Revista Odonto Ciência
Journal of Dental Science

ORIGINAL ARTICLE

Rev Odonto Cienc 2015;30(4):179-183
http://revistaseletronicas.pucrs.br/ojs/index.php/fo
http://dx.doi.org/10.15448/1980-6523.2015.4.17794

Open Access

In vitro antimicrobial activity of plants of the Amazon on oral biofilm micro-organisms

Nikeila Chacon de Oliveira Conde[a], Maria do Socorro Vieira Pereira[b], Maria Fulgência Costa Lima Bandeira[a], Gisely Naura Venâncio[a], Glauber Palma de Oliveira[a], Fábio Correia Sampaio[b]

[a] Federal University do Amazonas (UFAM), Manaus-Amazonas, Brasil
[b] Federal University of Paraíba (UFPB), Pessoa-Paraíba, Brasil

Abstract

Objective: Performing a screening of Amazonian species as antimicrobial action of oral biofilm micro-organisms in vitro.

Methods: Determining the Minimum Inhibitory Concentration (MIC) for solid medium diffusion and Minimum Inhibitory Concentration of Adherence (MICA). The strains tested were *S. sobrinus* (ATCC 27609), *S. mutans* (ATCC 25175), *S. mitis* (ATCC 9811); *S. sanguis* (ATCC10556), *L. casei* (ATCC 7469).

Results: Juca, Crajiru, Alfavaca and Copaiba presented MIC with halos of inhibition for all strains tested, with values of 62.5, 500, 1000 and 1000 mg/mL, respectively. In MICA, extracts of Juca, Jambu and Crajiru inhibited the adherence in all strains tested.

Conclusions: Extracts of Juca, Crajiru, Alfavaca and Copaiba essential oil had antimicrobial activity against the strains tested and extracts of Juca, Crajiru, Alfavaca, Jambu and Copaiba and Andiroba oils inhibited microbial adherence.

Key words: Biofilm; Micro-organisms; Medicinal plants

Atividade antimicrobiana *in vitro* de plantas da Amazônia sobre alguns micro-organismos formadores do biofilme dental

Resumo

Objetivo: Realizar um *screening* de espécies amazônicas quanto à ação antimicrobiana sobre micro-organismos formadores do biofilme dental *in vitro*.

Metodologia: Através da determinação da Concentração Inibitória Mínima (CIM) por difusão em meio sólido e a Concentração Inibitória Mínima de Aderência (CIMA). As cepas ensaiadas foram *S. sobrinus* (ATCC 27609); *S. mutans* (ATCC 25175); *S. mitis* (ATCC 9811); *S. sanguis* (ATCC10556); *L. casei* (ATCC 7469).

Resultados: Demonstraram que Jucá, Crajiru, Alfavaca e Copaíba apresentaram CIM com halos de inibição para todas as cepas ensaiadas, com valores de 62.5, 500, 1000 e 1000 mg/mL, respectivamente. Na CIMA, os extratos de Jucá, Jambu e Crajiru inibiram a aderência em todas as cepas ensaiadas.

Conclusão: Os extratos de Jucá, Crajiru, Alfavaca e o óleo essencial de Copaíba tiveram atividade antimicrobiana sobre as cepas ensaiadas e os extratos de Jucá, Crajiru, Alfavaca, Jambu e os óleos de Copaíba e Andiroba inibiram a aderência microbiana.

Palavras-chave: Biofilme; Micro-organismos; Plantas medicinais

Correspondence:
Gisely Naura Venâncio
ginaura@gmail.com

Received: June 26, 2014
Accepted: September 1, 2015

Conflict of Interests: The authors state that there are no financial and personal conflicts of interest that could have inappropriately influenced their work.

Introduction

The two worst health issues in Dentistry are cavities and periodontal disease, both with the same primary etiological factor – the accumulation of dental biofilm [1]. Among the measures used to control the dental biofilm, brushing and the use of antimicrobial agents are the most common [2].

Antimicrobial agents have been used in addition to mechanical procedures or for specific conditions [3,5], successfully releasing bactericide or bacteriostatic substances such as quaternary ammonia compounds, bisbiguanides, enzymes, metal salts, essential oils, and plant extracts [6].

