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Atividades anti-*Candida* e análise da citotoxidade do extrato da folha da *Schinopsis brasiliensis* Engl.

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VANESSA DE CARVALHO JOVITO

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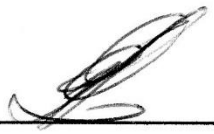

Prof. Dr. Lúcio Roberto Cançado Castellano

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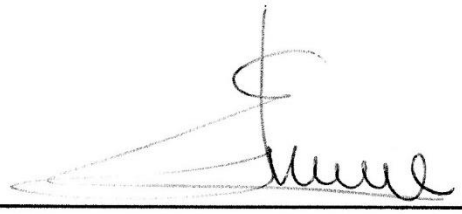

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DEDICATÓRIA

A Deus, meus pais, irmãs e minha família por todo apoio durante estes dois anos de esforços.

EPÍGRAFE

“Porque **nada no mundo é de graça**, Você pode até ter medo, mas ande, caminhe, e só não pare, não pare. NUNCA...” (ROSA de SARON)

RESUMO

Diante da necessidade de implementação de novos agentes terapêuticos para tratamento de infecções ocasionadas por *Candida* spp., a busca por produtos naturais cresce mundialmente. Frente a este cenário extratos obtidos a partir de *Schinopsis brasiliensis* Engl. representam potenciais fontes de agentes químicos com efeito antifúngico, considerando sua reconhecida atividade bacteriana. Objetivo: Avaliar o potencial antifúngico, anti-biofilme e citotóxico do extrato rotaevaporado da folha da *S. brasilienses* sobre *Candida* spp. Métodos: Foi determinada a Concentração Inibitória Mínima - CIM e Fungicida Mínima- CFM sobre 6 cepas de *Candida*, através da técnica da microdiluição em placas de 96 poços. Foi avaliada a cinética de inibição de crescimento da *C. albicans* ATCC 60193 em diferentes tempos nas concentrações de CIM, CIM x 2 e CIM X 4. A avaliação anti-biofilme foi determinada em placas de microdiluição com avaliação da inibição de formação e redução dos biofilmes maduros uniespécie ou multiespécie de 24 e 48 horas. Foi avaliado o sinergismo pelo método *checkerboard*. A citotoxidade foi realizada em células sanguíneas humanas. Os dados foram analisados através do teste T pareado, ANOVA e post hoc de Tukey, Mann- Wihitney, Kruskall Wallis com $p<0,05$ Resultados: Para a Concentração Inibitória Mínima o extrato da folha da *S. brasiliensis* apresentou atividade antifúngica frente a todas as espécies de *Candida* avaliadas. Houve redução na formação e nos biofilmes maduros uni e multiespécie para os períodos de 24 e 48h. Na cinética apenas a concentração CIM x 4 apresentou resultados, sendo o melhor resultado do extrato a partir de seis horas ($p<0,01$). Em todas as concentrações utilizadas no estudo até 1250 µg/ mL o extrato não apresentou citotoxidade em células humanas. Conclusão: O extrato da folha da *S. brasiliensis* possui atividade antifúngica sobre cepas do gênero *Candida*, apresentando, de modo geral, ação fungicida, especialmente após seis horas de contato com as células microbianas e efeito antibiofilme constituído por *Candida* spp. (uni e multiespécie), com habilidade para inibir a formação e reduzir o biofilme maduro, sem apresentar citotoxidade sobre células humanas de sangue periférico.

Palavras-chave: Plantas medicinais; *Candida*; Biofilme; Cinética microbiana.

ABSTRACT

Given the need to implement new therapeutic agents for the treatment of infections caused by *Candida* spp., the search for natural products grows worldwide. Against this background extracts obtained from *Schinopsis brasiliensis Engl*. represent potential sources of chemical agents with antifungal effect, considering their recognized bacterial activity. Objective: To evaluate the antifungal, anti-biofilm and cytotoxic potential of the rotavaporated extract of *S. brasilienses* leaf on *Candida* spp. Methods: Minimal Inhibitory Concentration - MIC and Minimum Fungicide - CFM were determined on 6 *Candida* strains by the microdilution technique in 96-well plates. The growth inhibition kinetics of *C. albicans* ATCC 60193 at different times in the MIC, MIC x 2 and MIC X 4 concentrations were evaluated. The anti-biofilm evaluation was determined in microdilution plates with evaluation of inhibition of formation and reduction of Biofilms ripe uniespecies or multispecies of 24 and 48 hours. The synergism was evaluated by the checkerboard method. Cytotoxicity was performed on human blood cells. The data were analyzed by Tukey, Mann-Wihitney, Kruskall Wallis, ANOVA and post-hoc paired T-test with $p < 0.05$. Results: For the Minimum Inhibitory Concentration the leaf extract of *S. brasiliensis* presented antifungal activity against all The *Candida* species evaluated. There was a reduction in formation and in the mature biofilms uni and multispecies for the periods of 24 and 48h. In the kinetics only the MIC concentration x 4 presented results, being the best result of the extract from six hours ($p < 0.01$). At all concentrations used in the study up to 1250 µg / mL the extract did not show cytotoxicity in human cells. Conclusion: The extract of the leaf of *S. brasiliensis* has antifungal activity on strains of the genus *Candida*, showing, in general, fungicidal action, especially after six hours of contact with the microbial cells and antibiofilm effect constituted by *Candida* spp. (Uni and multispecies), with ability to inhibit the formation and reduction of mature biofilm without cytotoxicity on peripheral blood mononuclear cells.

Keywords: Medicinal plants; *Candida*; Biofilm; Kinetics microbial .

LISTA DE ABREVIATURAS E SIGLAS

ATCC American Type Culture Collection

CBS CentralBureau voor Schimmelcultures

CIM- Concentração Inibitória Mínima

CFM- Concentração Fungicida Mínima

CSD- Caldo Sabouraud Dextrose

DMSO – Dimetilsulfóxido

TCT - 2,3,5-trifenil cloreto de tetrazólio

UFC- Unidade Formadora de Colônia

PBMC- Peripheral Blood Mononuclear Cells ou Células Mononucleares do Sangue Periférico

µg – microgramas

mL- mililitros

mg- miligramas

SUMÁRIO

1. INTRODUÇÃO

A microbiota oral é uma das mais diversificadas do organismo humano e tende a permanecer integra quando preservado o seu equilíbrio. Porém quebra da homeostase, pode ocasionar agressões inflamatórias e infecciosas ao hospedeiro (1-2).

Com este desequilíbrio alguns patógenos oportunistas podem expressar seus fatores de virulência e provocar danos aos tecidos. As espécies de *Candida* representam esses micro-organismos, especialmente por possuírem uma versatilidade, relacionada à capacidade de adaptação ambiental, muito grande, bem como exibirem habilidade para formação de biofilmes(3-4).

Por serem comensais, *Candida* spp são encontradas na microbiota anfibiôntica da cavidade bucal, mas tem sido bastante mencionada a presença desses patógenos nas superfícies de próteses dentárias e mucosa subjacente, ocasionado uma alteração neste tecido, caracterizada por hiperemia, edema, acompanhados algumas vezes por petéquias hemorrágicas, podendo a inflamação ser moderada ou intensa e raramente o processo é sintomático. Neste caso, quando a infecção fúngica está associada ao uso de prótese dentária, a mesma é denominada de estomatite protética (5-6).

A candidíase oral também pode se apresentar sob outras condições clínicas, acometendo regiões de tecido moles, especialmente em indivíduos imunocomprometidos, como em portadores da Síndrome da Imunodeficiência adquirida e pacientes que realizam terapia para o tratamento do câncer a partir da quimioterapia e radioterapia, com destaque para os tumores malignos da região de cabeça e pescoço (7).

