaldo Fechine[e], Edilson Martins Rodrigues Neto[a], Marta Maria de França Fonteles[a], Francineudo Oliveira Chagas[b], Patrícia Leal Dantas Lobo[a,b], Mary Anne Medeiros Bandeira[a]

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[c]Postgraduate Program in Dentistry, Department of Clinical Dentistry, School of Dentistry, Federal University of Ceará, Brazil
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ABSTRACT

Introduction: Dental caries is the most prevalent disease in humans and its incidence is particularly high during childhood. The use of medicinal plants is a common practice in Brazil.
Objective: To evaluate the optimal antimicrobial concentration of *Copaifera langsdorffii* (copaiba) oil-resin, in the form of dental varnish, against *Streptococcus mutans* (*S. mutans*) in children.
Methods: Twenty-four children, caries-free, aged until 6 years old, were selected to participate in this study. The varnish was applied to the occlusal surfaces of all deciduous molars. The antimicrobial activity was analyzed in saliva, whose collection was conducted in two phases: before applying the copaiba varnish and after use to verify the instantaneous effectiveness of *Copaifera langsdorffii* dental varnish in the reduction of *S. mutans*. The microbiological analysis was repeated twice, establishing dilutions of 1:10 mL and 1:100 mL.
Results: Comparisons between different times within the same dilution were carried out by repeated measures analysis of variance (ANOVA) associated with Tukey's multiple comparisons test. Comparisons of conditions prior to and after treatment were performed using the *t* test for paired samples and it indicated that the 1% formulation promoted a more significant decrease in the number of *S. mutans* colonies ($p = 0.0026$).
Conclusion: Copaiba oil-resin, in the form of dental varnish, has antimicrobial activity against *S. mutans* in all the concentrations studied. Further studies to identify the long-term activity and anticaries effect of this varnish are required to establish its use in caries prevention.

1. Introduction

Dental caries is currently considered a dysbiosis involving interactions between tooth structure, microbial biofilm and sugar exposure, where 35% of cases are not treated worldwide. The early childhood caries (ECC) is characterized by the presence of caries in children up to 6 years of age (Adams et al., 2017; Pitts et al., 2017).

When exposed to a high-sugar diet, diners in the streptococci group metabolize carbohydrates and produce acids that will demineralize the tooth structure. Among the agents that will participate in this process, *Streptococcus mutans* (*S. mutans*) is the primary pathogen, whose mechanism of virulence has been the most studied and known when compared to the other species (Pitts et al., 2017; Xiang et al., 2019).

Although it's not the etiological agent, an increase in the number of *S. mutans* can be considered a risk factor for the beginning of

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ANEXO V- COMPROVANTE DE SUBMISSÃO ARTIGO DO CAPÍTULO III

Gmail **Lídia Valadas <lidiavaladas@gmail.com>**

Submission Confirmation

Ingrid Maier" < <im_phytomedicine@t-online.de> 31 de maio de 2019 12:47
Para: lidiavaladas@gmail.com

Dear Lídia Audrey Rocha Valadas,

We have received your article "Clinical and microbiological evaluation of copaifera langsdorffii desf dental varnish in children: a randomized and double-blind study " for consideration for publication in Phytomedicine as Original Article

Your manuscript will be given a reference number once an editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

Ingrid Maier
Managing Editor
Phytomedicine

****
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ANEXO VI- COMPROVANTE DE DEPÓSITO DE PATENTE

Depósito de pedido nacional de Patente

(21) Nº do Pedido: BR 10 2016 021262 6
(22) Data do Depósito: 15/09/2016
(43) Data da Publicação: -
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(71) Nome do Depositante: UNIVERSIDADE FEDERAL DO CEARÁ (BR/CE)

Anuidades — Ver todas as anuidades

Tabela de Retribuição	3ª Anuidade ✓		4ª Anuidade ✗			
	Início	Fim	Início	Fim		
Ordinário	15/09/2018	15/12/2018	15/09/2019	15/12/2019		
Extraordinário	16/12/2018	15/06/2019	16/12/2019	15/06/2020		

Petições

Serviço	Pgo	Protocolo	Data	Imagens			Cliente	Delivery	Data
Serviços									
206	✓	870190017271	20/02/2019	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
206	✓	870180167817	26/12/2018	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
206	✓	870180151782	14/11/2018	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
203	✓	800180418532	02/10/2018	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
206	✓	870180131950	19/09/2018	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
206	✓	870180066661	01/08/2018	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
200	✓	013160000188	15/09/2016	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
Anuidade									
220	✓	800180418533	02/10/2018	-	-	-	UNIVERSIDADE FEDERAL DO CEARÁ		-
Outros									