In the Amazon region, there is a large biodiversity of medicinal plants used empirically, but whose prescription has been consolidated through centuries of cultural interaction. Among many medicinal plants, there are some used specifically as anti-inflammatory and antimicrobial agents, including: Andiroba (*Carapa guianense*), Alfavaca (*Ocimum micranthum*), Copaiba (*Copaifera multijuga*), Crajiru (*Arrabidaea chica*), Jambu (*Spilanthes acmella*) and Juca (*Libidibia ferrea*) [7].

The ethnobotanical variety and the antimicrobial potential of the medicinal plants used in popular medicine in the Amazon region justifies the study of those plants and how they work against the bacteria present in the mouth to find scientific proof of the effect of those plants or substances derived therefrom as antimicrobial agents in Dentistry [8].

The purpose of this study was to conduct a screening of Amazon species in terms of their antimicrobial action against some dental biofilm forming microorganisms *in vitro*.

Methodology

The antimicrobial activity of the Amazon plants was assessed by diffusion in a solid medium through Minimum Inhibitory Concentration (MIC), according to methodology proposed by Bauer et al. [9] as amended and the Minimum Inhibitory Concentration of Adherence (MICA) using the tilt tube method, according Gebara et al. [10], with different concentrations of the diluted extract solution ranging from 1:1 to 1:512.

Eight (8) Amazon plant species were studied: *Carapa guianensis* (Andiroba); *Aspidosperma nitidium* (Carapanauba); *Copaifera multijuga* (Copaíba); *Arrabidaea chica* (Crajiru); *Spilanthes acmella* (Jambu); *Eupatorium ayapana* (Japana-branca); *Libidibia ferrea* (Juca); *Ociumum micranthum* (Alfavaca). The selection followed a survey – in the literature – of the popular plants used in the city of Manaus.

The species used to prepare the extracts were collected from official research institutions to ensure their legitimacy (EMBRAPA, UFAM, and IBAMA) and were stored at the Lauro Pires Xavier Herbarium at the Systematics and Ecology Department/UFPB, according to the Genetic Heritage Component Sample Access and Delivery Authorization (No. 044/2004 – IBAMA/MMA).

The plants were dried in an oven, and then the extracts were prepared following the asepsis principles used to preserve the material quality. The choice of plant and part thereof to be used in the study followed the directions for use by the local people (Table 1).

The extracts were obtained using a methanol extracting solution (80% v/v) constantly renewed for 72 hours, in three maceration and decoction cycles, under reflow. After the extraction of the active ingredients, the final volume was reduced in a rotating evaporator to fully remove the alcohol and stored in amber-colored vials previously sterilized, dried, and stored in a cold chamber.

The chemical processes to fully extract the active ingredients from the vegetables yielded 100% of the crude extracts of the materials for maceration and decoction. Therefore, the start solutions were 500 mg/mL for crajiru, juca, japana-branca, and jambu and 100 mg/mL for carapanauba, andiroba, copaiba, and alfavaca.

Study of the antimicrobial activity of the vegetable extracts

The antimicrobial activity was assessed using the amended assay proposed by Bauer et al. [9] through the diffusion in a solid medium on Petri dishes. The MIC of the proposed extracts and essential oils was determined. For such, the *Streptococcus sobrinus* (ATCC 27609); *Streptococcus mutans* (ATCC 25175); *Streptococcus mitis* (ATCC 9811); *Streptococcus sanguis* (ATCC10556); *Lactobacilus casei* (ATCC 7469) strains were grown in a nutritious BHI (Brain Heart Infusion – DIFCO) broth, incubated at 98.6 ºF (37 ºC) for 18-20 hours in microaerophilia.

Tabela 1. Relação das plantas utilizadas, nome científico, nome popular, parte utilizada e concentração da solução de trabalho

Scientific Name	Popular Name	Part of the plant used	Work solution concentration (mg/mL)
Arrabidaea chica	Crajiru	Leaves	500
Aspidosperma nitidium	Carapanauba	Bark	1000
Libidibia ferrea	Juca	Pod	500
Carapa guianense	Andiroba	Essential oil	1000
Copaifera multijuga	Copaiba	Essential oil	1000
Eupatorium ayapana	Japana-branca	Leaves	500
Ocimum micranthun	Alfavaca	Leaves	1000
Spilanthes acmella	Jambu	Leaves	500

Agar Mueller Hinton (DIFCO) dishes were prepared and, after 24 hours for sterility control, inundated with a saline solution inoculated with the overnight microorganisms. Next, standard holes of approximately ¼" (6 mm) diameter were made. Each dish had five orifices that were numbered between 1 and 10 according to the dilution of the tested substance (1:1 to 1:512). After placing 50µL of the tested substances, the dishes were incubated in a bacteriological oven at 98.6 ºF (37 ºC) for 24 hours. Each assay was conducted twice for each strain selected. The same procedure was followed for the positive control, chlorhexidine gluconate (Periogard®).