As principais espécies do gênero *Candida* que acometem tanto indivíduos aparentemente sadios como imunocomprometidos são: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. guilliermondii*, *C. glabrata*, *C. kefyr*, *C. lusitaniae*, *C. viswanathii* e *C. famata* (8). Destas, destaca-se *C.albicans* pela expressiva capacidade de apresentar fatores de virulência relacionados ao aparecimento da doença (9-10), embora espécies de *Candida* não-*albicans*, como *C. tropicalis* e *C. krusei*, sejam reconhecidas pela capacidades de promover a doença (11).

Em relação aos fatores de virulência, *Candida* spp., de modo geral, expressam genes relacionados à formação de hifas e produção de proteinases e fosfolipases, que favorecem a invasão tecidual, adaptação ao estresse osmótico, plasticidade morfológica, formação de biofilmes, além de produção de mecanismos de resistências aos antifúngicos (bomba de efluxo, modificação do alvo molecular para o fármaco e produção de enzimas capazes de inativa-lo) (12-14).

Sobre a capacidade de formação de biofilmes orais, *Candida* spp. interagem entre si e com outros micro-organismos, a exemplo de bactérias cariogênicas e periodontopatogênicas, para sobreviver em uma diversidade ambiental, a exemplo das superfícies de tecidos moles e duros (esmalte dentário, materiais restauradores e protéticos), o que torna seu controle mais difícil (15-17).

Os métodos convencionais para o tratamento de candidíase, superficial ou sistêmica, são baseados na utilização de agentes antifúngicos, como: nistatina, anfotericina B, miconazol, cetoconazol, entre outros. Porém, são observados vários inconvenientes quando da utilização dos mesmos, representados por toxicidade, interações antagônicas fármaco-fármaco, falta de eficácia fungicida, alto custo e surgimento de espécies resistentes causado pela utilização frequente de alguns deles (18-19).

Além disso, apesar da introdução de novas drogas antifúngicas, elas ainda são em número limitado, evidenciando, portanto, uma grande demanda por novos agentes antifúngicos mais eficazes e menos tóxicos. Nesse contexto, desponta a utilização de metabólitos extraídos de plantas medicinais para este fim (18,19).

O uso de plantas medicinais para cura, tratamento ou prevenção das doenças é utilizado pela humanidade há milênios, e no Brasil, um país extremamente rico em sua biodiversidade, uma variedade de plantas ainda permanece inexplorada. Neste país, são encontrados 25% da flora mundial e, devido a isto, verifica-se uma vasta capacidade de desenvolvimento de novos produtos e medicamentos à base de plantas medicinais. A utilização popular e a troca das experiências com gerações anteriores impulsionam a realização de investigações científicas para comprovação dos efeitos relatados pela população (20-21).

Cerca de 80% da população de países em desenvolvimento se utilizam das plantas medicinais como forma de tratamento de suas enfermidades. Este fato amplia a perspectiva para o desenvolvimento de pesquisas com plantas com a

finalidade de propor alternativas que contribuam para qualificação e ampliação dos cuidados primários em saúde. O alto custo de medicamentos industrializados e a falta de acesso da população a serviços de saúde também impulsionam o crescente uso como métodos alternativos e complementares (22-23).

As plantas medicinais possuem em sua composição uma série de substâncias que são chamados de metabólitos secundários e estes são responsáveis em fornecer atividade biológica benéficas a saúde humana. Esses metabólitos são classificados em alcaloides, cumarinas, esteroides, flavonoides, glicosídeos cardioativos, lignanas, óleos essenciais, saponinas, terpenos, entre outros. Cada um destes grupos com características e ações específicas sobre os sistemas biológicos (24-25).

Muitas destas plantas, em forma de uma diversidade de tipos de extratos, tiveram comprovação de atividades biológicas, com aplicação em odontologia, a partir de investigações científicas. Destas destacam-se: *Rosmarinus officinalis* (Alecrim) - antimicrobiana*; Psidium guajava* Linn (Goiaba) -antimicrobiana; *Eugenia uniflora* L. (Pitanga) - antimicrobiana*; Punica grantum* Linn (Romã) - bactericida e bacteriostática, antifungico; *Syzygium jambolanum (*Jambolão*)* - antibacteriano*, Chinus terebinthifolius* Raddi (Aroeira*)*- antifúngico e antibacteriano*;* Própolis, e outras (26; 27; 28; 29, 30).

A *Schinopsis brasilienses*, objeto de interesse deste estudo, pertence à família Anacardiaceae, sendo encontrada em áreas tropicais de todos os continentes. No Brasil pode-se encontrar 13 gêneros e 80 espécies da *Schinopsis*, dentre elas a *S. brasilienses* conhecida popularmente por braúna, baraúna, braúna parda, braúna-sertão ou ainda na região de Mato Grosso chamada de chamacoco. É uma planta muito presente no nordeste do Brasil na região de caatinga (31; 32).

A árvore da *S. brasilienses* pode chegar a atingir 15 metros de altura e 60 cm de diâmetro (Figura 1). Possui um caule forte muito usado na indústria madeireira, suas cascas são ásperas, folhas são de coloração verde escura na parte superior e mais clara na inferior. As flores são pequenas de coloração branca e um pouco perfumadas. Os frutos são compostos por vagens em formato de foice e que possui apenas uma semente (33).

Como mencionado anteriormente *S. brasiliensis* por possuir um caule muito forte e resistente a decomposição é muito usada pela população na fabricação de móveis e objetos da indústria madeireira. Porém, também suas cascas, folhas e

frutos são utilizados pela medicina popular como tratamento de gripe, processos inflamatórios, febre, tosse, diarreia, fraturas e infecções (34; 35).

Através da cromatografia líquida realizada com o extrato da *S. brasiliensis* para identificação de seus componentes químicos, foi encontrado que o ácido gálico (ácido 3,4,5- tri-hidroxibenzóico), é o componente majoritário presente na planta. E este caracteriza- se por demonstrar atividade antioxidante, anti-inflamatória e antibacteriana (36).

Figura 1: *Schinopsis brasilienses* Engl.

Fonte: http://belezadacaatinga.blogspot.com.br Acesso em:10 de outubro de 2016

Na medicina popular o uso da *S. brasiliensis* pela população ocorre em maior parte na forma de chá das folhas e da casca. Algumas pequisas científicas comprovaram atividade antibacteriana frente a *Staphylococcus aureus* multirresistente, *Enterococcus faecalis, Pseudomonas aeruginosa*, bem como sobre bactérias cariogênicas, como *Streptococcus mutans*, *S. oralis*, *S. mitis* e *S. salivarius* (34; 37; 38;39).

Estes achados impulsionaram a realização desta pesquisa, que apresenta como hipótese alternativa (H_0) a existência do efeito antifúngico do extrato obtido a partir das folhas de *S. brasiliensis* Engl. sobre espécies de *Candida* potencialmente ativas em infecções fúngicas da cavidade bucal e avaliar o potencial citotóxico do extrato as células mononucleares do sangue periférico.

2. CAPÍTULO 1

O manuscrito a seguir será submetido para publicação no periódico ¨ Archives of oral biology ” qualis A2 – Odontologia

Títle: Anti-*Candida* activities and cytotoxicity analysis of leaf extract from *Schinopsis brasiliensis* Engl.