Publicações

RPI	Data RPI	Despacho	Img	Complemento do Despacho
2515	**19/03/2019**	2.5	-	
2507	**22/01/2019**	2.5	-	
2500	**04/12/2018**	2.5	-	
2493	**16/10/2018**	2.5	-	
2485	**21/08/2018**	2.5	-	

APÊNDICE I- FICHA DE ANAMNESE

FICHA DE ANAMNESE

DADOS PESSOAIS E EXAME DENTÁRIO

NOME:_____

*IDADE_____ DATA DE NASCIMENTO*_____
NOME DO PAI_____

NOME DA MÃE_____

RESPONSÁVEL LEGAL_____

ENDEREÇO_____

TELEFONE PARA CONTATO_____

NOME DA ESCOLA_____

ENDEREÇO DA ESCOLA_____

ESTADO DE SAÚDE GERAL DA CRIANÇA

FAVOR LER E RESPONDER COM ATENÇÃO.

1) O seu filho ou filha se encontra sob tratamento médico? SIM NÃO

Especifique. Caso a sua resposta tenha sido SIM._____

2) O seu filho ou filha tem alguma doença crônica? SIM NÃO

Especifique. Caso a sua resposta tenha sido SIM. _____

3) O seu filho ou filha está tomando algum medicamento (remédio)? SIM NÃO

Especifique. Caso a sua resposta tenha sido SIM. _____

4) O seu filho ou filha tem algum tipo de doença alérgica? SIM NÃO

Especifique. Caso a sua resposta tenha sido SIM.
_____

5) O seu filho ou filha já apresentou alergia a algum tipo de medicamento? SIM NÃO

Identifique o(s) medicamento(s). Caso sua resposta tenha sido SIM.

_____

5) O seu filho ou filha já esteve hospitalizado (a)? SIM NÃO

Especifique o motivo. Caso a sua resposta tenha sido SIM.

_____

Afirmo que as informações acima são verdadeiras.

Data_____

Assinatura _____

RG: _____

Testemunha1: _____

Testemunha2:_____

Pesquisador:_____

EXAME DENTÁRIO

NOME DA CRIANÇA_____

IDADE_____ DATA DE NASCIMENTO_____

DATA_____

EXAME EXTRA-ORAL

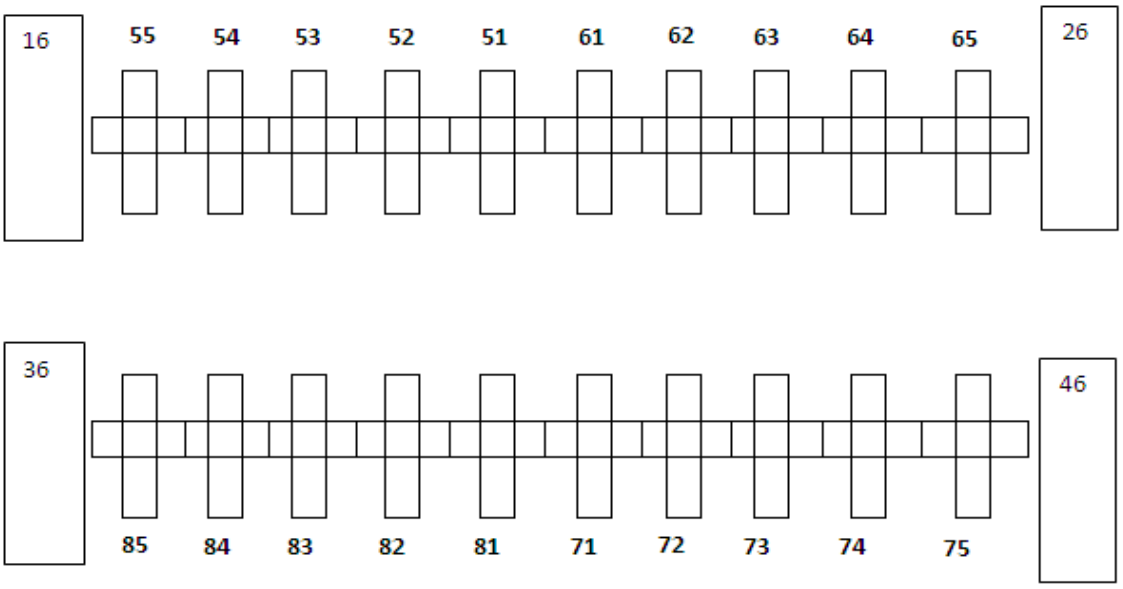
LINFADENOPATIA: PRESENTE ☐ AUSENTE ☐

ASSIMETRIA FACIAL POR INFECÇÃO: PRESENTE ☐ AUSENTE ☐

EXAME INTRA – ORAL

TECIDOS MOLES: NORMAIS ☐ PATOLÓGICOS ☐

EXAME INTRA-ORAL



COMENTÁRIOS:_____