The antimicrobial activity assay using Andiroba and Copaiba essential oils required the use of Tween 80 (Sigma®), which is an oleic acid ester used as emollient to allow the dispersion of the oil in the solid medium. For that, 60 µL of Tween 80 per 1 mL of essential oil were used.

The resulting MIC was the smallest concentration of the substance capable of completely inhibiting bacterial growth, that is, the presence of a halo >12 mm defined the average for the two readings of each microorganism.

Minimum Inhibitory Concentration of Adherence (MICA)

The MICA of the bacteria in vitro was determined in the presence of 5% according to Gebara et al.[10] using concentrations of the substance diluted from the extract, varying between 1:1 and 1:512.

From the overnight growth, the strains were sub-cultivated at 98.6 ºF (37 ºC) in a Mueller Hinton broth (DIFCO) for 24 hours, to obtain a 10^6 UFC/ml inoculate. 1.8 mL of the sub-culture were distributed in hemolysis tubes and 0.2 ml of the solution corresponding to the extract scale were added. A variation of 0.97 mg/mL to 500 mg/mL was used for the crajiru, juca, japana-branca, and jambu extracts. While concentrations were 1.95 mg/mL to 1,000 mg/mL for the carapanauba, andiroba, copaiba, and alfavaca extracts.

The sub-cultures were incubated at 98.6 ºF (37 ºC) in microaerophilia for 24 hours with the tubes at 30 degrees inclination. The reading consisted of the visual observation of the adherence of the bacteria to the tube walls upon agitation. The resulting MICA was determined from the tube with the smallest concentration of the diluted extract with sucrose added to prevent the microorganism from adhering to the glass.

The findings are shown in simple numerical form according to the MIC and MICA for the respective inhibition halos and extract concentrations (mg/mL) using descriptive statistics.

Results

Among the eight plants under study, four species, namely *Libidibia ferrea* (Juca), *Arrabidaea chica* (Crajiru), *Ocimum micranthum* (Alfavaca), and *Copaifera multijuga* (Copaiba), had a positive MIC, with inhibition halos in different concentrations for all the strains used, especially Juca and Copaiba essential oil (Table 1).

The *Libidibia ferrea* (Juca) showed inhibition halos from 21 to 24 mm at a concentration of 500 mg/mL and halos from 14 to 15 mm at a concentration of 62.5 mg/mL (1:8) for the tested microorganisms (Table 2). Their best results were achieved against *Streptococcus mutans* (*S. mutans*), *Streptococcus sobrinus* (*S. sobrinus*), and *Lactobacillus casei* (*L. casei*), with 15 mm halos at 1:8 dilution.

The Copaiba essential oil exhibited excellent behavior against the tested microorganisms. The MIC was 39 mg/mL, with inhibition halos of 12 to 15 mm. The best result was against the *S. mutans*, whose growth was inhibited with halos of 15 mm in the 7.8 mg/mL crude oil dilution.

The other plants studied, Carapanauba (*Aspidosperma nitidum*), Jambu (*Spilanthes acmella*), and Andiroba (*Carapa guianensis*) essential oil did not exhibit any antimicrobial activity against the microorganisms tested at MIC (Table 2) in this study.

Table 2. Diameter of the inhibition halos of the substances against the microorganisms

Substances Tested / MIC	Microorganism				
	S. mutans	*S. mitis*	*S. sanguis*	*S. sobrinus*	*L. casei*
L. ferrea (Juca) 1:8 (62.5mg/mL)	15	14	14	15	15
A. chica (Crajiru) (500mg/mL)	0	0	0	13	13
O. micranthum (Alfavaca) (1000mg/mL)	0	0	16	12	0
C. multijuga (Copaiba) (1000mg/mL)	20	19	19	20	22
Chlorhexidine 0.12% 1:8 (0.15mg/mL)	12	13	13	12	13
A. nitidum (Carapanauba)	0	0	0	0	0
C. guianense (Andiroba)	0	0	0	0	0
E. ayapana (Japana-branca)	0	0	0	0	0
S. acmella (Jambu)	0	0	0	0	0

