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The influence of Brazilian plant extracts on *Streptococcus mutans* biofilm

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ABSTRACT

Nineteen plant extracts obtained from plants from the Brazilian Amazon showed activity against planktonic *Streptococcus mutans*, an important bacterium involved in the first steps of biofilm formation and the subsequent initiation of several oral diseases. Objective: Our goal was to verify whether plant extracts that showed activity against planktonic *S. mutans* could prevent the organization of or even disrupt a single-species biofilm made by the same bacteria. Material and Methods: Plant extracts were tested on a single-bacteria biofilm prepared using the Zürich method. Each plant extract was tested at a concentration 5 times higher than its minimum inhibitory concentration (MIC). Discs of hydroxyapatite were submersed overnight in brain-heart infusion broth enriched with saccharose 5%, which provided sufficient time for biofilm formation. The discs were then submersed in extract solutions for one minute, three times *per* day, for two subsequent days. The discs were then washed with saline three times, at ten seconds each, after each treatment. Supports were allowed to remain in the enriched medium for one additional night. At the end of the process, the bacteria were removed from the discs by vortexing and were counted. Results: Only two of 19 plant extracts showed activity in the present assay: EB1779, obtained from *Dioscorea altissima*, and EB1673, obtained from *Annona hypoglauca*. Although the antibacterial activity of the plant extracts was first observed against planktonic *S. mutans*, influence over biofilm formation was not necessarily observed in the biofilm model. The present results motivate us to find new natural products to be used in dentistry.

Keywords: Dental plaque. Plant extracts. Amazonian ecosystem. *Streptococcus mutans*.

INTRODUCTION

Previous studies performed on more than 2,000 extracts from plants from the Amazon rain forest reported that only nineteen showed activity against *S. mutans*[18] and that twenty-six were active against *S. sanguinis*[19]. Streptococci are commensal bacteria that pioneer oral biofilm colonization and may be involved in severe systemic diseases, such as infective endocarditis[15] and atherosclerosis[13]. For that reason, it is crucial that the first steps of biofilm formation be controlled for the effective maintenance of general health balance based on oral health. Brazilian forests are considered the most biodiverse forests in the world and may be a source of new products to be used in biofilm control.

Nature plays a significant role in the search of new lead compounds to be introduced in Medicine to defeat pathogenic microorganisms. The first decades of the 20th century witnessed the discovery and introduction of a new molecule in therapeutics that would revolutionize the fight against infectious diseases, penicillin, isolated from a filamentous fungus named *Penicillium notatum*. After that, research relied on the search of new molecules from fungi, from which cephalexin, cyclosporine, erythromycin and other important antibacterials were discovered. Although much has been done in the search for new leads from plants, it is estimated that only 6% of the species have been studied so far[5]. In Brazil, strong efforts are made in order to search for new antibacterials from local plants, using

not only information regarding popular uses[23], but also the establishment of throughput assays aiming at the rapid analysis of a large number of plant extracts against a variety of microorganisms[1,18,22,26].

The process of biofilm formation begins with the coating of the tooth surface through the salivary pellicle, formed by salivary components, and is the basis for microorganism-induced biofilm formation in the oral cavity. Single *S. mutans* cells or their aggregates fuse with pellicle via sucrose-dependent or sucrose-independent mechanisms. The ability to fuse with the pellicle made the *S. mutans* the main initiator of the process to form the framework that is the basis for the colonization of new bacteria. Other bacteria were then anchored to solid surfaces as enamel tooth and then were embedded in an exopolysaccharide matrix. Over 700 different bacterial species incorporated into biofilms have been identified. Since *S. mutans* provides the aggregation of other bacteria, antimicrobial actions aiming at *S. mutans* would be useful to retain the progression of caries[12].

The wide range of bacteria and oral factors involved in the caries development leads researches to investigate methods, besides antibiotics, which deal with pathogens that occur in the oral cavity. Chlorhexidine[14] is the most used antibiotic, but constant therapy finds undesirable effects, which, in a long-term clinical requirement, asks for the introduction of new therapeutic options. The use of plant extracts might be an option that could be effective, particularly those plants that can effectively interfere with biofilm formation.