1- Introduction:

Brazil is a country with a huge diversity of plants, and these because they have biological effects on the health of the population has aroused interest in science. Approximately 80% of developing countries use some alternative means to overcome their pathologies (Calixto, 2003; Bochner, 2012).

Medicinal plants have been an easily accessible and low cost alternative that has been gaining ground amid conventional treatment methods in the market that besides the high cost have undesirable effects such as toxicity, microbial resistance and antagonistic interaction with other drugs (Ribeiro et al, 2014; Yamada et al, 2016)

The extracts of medicinal plants are composed of metabolites that provide therapeutic effects to health. *Schinopsis brasiliensis*, belonging to the family Anacardiaceae, is a plant very common in all continents and used by popular medicine as treatment of influenza, inflammatory processes, fever, cough, diarrhea, fractures and infections (Saraiva, 2013).

Fernandes et al. (2015) identified gallic acid (3,4,5-trihydroxybenzoic acid) as the main chemical marker present in *Schinopsis brasiliensis* through liquid chromatography. An important polyphenol present in plants and with antioxidant, anti-inflammatory and antimicrobial properties (Corradini et al., 2011). Through the liquid chromatography of the *S. brasiliensis* extract the polyphenols present were quite high when compared to flavonoids also present in the plant (Fernandes et al, 2015).

Candida spp is an important opportunistic pathogen commonly found in immunocompromised patients and is responsible for high rates of morbidity and mortality and an increase in the cost of health care (Navarathna et al, 2016).

In view of the need to implement new therapeutic agents for the treatment of infections caused by *Candida* spp., Which has versatility related to the ability to adapt very environmentally, as well as exhibit the ability to form biofilms and knowing that extracts obtained from *Schinopsis brasiliensis* are potential sources of chemical agents with an antifungal effect (Seneviratne and Samaranayake, 2008, Sesma and Morimoto, 2011, Figueiral et al, 2007, De Rossi et al., 2011). The objective of this work was to evaluate the anti-*Candida* potential and to analyze the cytotoxicity of the leaf-rotavaporated extract of *Schinopsis brasilienses* Engl.

2- Materials e Methods:

2.1 Location of the experiments:

The antifungal, anti-biofilm and kinetics assays were performed at the Oral Microbiology Laboratory of the Nucleus of Tropical Medicine - NUMETROP and at the Laboratory of Microbiology of the Technical School of Health - LABIMIC. The evaluation of the cytotoxicity was carried out in the Laboratory of Culture and Cell Analysis - LACEC. All located in the Health Sciences Center / Federal University of Paraíba.

2.2 Plant Extract

The leaves of *Schinopsis brasiliensis* Engl. were collected in the region of Campina Grande, Paraíba, Brazil, (7 ° 13 50 S, 35 ° 52 52 W), respecting the time and period of ideal collection, a sample was prepared and deposited in the herbarium Professor Jayme Coelho de Morais (Herbarium Code EAN) of the Federal University of Paraíba under the number EAN-14049. The plant material was dried in an oven with air circulation at 40 ± 1 ° C and ground in a mill having a particle size of 10 mesh. The powder of the ground plant (100 g) was extracted exhaustively

with 96% ethanol by percolation, and subsequently the concentration was conducted on a rotary evaporator (Chaves et al, 2015).

2.3 Microorganisms Used:

Candida strains from the American Type Culture Collection (ATCC) - *Candida albicans* ATCC 60193; *Candida krusei* ATCC 34135; *Candida tropicalis* ATCC 750; from the Dutch Central Bureau voor Schimmelcultures (CBS) - *Candida albicans* CBS 562, *Candida tropicalis* CBS 94,of the Zimotécnico Institute, Banco de Luiz de Queiroz "of Unicamp- Campinas-SP, Brazil - *Candida glabrata* IZ 07 and Clinical strain of *Candida albicans* (CAM) isolated from the oral cavity and provided by the Clinical Mycology Laboratory of the Department of Pharmaceutical Sciences of the UFPB .

2.4 Determination of Mínimum Inhibitory Concentration (MIC)

The MIC was determined by the microdilution technique in Sabouraud Dextrose broth (CSD, KASVI, Curitiba, Brazil), according to the protocol proposed by CLSI (2002), where the antifungal activity of the extract and gallic acid produced at an initial concentration of 2000 µg / mL, compared to fungal strains of the genus *Candida*.

For this purpose, 96-well microtiter plates (Cellstar®) were used and 100 µL of CSD were initially inserted into each well. Next, 100 µL of the solution of the substance was inserted into the first well of each column and serially diluted by withdrawing a 100 µL aliquot from the more concentrated well into the successor well. At the end of each column, the last aliquot was discarded.

Then, 100 µL of the inoculum of the fungal strain, prepared according to CLSI (2002) standards in CSD with final concentration equivalent to 2.5 x 103 CFU / mL, were inserted into each well. The plates were incubated in an oven for 24 h at 37 ° C and then visual readout of the results from observation of the formation of fungal cell agglomerates at the bottom of the wells.

To confirm the presence of viable microorganisms, 50 µL of the 2,3,5-triphenyl dye tetrazolium chloride (TCT) dye at 1g / 100mL concentration was inserted into each well of the plate and incubated again in an oven for 24h. In this

sense, in the second reading, the presence of viable microorganisms was considered when the wells were stained red. The MIC was defined as the lowest concentration of the substance capable of visibly inhibiting fungal growth.

The assays were performed in triplicate and the MIC calculated by the fashion of the results obtained. The positive control used for this test was nystatin (Sigma-Aldrish, São Paulo, Brazil) at an initial concentration of 12 µg / mL. Strain viability control, culture medium sterility control and dimethylsulfoxide control (DMSO), used to prepare the product solution, were carried out simultaneously with the assay.

2.5 Determination of Minimum Fungicide Concentration (CFM)

From the results obtained in the MIC assay, 50 µL of MIC contents and the two most concentrated concentrations (CIMx2 and CIMx4) were seeded in Petri dishes containing Sabouraud Dextrose agar (ASD, KASVI, Curitiba, Brazil). Plates were incubated in an oven at 37 ° C for 24h. Visual reading was performed by observing fungal growth in the culture medium. CFM was considered the lowest concentration capable of inhibiting the visible growth of the subculture (CLSI, 2002).

The CFM / MIC ratio was calculated to determine if the substance had a fungistatic activity (CFM / MIC ≥ 4) or fungicide (CFM / MIC <4) (Siddiqui et al, 2013).

According to studies by Souza, 2015, Fernandes et al, 2015, the major chemical component of the leaf extract of *Schinopsis brasiliensis* Engl. Is the gallic acid that has great antioxidant and antimicrobial potential (Rúa et al, 2011), then the CIM against some strains of *C.Krusei* ATCC 34135; *C. glabrata* IZ07; *C. Tropicalis* ATCC 750 and *C. albicans* ATCC 60193 performed in triplicate at a concentration of 2000 µg / ml.

2.6 Evaluation of kinetics of inhibition of fungal growth

The study of the interference of the extract in the growth and multiplication of fungal cells of *C. albicans* ATCC 60193 was carried out by the counting of colony forming units (CFU), based on previous studies (Castro et al., 2013; Leite et al., 2014). The evaluation times defined for this test correspond to T0 (initial); T1 (1

hour after onset), T2 (2 hours); T6 (6 hours); T8 (8 hours); T12 (12 hours) and T24 (24 h after the start of the assay).

The assay was performed in a 96-well plate, using the same protocol proposed in the microdilution technique (CLSI, 2002), using the extract concentrations for MIC, MIC x 2 and MIC x 4. Nystatin was the positive control used. In parallel, growth control of the tested strain and control of sterility of the culture medium was performed.