Table 3. MICA of the Juca, Jambu, Alfavaca, and Crajiru Extracts (mg/mL)

Microorganism	Tested Substances / MICA			
	Libidibia ferrea (Juca)	*Spilanthes acmella* (Jambu)	*Ocimum micranthum* (Alfavaca)	*Arrabidaea chica* (Crajiru)
S. mutans	31,2	125	500	500
S. mitis	31,2	250	0	500
S. sanguis	31,2	250	250	500
S. sobrinus	31,2	250	250	250
L. casei	31,2	125	0	500

Table 4. MICA of the Andiroba and Copaiba Essential Oils and the Positive Control – Chlorhexidine 0.12% (mg/mL)

Microorganism	Tested Substances / MICA		
	Carapa guianense (Andiroba)	*Copaifera multijuga* (Copaiba Oil)	Chlorhexidine 0.12% 1:8
S. mutans	1000	1,95	4,1
S. mitis	1000	1,95	4,1
S. sanguis	1000	1,95	4,1
S. sobrinus	500	1,95	4,1
L. casei	1000	1,95	4,1

At MICA, the Juca, Jambu, and Crajiru extracts inhibit adherence in all strains tested, especially Juca and Copaiba oil (Tables 3 and 4).

The MICA observed for Andiroba varied between 100 and 500 mg/mL, and the Jambu MICA varied between concentrations of 1:1 and 1:4 according to the microorganism tested, with inhibition of the microorganisms' adherence to the glass.

The Crajiru extract showed MICA numbers similar to the Andiroba essential oil. The best results in this test were from the Juca extract and the Copaiba essential oil, with MICA of 1.95 mg/mL for the latter with all strains tested.

Discussion

The study represents – for some medicinal plants – the first attempt to observe their antimicrobial effect against biofilm forming bacteria in the mouth.

Similar findings were described by Pereira et al. [3], which studied the in vitro antimicrobial activity, MIC, MICA, and MBC (Minimum Bactericide Concentration) of the pomegranate extract (*Punica granatum* Linn.) against the strains of dental biofilm forming bacteria: *S. mutans, S. mitis, S. sanguis, S. sobrinus*, and *L. casei*, compared to chlorhexidine, concluding that both substances exhibit an effective inhibitory action.

Carvalho et al. [11] had already described the effect of the Juca aqueous extract as an anti-inflammatory and analgesic substance in rats, compared to standard substances in terms of their action. According to the author, those findings support the popular use of the Juca tincture, as this plant is used by the Amazon people as an antifungal, antianemic, antihemorrhagic, healing, antimicrobial, anti-inflammatory, and antidiarrheal substance [11-14].

The results of those tests show a strong potential of the Juca against microorganisms of the dental biofilm, with its best results in the 62.5 mg/mL concentration, similar to Chlorhexidine (positive control), which exhibited antimicrobial effects as expected, with halos between 12 and 13 mm appearing for all microorganisms tested up to the 8.3 mg/mL concentration.

Marreiro et al. [15] proposed the formulation of a mouth rinse using the Juca extract, and concluded that this new product is feasible considering the good results achieved in the research in terms of the antimicrobial activity against *S. mutans* and *S. oralis*.

Soares et al. [16] studied the antimicrobial activity of the juca, aroeira, ginger, alfavaca, propolis, pomegranate, and large leaf mint tinctures against strains of *S. aureus*, *S. mutans*, *S. sobrinus*, *S. mitis*, *S. sanguis*, and *L. casei*, using chlorhexidine 0.12% as positive control. The maximum inhibitory concentration (MIC) was determined in an Agar Mueller Hinton medium for pure (1:0) and diluted (1:1 to 1:32) concentrations of the tinctures. They concluded that, among all tinctures, juca, aroeira, and propolis exhibited significant antimicrobial activity against *S. mutans, S. sobrinus, S. mitis, S. sanguis*, and *L. casei*, with ginger and alfavaca exhibiting the weakest action against the bacteria strains tested.

As for the Copaiba oil, the findings of this study agree with the findings of Bandeira et al. [17], in which the Copaiba essential oil exhibited bacteriostatic and bactericide activity against *S. mutans*. The authors also observed that, when studied separately, the crude oil had better results than the *Copaifera multijuga.*

With respect to the other plants, despite the lack of antibacterial activity, Añez [18] conducted an ethnobotanical research using phytochemical screening and concluded that the *Aspidosperma nitidum* (Carapanauba) husk contains coumarin, which has been considered a promising substance for the treatment of cancer as an anti-inflammatory, vaso-dilator, and other treatments, according to Kuster, Rocha [19].