For evaluation of fungal growth inhibition kinetics, 10 µl of the well contents after homogenization were seeded into Petri dishes containing ASD, at the predefined time intervals, and incubated at 37 ° C for 24 h for Subsequent UFC count. After the incubation, the count and the values transformed into log 10 CFU / mL were performed and presented in the form of death curve of the fungal cells.

2.7 Evaluation of the antifungal activity of the extract on the biofilm

The assays evaluated the anti-biofilm activity of the products at three different times: inhibition of biofilm formation and reduction of mature *Candida* biofilm, with extract action for a period of 24 and 48 hours.

The tests were performed on the biofilms of *C. albicans* ATCC 60193 and *C. tropicalis* ATCC 750 and biofilm multispecies (*C. albicans* + *C. tropicalis*).

The assays were performed in triplicate. Nystatin was the commercial antifungal used as a control in all groups. In parallel, growth control of the tested strain and control of sterility of the culture medium was performed.

2.7.1 Evaluation of the anti-biofilm activity of the extract in the formation of fungal cells

In a flat bottom 96-well microtiter plate containing 100 µl of Gibco® RPMI 1640 (USA), 100 µl of the inoculum prepared in RPMI plus 2% sucrose containing 2.5 x 10^5 CFU / ml were transferred to each well of the plate with the aid of a pipettor. Then, 100 µL of the extract, in different concentrations, were added to the

corresponding wells. The plate was incubated for 48 h at 37 ° C, allowing the yeast to remain adhered to the bottom of the wells.

To perform the reading and quantification of the biofilm formed, after the incubation time, the wells were washed twice with 200 µl of PBS and air dried for 45 min. In each well was added 100 µL of 0.4% aqueous crystal violet solution, which remained in contact with the biofilm for 45 min. After incorporation of the dye, the wells were washed three times with 200 µL of sterilized distilled water and immediately bleached with 200 µL of 95% ethanol. After 45 minutes of the latter procedure, 100 µL of the bleached solution were transferred to a well of a new plate and the amount of the violet crystal measured at 600 nm in an absorbance reader (GloMax-Multi, Promega -USA) (adapted from FURLETTI et al, 2011).

The absorbance values obtained in the wells of the tested concentrations and the growth control were used to calculate the percentage of inhibition (% inhibition) of the biofilm formation due to the action of the substance.

2.7.2 Evaluation of the anti-biofilm activity of the extract in the reduction of the mature biofilm of *Candida*

In a 96 well flat bottom microtiter plate containing 100 µl RPMI, 100 µl of the RPMI-prepared inoculum plus 2% sucrose containing 2.5 x 10^5 CFU / ml were transferred to each well of the plate with the aid of a pipettor, and incubated in an oven at 37 ° C for 48 hours to form the mature biofilm.

After this period, the biofilm reduction assays were started, with the substance being inserted at different concentrations. Initially, the culture medium was aspirated from the wells to remove planktonic cells. They were washed twice with 200 µl of PBS. After washing, 100 µL of the RPMI medium was transferred to each well.

Then, 100 µL of the extract, at the concentrations tested, were added to the wells, staining in contact with the biofilm for a period of 24 and 48 hours

Controls and quantification procedures of the formed biofilm were performed in the same manner as described in the previous assay.

2.8 Synergism evaluation - *Checkerboard* method

The combined effect of the two substances (nystatin with leaf extract of *S. brasiliensis*) and (nystatin with Galic acid) was determined from the microdilution - *checkerboard* technique for derivation of the fractional inhibitory concentration index (CIF Index).

The turbidity of the fungal suspensions was adjusted in a spectrophotometer at a concentration of 10^5 colony forming units / mL (CFU / mL). Solutions of the tested products were used in concentrations determined from their respective MICs. Initially, 100 µL of the Saboraud dextrose culture medium was added to the wells of the 96-well, U-bottomed (Cellstar®) 96-well microplate. Then, 50 µL of each product tested at various concentrations (MIC-8, CIM ÷ 4, MIC ÷ 2, MIC, MIC x 2, MIC x 4 and MIC x 8) were added vertically (nystatin) and horizontal (extract) of the microplate. Finally, the culture medium was inoculated with 10 µL of the fungal suspension. Fungal growth was evidenced by the use of the TCT dye. The assay was performed in triplicate, and the microplates were incubated at 37 ° C for 48 hours (ELIOPOULOS; MOELLERING, 1991; DUTTA et al., 2004; CASTRO, 2010).

The FIC index was calculated by adding $FIC^A + FIC^B$, where A represents the extract and B to nystatin. The FIC^A, in turn, calculated through the combined CIM^A / CIM^A ratio alone, while the FIC^B = combined CIM^B / CIM^B alone. This index was interpreted as follows: synergism (<0.5), additivity (0.5-1.0), indifference (> 1 and <4) or antagonism (> 4.0).

2.9 Evaluation of the cytotoxicity of the extract on peripheral blood mononuclear cells

Three (03) healthy volunteers, older than 18 years, with no history of infectious or metabolic disease, without the use of immunomodulatory medication were invited to sign the Free and Informed Consent Form for blood donation, in accordance with resolution 466/2012, With respect to ethical issues in human research.

2.9.1 Peripheral blood collection

One 12mL tube of peripheral blood was collected by vacuum venous puncture with sterile heparinized plastic bottles of the Vacutainer (Becton Dickinson) line.

2.9.2 Isolation and stimulation of peripheral blood mononuclear cells (PBMC) and cytotoxicity

Heparinized whole blood was processed by Ficoll-Paqueâ "¢ 1077 density gradient centrifugation (GE Healthcare, USA). Peripheral blood mononuclear cells (PBMC) were collected and washed three times with phosphate buffer. Viability was determined by exclusion of Trypan blue (Sigma-Aldrich, St. Louis, USA) in Neubauer chamber. Cells were resuspended in aliquots of 2x10 6 PBMC / ml in RPMI 1640 (Gibco, Life Technologies, UK) supplemented with 5 µg / ml PHA (Sigma-Aldrich, St. Louis, USA) and 10% fetal bovine serum Inactivated (Gibco, USA) second (Kutscher et al, 2013).

2.9.3 Cell viability assay of PBMC against the extract

The cell viability assay was based on 100 µl of 1: 1 PBMC suspension incubated with the leaf extract of *S. brasiliensis* at concentrations up to 1250 µg / mL (Sigma Aldrich, USA) and serial suspensions in culture medium in 96-well black polystyrene microplates (Greiner Bio-One, USA) for 24 h, 37 ° C, humidified atmosphere and 5% CO2. After the period, 20µl of resazurin solution was added according to the alamarBlue® protocol by (Bio-Rad, Hercules, USA). The plate was read on microplate fluorescence reader using specific filters for the desired wavelength (GloMax multi, Promega, USA). The viability percentage was calculated as follows:

% Cytotoxicity = [(FI 590 from treated samples / FI 590 from untreated cells) (100)]

Where: FI 590 = fluorescence intensity at 590 nm emission (560 nm excitation). The lethal concentration 50 (LC50) was determined by log plot plotted with percentage of untreated control for each dilution of the leaf extract of *S. brasiliensis*.

2.10 Análise Estatística:

The data were analyzed by paired T test, ANOVA and post hoc by Tukey, Mann-Whitney and Kruskal-Wallis with p <0.05. The Statistical Package for Social Sciences (SPSS) version 2.1 (IBM Corp, New York, USA). (A = 0.05).

2.11 Considerações éticas:

The project was approved by the Committee of Ethics in Research with Human Beings of CCS / UFPB.

3 Results:

3.1 Minimum Inhibitory Concentration - MIC and Minimum Fungicide Concentration - CFM:

It was observed that the extract obtained from the leaves of S. brasiliensis showed antifungal effect on all the strains tested, with MIC and MFC values varying between 31.25 and 250 µg / mL (Table 1), being the strains of *C.albicans* (ATCC 60193 and CBS 562), with the highest MIC and MFC values between 0.375 and 3 µg / mL, with higher resistance of C. krusei ATCC 34135.

Table 1: MIC and MFC of leaf extract of *S. brasiliensis* versus *Candida* spp.

Substances	***S. brasiliensis***			***Nystatin***		
Strains	**MIC µg/mL**	**MFC µg/mL**	**MFC/MIC µg/mL**	**MIC µg/mL**	**MFC µg/mL**	**MFC/MIC µg/mL**
C,albicans ATCC 60193	31,25	125	4 (Fungistatic)	0,375	0,375	1 (Fungicide)
C. krusei ATCC 34135	125	125	1 (Fungicide)	3	3	1 (Fungicide)
C tropicalis ATCC 750	250	250	1 (Fungicide)	1,5	1,5	1 (Fungicide)
C albicans CBS 562	31,25	31,25	1 (Fungicide)	0,375	0,75	2 (Fungicide)
C tropicalis CBS 94	250	250	1 (Fungicide)	1,5	1,5	1 (Fungicide)
C. albicans CAM	250	250	1 (Fungicide)	0,375	0,75	2 (Fungicide)

Following the proposed by SIDDIQUI et al (2013) the MFC / MIC ratio for leaf extract of *S. brasiliensis*. Showed fungicidal activity for all *Candida* species analyzed, except for *C. albicans* ATCC 60193 which showed to be fungistatic.

Gallic acid with high antioxidant and antimicrobial potential (9), does not present antifungal activity when analyzed the same concentration of *Schinopsis brasiliensis* extract that was 2000µg / mL, but the antifungal activity can be attributed to another chemical component present in its composition (Table 2).

Table 2: MIC of gallic acid against *Candida* spp.

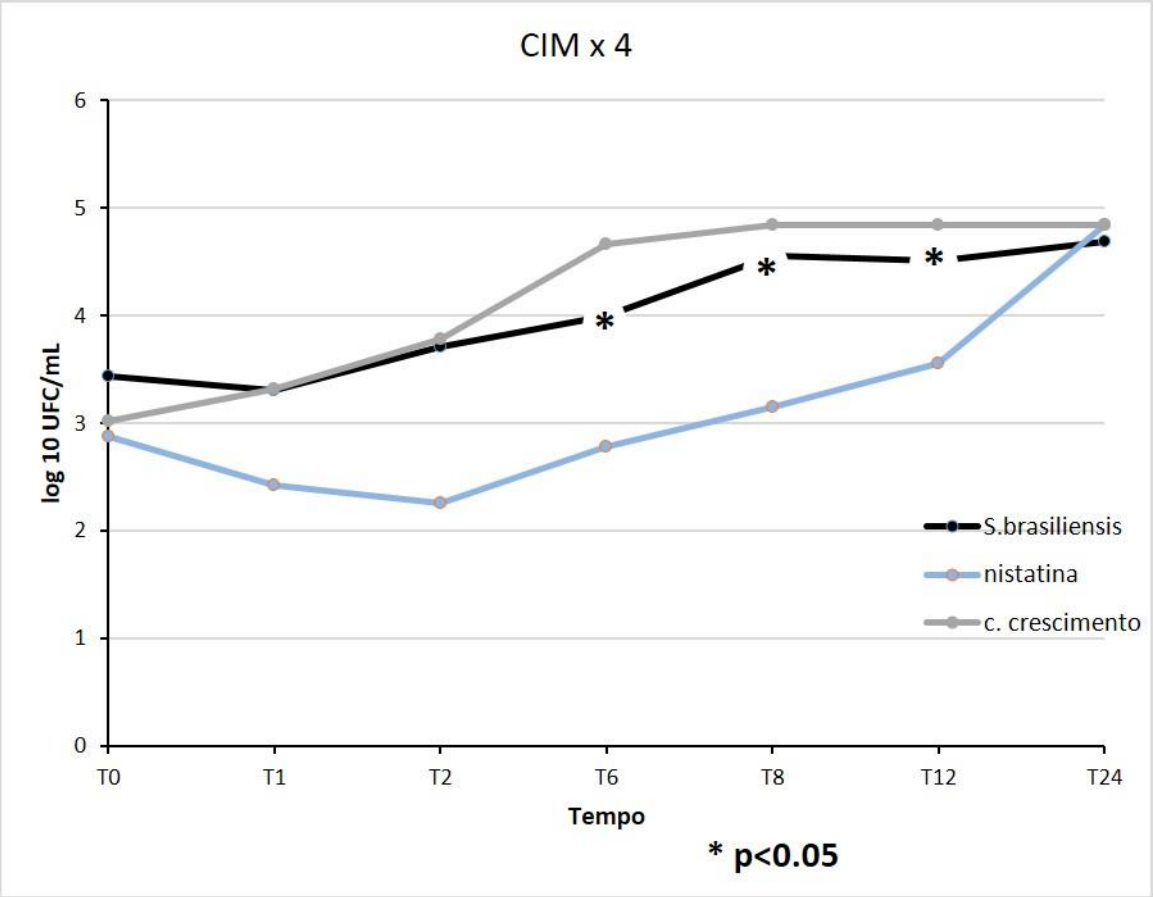
Cepas	**CIM**
C.Krusei ATCC 34135	+
C. Tropicalis ATCC 750	+
C. albicans ATCC 60193	2000µg/ml
C. glabrata IZ 07	2000µg/ml

+ Fungal growth

3.2 Effect of the *S. brasiliensis* extract on the growth kinetics of *C. albicans*

In the concentrations related to CIM and CIMx2, the *S. brasiliensis* extract did not significantly reduce the number of *C. albicans* ATCC 60193 colony forming units at the times evaluated when compared to the growth control. However, at the concentration of 125 µg / mL, the extract evaluated significantly reduced growth after 6 hours of incubation ($p < 0.05$). Nystatin significantly reduced fungal growth ($p < 0.05$) when purchased at growth control from the first hour of incubation, and this effect was prolonged up to 12 hours (Figure 1).

Figure 1: Kinetics test showing the behavior of the extract *S. brasiliensis*, Nystatin and growth control in the CIMx4 concentration, during T0, T1, T2, T6, T8, T12 and T24 times.



3.3 Evaluation of the anti-biofilm activity of the leaf extract of *S. brasiliensis* Engl.

For the biofilm of *C. albicans* ATCC 60193, there was a statistically significant reduction of biofilm between the groups of *S. brasiliensis* extract and nystatin with ($p < 0.05$, Mann-Whitney Test). Among the concentrations, there were statistically significant differences ($p < 0.05$, Kruskal-Wallis test) for CIMx8 (250 µg / mL) of the extract in groups G1 and G3, and for CIMx4 (125 µg / mL) and CIMx8 (250 µg / mL) for the G2 group.

The biofilm of *C.tropicalis* ATCC 750 showed a statistically significant reduction between extract and nystatin in groups G1 and G2 with $p < 0.05$. The concentration of CIMx2 (500µg / mL) and CIMx4 (1000µg / mL) at $p < 0.05$ was observed in the concentrations of CIMx8 (2000µg / mL) in G1 and G2.