Spelman et al. [20] studied the jambu (*Spilanthes acmella*), and the findings provided the first evidence supporting the use of the plant against malaria and showing that it has active ingredients against *Plasmodium falciparum.*

The andiroba (*Carapa guianensis*) essential oil exhibits peculiar physical and chemical characteristics that promote anti-inflammatory action [21], and despite not exhibiting antimicrobial activity in this study, it can be used for other purposes.

The MICA findings may raise the hypothesis that the Jambu extract and the andiroba essential oil may be able to control cavities, not by inhibiting bacterial growth but by somehow preventing the microorganisms from adhering to the teeth.

The use of the Tween 80 emollient did not interfere with the findings of the Copaiba and Andiroba oils. The findings of Domingues et al. [22] describe that the Tween used as vehicle was toxic in the cell culture. This is possibly due to its ability to change the morphology and the surface of the cell wall because of its detergent action that contributed to the toxic outcome.

Evangelista et al. [23] conducted an ethnobotanical study to diagnose the prescription and use of medicinal plants in pathological oral changes at dental clinics in the city of Manaus. The findings show that medicinal plants are sold for oral pathologies, the following species in particular: Pedra ume caã (*Aulomyrcia sphareocarpa*), Crajiru (*Arrabidae chica*), and the plant – without a botanical identification – known as Sara tudo (heal all). The authors concluded that the medicinal plants sold in the city of Manaus empirically and the ones most used by the healers are Sara tudo, Andiroba (*Carapa guaianensis*), and Carapanauba (*Aspidosperma discolor*), and the ones most sought after by consumers are Sara tudo and Pedra ume caã (*Myrcia sphaerocarpa*). The Cashew plant was the one most used by users, and the medicinal plant most prescribed by dentists was Crajiru (*Arrebidaea chica Verlot*).

Natural products remain a largely unexplored source of effective anti-biofilm molecules of potentially low toxicity that could be used in alternative or adjunctive anti-cavity therapies [24].

Conclusions

We can conclude that the Juca, Crajiru, and Alfavaca extracts and the Copaiba oil exhibited antimicrobial activity against the strains tested, and that the Juca, Crajiru, and Alfavaca extracts and the Copaiba and Andiroba oils inhibited microbial adherence.