In the multispecies biofilm the difference of reduction was observed between the extract and the nystatin in G1 and G3, and between the extracts concentrations there was a difference in the CIMx8 of G1 and G3, both with $p < 0,05$.

Table 3: Effect of leaf extract of *S. brasiliensis* Engl. On the inhibition of biofilm formation - G1 and reduction of biofilm mature 24h in contact with extract - G2 and 48hrs in contact with extract - G3 of the mature biofilm of *Candida albicans* ATCC 60193. Values expressed in percentage (%).

Concentration	G1				G2				G3			
	S. brasiliensis		Nystatin		*S. brasiliensis*		Nystatin		*S. brasiliensis*		Nystatin	
CIM	42%	Aa	Sem inibição	Ba	76%	Aa	Sem inibição	Ba	55%	Aa	10%	Ba
2 CIM	73%	Aa	Sem inibição	Ba	86%	Aa	Sem inibição	Ba	68%	Aa	35%	Ba
4 CIM	63%	Aa	Sem inibição	Ba	100%	Ab	6%	Ba	85%	Aa	41%	Bb
8 CIM	100%	Ab	Sem inibição	Ba	100%	Ab	Sem inibição	Ba	100%	Ab	24%	Ba

Different upper-case letters in the lines represent statistically significant differences (Mann-Whitney Test, $p < 0.05$) between substances (Extract of leaf of *S. brasiliensis* and nystatin) in each group and at the same concentration; Different lowercase letters in each column represent statistically significant differences (Kruskal-Wallis test, $p < 0.05$) between different concentrations of the same substance.

Table 4: Effect of leaf extract of *S. brasiliensis* Engl. On the inhibition of biofilm formation - G1 and reduction of biofilm mature 24h in contact with extract - G2 and 48hrs in contact with the G3 extract of the mature biofilm of *Candida tropicalis* ATCC 750. Values expressed in percentage (%).

Concentration	G1				G2				G3			
	S. brasiliensis		Nystatin		*S. brasiliensis*		Nystatin		*S. brasiliensis*		Nystatin	
CIM	Sem inibição	Aa	47%	Ba	64%	Aa	21,22%	Aa	78%	Aa	2%	Ba
2 CIM	Sem inibição	Aa	34%	Ba	66%	Aa	32,84%	Aa	89%	Aa	23%	Bb
4 CIM	23%	Aa	40%	Ba	80%	Aa	21,69%	Aa	82%	Aa	21%	Bb
8 CIM	100%	Ab	44%	Ba	84,59%	Ab	22,09%	Aa	99%	Ab	17%	Bb

Table 5: Effect of leaf extract of *S. brasiliensis* Engl. On the inhibition of biofilm formation - G1 and reduction of biofilm 24h in contact with extract - G2 and 48hrs in contact with the G3 extract of the mature multispecies biofilm (*Candida albicans* ATCC 60193 + *Candida tropicalis* ATCC 750). Values expressed as a percentage (%)

Concentration	G1				G2				G3			
	S. brasiliensis		Nystatin		*S. brasiliensis*		Nystatin		*S. brasiliensis*		Nystatin	
CIM	63%	Aa	38%	Ba	39%	Aa	79%	Ba	76%	Aa	3%	Ba
2 CIM	73%	Aa	14%	Bb	47%	Aa	76%	Aa	88%	Aa	13%	Bb
4 CIM	89%	Aa	22%	Bb	56%	Aa	81%	Bb	92%	Aa	27%	Bb
8 CIM	100%	Ab	26%	Bb	72%	Aa	69%	Aa	96%	Ab	25%	Bb

3.4 Synergism evaluation - *Checkerboard* method

The FIC index was calculated by adding FIC^A + FIC^B, where A represents the extract and gallic acid, and B to nystatin. The FIC^A, in turn, calculated through the combined CIM^A / CIM^A ratio alone, while the FICB = combined CIM^B / CIM^B alone. The FIC of the *S. brasiliensis* extract in association with nystatin was the same as the FIC of gallic acid in combination with nystatin = 1.125µg / mL (Interpreted as indifferent, according to the methodology adopted).

3.5 Cytotoxicity in human peripheral blood mononuclear cells

In the interesting analysis of cytotoxicity, we observed induction of cell proliferation by the extract of the leaves of *S. brasiliensis* tested in the concentrations of interest of our study, when compared to the percentage of cellular metabolism induced by the control treatment of the cytotoxicity test. Significant differences in cell viability between the concentrations of the *S. brasiliensis* extract were determined by the one-way ANOVA with Tukey post hoc (α = 0.05) using the Statistical Package for Social Sciences (SPSS) version 2.1 (IBM Corp., New York, USA). (A = 0.05)

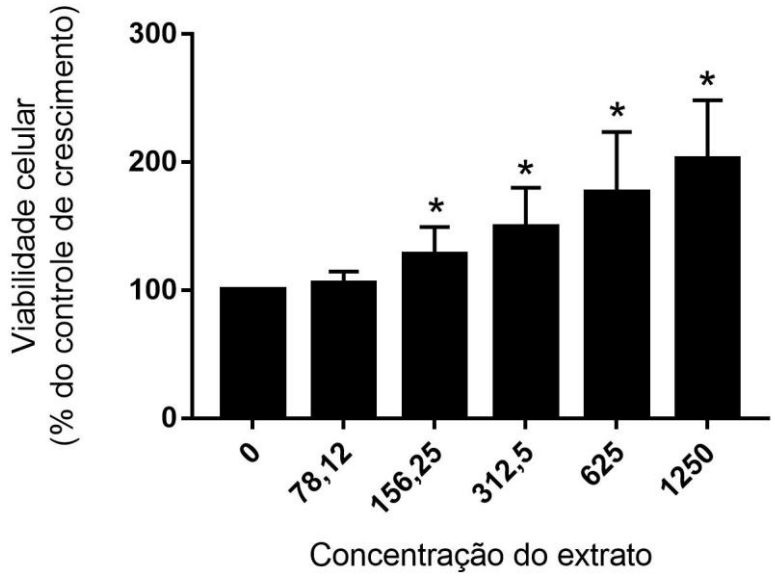


Figure 2: The viability of PBMC cells after treatment with increasing concentrations in µg / mL of leaf extract from *Schinopsis brasiliensis* Engl. Expressed as a percentage. Results shown as mean values ± standard deviation of a triplicate experiment (* P <0.05 compared to growth control).

4 Discussion:

Candida spp, through its versatility in adapting to surfaces has been a challenge to the scientific community to maintain its balance, and prevent greater consequences to the health of the population, thus medicinal plants such *as S. brasiliensis* has shown antifungal potential Through studies.

The antimicrobial activity of the leaf of *Schinopsis brasiliensis* Engl. was analyzed by Chaves et al (2011) against *Staphylococcus aureus* (29213), *Pseudomonas aeruginosa* (27563), *Escherichia coli* (25922), *Klebsiella pneumoniae* (13883) and *Candida albicans* (10231) strains showing antifungal activity in all strains. In this study the data corroborate with Chaves et al. (2011), where we also found activity in all strains of *Candida* spp analyzed, being the effect on *C. albicans* that presented better results with MIC of 31.25 µg / mL.

SILVA et al, 2012 evaluated the antifungal and antibacterial activity of *S. brasiliensis* extract in *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus mutans* (ATCC 25175), *S. salivarius* (ATCC 7073), *S. oralis* (ATCC 10557), *Lactobacillus casei* (ATCC 7469), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 18804), *C. tropicalis* (ATCC 13803), *C. krusei* (ATCC 34135). The best result observed in this study was the *S. aureus*. *Candida albicans* (ATCC 18804), *C. tropicalis* (ATCC 13803), and *C. krusei* (ATCC 34135) had no antifungal activity at any of the concentrations used against the *Candida* strains evaluated by Silva et al. *S. brasiliensis*. However, the CIM technique was performed through solid media dilution, a differential in relation to our study, where we used the liquid microdilution technique.

Santos et al., 2013 evaluated the antimicrobial activity of the nebulized extract of *S. brasiliensis* leaves at a concentration of 50 to 500 mg / mL in agar dilutions against *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, *S. oralis* and *S. parasanguis*. zone of inhibition in all microorganisms tested.

When it comes to plant extracts, when they exhibit MIC values between 100 and 500 µg / mL, they are considered promising for possible clinical use (Alves et al, 2000). In this study, the MIC values observed reinforce the potentiality of the leaf extract of *Schinopsis brasiliensis* Engl. for treatment of infections caused by *Candida* spp

According to Fernandes et al., (2015) the major component present in the *S. brasiliensis* extract is gallic acid, a phenolic compound very present in plants and demonstrating antioxidant, anti-inflammatory and antimicrobial activity. In this study, gallic acid tested at a concentration equivalent to the extract of *S. brasiliensis* did not obtain antifungal activity, but can be explained by the presence of secondary metábolites present in plants such as rutin, gallic acid, quercetin and caffeic acid at low concentrations acting synergistically potentiating The antimicrobial activity, or even that this activity found in the present study may be related to the presence of other active substances such as flavonoids and tannins present in the extract. (Rúa et al, 2011).

In view of the antifungal activity of the *S. brasiliensis* extract found in this study, and knowing that the dental biofilm is defined as a complex community of

microorganisms, surrounded by an extracellular matrix of polysaccharides, adhered to one another on a surface or interface, and that most of the persistent infections associated with this biofilm are mainly caused by fungi of the *Candida* genus (Sardi et al, 2014), the formation and reduction of uni and multispecies biofilms of *Candida* spp.

For the biofilm technique RPMI 1640 was chosen as the culture medium, since it is rich in nutrients and known to simulate the composition of human fluids (Kucharíková et al, 2011). In addition, according to studies conducted by Weerasekera et al., 2016, this medium was considered the best for facilitating adhesion and biofilm formation for strains of *C. albicans* and *C. tropicalis*.

Other studies with extracts and essential oils from herbal anti-biofilms have been reported in the literature (Peixoto et al, 2017, Rane et al, 2013), but this is the first to make use of this plant against biofilms of opportunistic pathogens. *Candida* spp have important characteristics in the adhesion to this biofilm, which serves as protective deposits of the microorganisms, which makes them more resistant to the activity of antimicrobial agents (Sardi et al, 2013). Therefore, the interruption of the formation and maturation of this biofilm in the early stages is so important (Rane et al, 2013). Due to this, we analyzed the anti-biofilm activity of the leaf extract of *Schinopsis brasiliensis*, which showed a statistically significant reduction between the inhibition groups of biofilm formation and reduction of mature biofilm when compared to nystatin at the same concentrations.

Nystatin was the anti-fungal chosen because it is the product most commonly used by the population in the treatment of oral candidiasis. (Williams and Lewis, 2011). Some studies state that the antibiofilm concentration that becomes effective to be used for antimicrobial agents would be 10 to 1000 times higher than MIC in conventional liquid media in planktonic cells (Rochelli et al, 2016; Et al, 2014).

The behavior of *S. brasiliensis* extract against *C. albicans* ATCC 60193 during a 24 hour period and at the concentrations of 31.25 µg / mL and 62.5 µg / mL (MIC and MIC x 2, Respectively), no death of *C. albicans* ATCC 60193 was found. However, at the concentration of 125 µg / mL, the extract showed to be significantly different from the growth control after 6 hours of contact with the substance. No study was found in the literature that evaluated the kinetics of the leaf extract of *S. brasiliensis*, emphasizing, once again, the innovative character of this work.

Due to the antifungal potential of the *S. brasiliensis* extract, we decided to analyze the synergistic / antagonistic interaction of the extract and the gallic acid (main component of this extract) associated to nystatin through the fractionated inhibitory concentration index (CIF Index). Where our results showed that the association is not necessary because both the extract and the acid gallic were indifferent when associated with nystatin.

Saraiva et al, 2013 evaluated the synergism of the *S. brasiliensis* leaf at a concentration of 25 and 50 µg / mL, which were associated with antibiotics (tetracycline and oxacillin), the results showed additive and synergic actions for the concentration of 50 µg / mL, although not enough for MIC to reach values below 2 and 4 µg / mL, necessary to be classified as sensitive strains CLSI (2005) to oxacillin and tetracycline, respectively. Therefore, it was concluded that in the concentration of 50 µg / mL of the leaves of *S. brasiliensis* presented antimicrobial potential against the multiresistant strains of *S. aureus* MRSA and that the associations of the fractions with the antibiotics tested did not present benefits, not justifying the concomitant use. This was the only study in the literature found to perform the association with the *S. brasiliensis* extract.

When handling medicinal plants some care should be observed as they may possess extremely toxic substances the cells, because of this, it is important to analyze the toxicity of the plant, which is defined as, the ability to cause serious damage or death of a organism or cells. (Ferreira and Nardi, 2015).

The toxicity of the leaf extract of *S. brasiliensis* was performed by Santos et al, 2013 at a concentration of 2000 to 250 µg / mL of 24 and 48 h in *Artemia salina* and showed the LC50 value of 511.90 µg / mL , Considered a moderate toxicity because it is between 500-1000µg / mL.

Silva et al, 2012 also performed the toxicity in *Artemia salina* but with the extract of the shell of *S. brasiliensis*. and obtained a LC50 of 428µg / mL which calls it toxic. Acute toxicity in mice was also evaluated by (Santos, 2003) and by (Chaves et al, 2015) at a concentration of 2000mg / mL and no organ damage or death was observed considering leaf extract with low toxicity. Silva et al, 2012 also performed the toxicity in mice only from the shell of the extract and also found the same results as Santos et al, 2013.The cytotoxicity was performed by Saraiva, 2012 against healthy macrophages and cancer cells where the methanolic extract of *S.*

brasiliensis leaf presented low toxicity to healthy cells, which corroborates with our study where the human blood cells were biocompatible.

There is a diversity of researches in the area of natural products and it is very difficult to compare the studies, due to the divergence of methodologies, part of plants used, antimicrobial agents, among others. In addition, when talking about *S. brasiliensis*, few studies have reported in the literature about its effects in favor of dentistry. Therefore, from the results found in this study and considering the great antifungal potential, and antibiofilm activity, with no cytotoxicity in the peripheral blood cells, an advance regarding the use of *S. brasiliensis* extract, this study tends to aggregate knowledge and propose that new studies such as immunomodulation, and confection of products to be used in the treatment of oral candidiasis, especially in prosthetic stomatitis

5 Conclusion:

We can say that the leaf extract of *S. brasiliensis* has an antifungal activity on strains of the genus *Candida*, presenting, in general, fungicidal action, especially after 6 hours of contact with the microbial cells and antibiofilm effect constituted by *Candida* spp. (uni and multispecies), with ability to inhibit the formation and reduction of mature biofilm, in association with nystatin behaved indifferently and did not present cytotoxicity on peripheral blood mononuclear cells. It is considered a promising extract for the development of oral candidiasis products.