References

1. Añez RBS. Análise morfoanatômica das folhas e casca de *Aspidosperma nitidum* Benth e *Aspidosperma marcgravianum* Woodson (Apocynaceae) com abordagem farmacognóstica e etnofarmacológica. [tese]. Manaus (AM): Programa de Pós-graduação em Biologia Tropical e recursos naturais, Instituto Nacional de Pesquisas da Amazônia (INPA)/ Universidade Federal do Amazonas (UFAM); 2009.
2. Bandeira MFCL, Oliveira MRB, Benatti Neto C, Comelli LRC. Estudo preliminar da atividade antibacteriana do óleo essencial e da resina da *Copífera multijuga* (óleo de copaíba), associado ao óxido de zinco e ao hidróxido de cálcio. J Bras Clín Est Odontol. 1999;3(17):46-52.
3. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966;45(4): 493-6.
4. Borghi WMMC, Moimaz SAS, Saliba NA. Métodos alternativos para higienização bucal e terapêutica odontológica. Rev Inst Ciênc Saúde. 2005;23(4):309-14.
5. Borrás MRL. Plantas da Amazônia: Medicinais ou mágicas? – Plantas comercializadas no mercado Adolpho Lisboa. 1.ed. Manaus: Editora Valer/Governo do Estado do Amazonas; 2003.
6. Bugno NA, Nicolleti MA, Almodóvar AAB, Pereira TC, Auricchio MT. Enxaguatórios bucais: Avaliação da eficácia antimicrobiana de produtos comercialmente disponíveis. Rev Inst Adolfo Lutz. 2006;65(1):40-5.
7. Carvalho JC, Teixeira JR, Souza PJ, Bastos JK, Santos Filho D, Sarti SJ. Preliminary studies of analgesic and anti-inflamatory properties of *Caesalpinia ferrea* crude extract. J Ethnopharm. 1996; 53:175-8.
8. Diniz MFFM, Oliveira RAG, Medeiros ACD, Malta-Junior A. Memento Fitoterápico: As plantas como alternativa terapêutica - Conhecimentos populares e científicos. João Pessoa: Universitária/ Universidade Federal da Paraíba (UFPB); 1997.
9. Domingues FC, Queiroz JA, Cabral JM, Fonseca LP. The influence of culture conditions on mycelial structure and cellulase production by Trichoderma reesei Rut C-30. Enzyme Microb Technol. 2000;26(5-6): 394-401.
10. Evangelista SS, Sampaio FC, Parente RC, Bandeira MFCL. Fitoterápicos na Odontologia: Estudo etnobotânico na cidade de Manaus. Rev Bras Plantas Med. 2013;15(4): 513-9.
11. Gebara ECE, Zardetto, CGDC, Mayer MPA. Estudo *in vitro* da ação antimicrobiana de substâncias naturais sobre *S. mutans* e *S. sobrinus*. Rev Odontol Univ São Paulo. 1996; 10(4):251-6.
12. Kuster RM, Rocha LM. Cumarinas, cromonas e xantonas. In: Simões CMO et al. Farmacognosia: Da planta ao medicamento. 5.ed. Porto Alegre: Editora UFRGS; 2003. p.537-56 .
13. Marreiro RO, Bandeira MFCL, Souza TP, Almeida MC, Bendaham K, Venâncio GN, et al. Evaluation of the stability and antimicrobial activity of an ethanolic extract of *Libidibia ferrea.* Clin, Cosmet Investigat Dent. 2014 Jan;6:9-13.
14. Moreira ACA, Santos TAM, Carneiro MC, Porto MR. Atividade de um enxaguatório bucal com clorexidina a 0,12% sobre a microbiota sacarolítica da saliva. Rev Cienc Méd Biol. 2008;7(3):266-72.
15. Moreira NA, Ferreira RC, Vieira PA, Valadares HAC. Agentes antimicrobianos no controle da placa supragengival parte I. Arq Odontol. 2001;37(1):87-98.
16. Oliveira ACM, Fontana A, Negrini TC, Nogueira MNM, Bedran TBL, Andrade CR, et al. Emprego do óleo de *Melaleuca alternifolia* Cheel (*Myrtaceae*) na Odontologia: Perspectivas quanto à utilização como antimicrobiano alternativo às doenças infecciosas de origem bucal. Rev Bras Pl Med. 2011;13(4):492-9.
17. Orellana BJP, Kobayachi ES, Lourenço GM. Terapia alternativa através do uso da andiroba. Lato & Sensu. 2004;5(1):136-41.
18. Pereira JV, Pereira MSV, Higino JS, Sampaio FC, Alves PM, Araújo CRF. Estudos com o extrato da *Punica granatum* Linn. (romã): Efeito antimicrobiano *in vitro* e avaliação clínica de um dentifrício sobre micro-organismos do biofilme dental. Rev Odonto Ciênc. 2005;20(49):262-9.
19. Pereira LP, Silva RO, Bringel PH, Silva KE, Assreuy AM, Pereira MG. Polysaccharide fractions of *Caesalpinia ferrea* pods: Potential anti-inflammatory usage. J Ethnopharmacol. 2012;139(2):642-8.
20. Sampaio FC, Pereira MS, Dias CS, Costa VC, Conde NC, Buzalaf MA. *In vitro* antimicrobial activity of *Caesalpinia ferrea* Martius fruits against oral pathogens. J Ethnopharmacol. 2009;124(2):289-94.
21. Santos RI, Pereira DFA, Teodoro GR, Del Ciampo JO, Ogasawara MS, Canettieri ACV et al. Óleo essencial de *Thymus vulgaris*: Elaboração de enxaguatório bucal e avaliação do efeito *in vitro* na formação da placa bacteriana. Lat Am J Pharm. 2010;29(6):941-7.
22. Soares DGS, Oliveira CB, Leal C, Drumond MRS, Padilha WWN. Susceptibilidade *in vitro* de bactérias bucais a tinturas fitoterápicas. Rev Odonto Ciênc. 2006;21(53):232-7.
23. Spelman K, Depoix D, McCray M, Mouray E, Grellier P. The traditional medicine *Spilanthes acmella*, and the alkylamides spilanthol and undeca-2*E*-ene-8,10-diynoic acid isobutylamide, demonstrate *in vitro* and *in vivo* anti-malarial activity. Phytother Res. 2011;25(7):1098-101.