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3. CONSIDERAÇÕES GERAIS

Este trabalho tem um caráter inovador por apresentar informações ainda não encontradas na literatura com propriedades voltadas a um potencial uso desta parte da planta com atividade antifúngica na odontologia. Neste trabalho foi avaliado a atividade antifúngica frente a cepas de *Candida* que possui uma grande importância clínica na odontologia principalmente na candidíase oral, que podem estar associadas ou não ao uso de prótese dentária, como é o caso da estomatite protética. Além disso, nenhum trabalho anteriormente foi encontrado que realizasse a atividade antibiofilme e cinética entre espécies de *Candida* e a folha da *Schinopsis brasiliensis* Engl.

A *S. brasiliensis* por muito tempo só era utilizada na indústria de marcenaria, por possuir um caule muito rígido e de difícil decomposição, e suas propriedades biológicas advinham de alguns trabalhos com a utilização da casca da Braúna, como é popularmente chamada.

Poucos estudos foram encontrados com a utilização das folhas da *S. brasiliensis* porém com atividades antibacteriana, e um apenas com atividade contra fungos do gênero *Candida* mas que não tiveram ação. Neste estudo tivemos resultados de CIM em todas as cepas testadas e com concentrações que variam de 31,25 a 250µg/mL valores de CIM considerado muito ativo até 100µg/mL e ativos de 100- 500 µg/mL de acordo com Alves et al, 2000. A relação CFM/CIM mostrou uma atividade fungicida da substância para a maioria das cepas, exceto para *C. Albicans* ATCC 60193.

O estudo com plantas medicinais apesar de crescente apresenta algumas limitações de comparação entre estudos por apresentar técnicas diferentes para obter os resultados, parte de plantas diferente, concentrações diferentes, preparação de extrato ou óleo diferentes, componentes químicos das partes da planta que depende de diversos fatores ambientais, de colheita, etc. Todas essas divergências impedem a comparação de um estudo com outro.

A avaliação do sinergismo foi proposta após comprovada atividade antifúngica do extrato da *S. brasiliensis* para verificar se ao associa- ló a nistatina, que foi o produto comercial utilizado como padrão ouro, por ser o antifúngico mais comumente usado pela população no tratamento de candidíase oral e estomatite

protética, se teríamos uma ação sinérgica. Porém o que observamos foi que não existe necessidade de associação pois comportaram –se indiferentemente. Da mesma forma com o ácido gálico, componente majoritário deste extrato. Essa análise também foi de extrema importância pois até o momento apenas um estudo relatava a associação deste extrato, porém com antibióticos e não antifúngico.

Diante de todos estes resultados e de tanto acúmulo de conhecimentos inovadores, este estudo ainda se propõe a realizar a imunomodulação e indução de citocinas, etapa em andamento para um fechamento e comprovação de sua atividade anti-inflamatória e após isso sugerir a confecção de um produto a ser utilizado pela odontologia no tratamento de candidíase oral e estomatites protéticas.

4. CONCLUSÃO

O extrato da folha da *S. brasiliensis* possui atividade antifúngica sobre cepas do gênero *Candida*, apresentando, de modo geral, ação fungicida, especialmente após 6 horas de contato com as células microbianas e efeito antibiofilme constituído por *Candida* spp*.* (uni e multiespécie)*,* com habilidade para inibir a formação e redução do biofilme maduro, e quando usado em associação com a nistatina comportou-se indiferente e sem apresentar citotoxidade sobre células mononucleares de sangue periférico.

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* De acordo com as normas do PPGO/UFPB, baseadas na norma do International Committee of Medical Journal Editors - Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

APÊNDICE: TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Prezado (a) Senhor (a)
Esta pesquisa fala do Potencial citotóxico e imunomodulador de extratos de plantas medicinais e está sendo desenvolvida por Vanessa de Carvalho Jovito, mestranda em Ciências Odontológicas pela Universidade Federal da Paraíba, com orientação do Professor Dr. Lúcio Roberto Cançado Castellano..A finalidade deste trabalho é contribuir para o esclarecimento acerca dos efeitos de extratos das plantas: braúna e canela sobre as células do sangue humano. Se você concordar em participar deste estudo serão coletadas 1 amostra 12 mililitros de seu sangue por punção periférica venosa e este será submetido a teste de avaliação anti-inflamatória. A partir desta amostra serão realizados testes (exames com o sangue) para determinação da toxicidade dos extratos. A coleta será realizada no próprio laboratório.

Ao ser retirado o seu sangue através de punção venosa periférica para exames laboratoriais com seringas e agulhas descartáveis e estéreis podem, embora sejam raros, resultarem em dor no local da punção, manchas rochas transitórias chamadas de equimoses, desconforto e a possibilidade de infecção.

Solicitamos sua autorização para apresentar os resultados deste estudo em eventos da área de saúde e publicar em revista científica. Por ocasião da publicação dos resultados, seu nome será mantido em sigilo.

Esclarecemos que sua participação no estudo é voluntária e, portanto, o (a) senhor (a) não é obrigado (a) a fornecer as informações e/ou colaborar com as atividades solicitadas pelo Pesquisador (a). Caso decida não participar do estudo, ou resolver a qualquer momento desistir do mesmo, não sofrerá nenhum dano, nem haverá modificação na assistência que vem recebendo na Instituição.
Os pesquisadores estarão a sua disposição para qualquer esclarecimento que considere necessário em qualquer etapa da pesquisa.

Diante do exposto, declaro que fui devidamente esclarecido (a) e dou o meu consentimento para participar da pesquisa e para publicação dos resultados. Estou ciente que receberei uma cópia desse documento.

_____

Assinatura do Participante da Pesquisa ou Responsável Legal

Espaço para impressão dactiloscópica

_____

Assinatura da Testemunha

OBS: O participante da pesquisa e o pesquisador responsável deverão rubricar todas as folhas do TCLE apondo suas assinaturas na última página do referido Termo.

Contato com o Pesquisador (a) Responsável: Caso necessite de maiores informações sobre o presente estudo, favor ligar para o pesquisador Lúcio Roberto Cançado Castellano.

Endereço (Setor de Trabalho): Universidade Federal da Paraíba – Campus I / Cidade Universitária - João Pessoa - PB - Brasil
Telefone: (83) 3216-7189

Comitê de Ética em Pesquisa- CCS- UFPB
Endereço: Universitário S/N Castelo Branco – João Pessoa- PB CEP: 58051-900
fone: (83)3216-7791
Atenciosamente,_____

Assinatura do Pesquisador Responsável

_____

Assinatura do Pesquisador Participante

UNIVERSIDADE FEDERAL DA PARAÍBA
CENTRO DE CIÊNCIAS DA SAÚDE
COMITÊ DE ÉTICA EM PESQUISA

C E R T I D Ã O

Certifico que o Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federal da Paraíba – CEP/CCS aprovou por unanimidade na 8ª Reunião realizada no dia 29/09/2016, o Projeto de pesquisa intitulado: **"POTENCIAL ANTIMICROBIANO E IMUNOMODULADOR DE EXTRATOS DE PLANTA PARA POSSÍVEL USO EM UM PRODUTO NO TRATAMENTO DE ESTOMATITE PROTÉTICA"**, da pesquisadora Vanessa de Carvalho Jovito. Prot. nº 0412/16. CAAE: 57854716.8.0000.5188.

Outrossim, informo que a autorização para posterior publicação fica condicionada à apresentação do relatório final do estudo proposto à apreciação do Comitê.

Drª Elizne Marques D. Sousa
Coordenadora CEP/CCS/UFPB
Mat. SIAPE: 0332618

Comitê de Ética em Pesquisa do Centro de Ciências da Saúde da Universidade Federa da Paraíba
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