As part of our ongoing effort to find new products to be used in dentistry, nineteen anti-*Streptococcus* plant extracts were selected to be tested in a biofilm model that was based on Guggenheim's Zürich biofilm model[7,8] and used *S. mutans*.

MATERIAL AND METHODS

Plant collection and extraction

Plants were randomly collected from the Amazon and Atlantic rain forests from 1997 to 2002 (license for plant collection and access to biodiversity: MMA 012A/2010). Different organs were collected from each plant, such as leaves, stems, flowers, fruits and bark, according to their biomass availability. If biomass was not available to be collected alone, organs were collected together and referred to as the "aerial organs". Each type of plant material was dried in an air-circulating incubator (Fanem, Diadema, SP, Brazil) at 40°C and was ground in a hammer mill (Holmes, Dan Ville, Illinois, USA)[26]. The ground plant material was placed into a glass percolator (Kontes, Vineland, NJ, USA), and a solvent system composed of a 1:1 mixture of dichloromethane:methanol (Synth, Diadema, SP, Brazil) was used to perform 24 h maceration. Afterward, the extract was drained, and the solvents were rotavaporated (Buchi, Flawil, Switzerland). Milli-Q-grade water (Millipore, São Paulo, SP, Brazil) was added to the ground plant material that remained in the percolator, and a second 24 h maceration was performed. The aqueous extract was drained from the percolator and lyophilized (Virtis, Warminster, PA, USA). Organic and aqueous extracts were kept at -20°C until use[17].

Extracts, fractions and standard drug preparation

Organic extracts were solubilized in dimethylsulfoxide 50% (Merck, Darmstadt, Germany) in water, and aqueous extracts were solubilized in Milli-Q water[21]. Screening via the disk diffusion assay (DDA) was performed with the prepared extracts, and 200 mg/mL chlorhexidine digluconate (CHX; Biodinâmica®, Ibiporã, PR, Brazil) was used as a standard at concentrations of 0.12%, 1% and 2% in the assays, as well as Periogard® (Colgate, São Paulo, SP, Brazil). The procedure resulted in more than 2,000 plant extracts, obtained from more than 660 plant species. Odd numbers were assigned to the organic extracts, whereas even numbers were assigned to the aqueous extracts. Figure 1 shows the botanical sources for the extracts, as well as the concentrations, prepared in dimethylsulfoxide 50% or in water, used in the assays.

Bacteria

All procedures were performed under sterile conditions. *Streptococcus mutans* (ATCC® 25175™, Microbiologics®, St. Cloud, MN, USA) was used in the 4th passage in all experiments. The bacteria were resuspended in saline solution at a concentration of 0.5 McFarland (corresponding to $1.5x10^8$ CFU/mL) for the DDA[19].

Culture medium

Brain-heart infusion agar with blood (BHIAB; Oxoid Ltd, London, England) was prepared according to the manufacturer's instructions in 12 mm-diameter Petri dishes (J. Prolab, São José dos Pinhais, PR, Brazil), and defibrinated cattle blood at a concentration of 5% (Lonza, Basel, Switzerland) was added as a complement to the agar medium. These plates were kept in a microaerophilic environment.

Brain-heart infusion broth (BHIB; Oxoid Ltd, London, England) was also prepared according to the manufacturer's instructions, and saccharose 5% (União, São Paulo, SP, Brazil) was added to the broth.

In vitro biofilm analysis

The technique described here was adapted from Guggenheim[7,8] and was performed in 24-well microplates (Costar, Tewksbury, MA, USA). On day 1, 1 mL of BHIB complemented with saccharose 5% was inoculated with *S. mutans* up to a concentration of 0.5 McFarland. A 1 ml sample of the suspension was added to each well of each microplate. A support for biofilm formation was then added to each well. Sterilized hydroxyapatite discs measuring 5.0 mm in diameter and 2.0 mm in height (Clarkson Chromatography Prod. Inc., South Williamsport, PA, USA) were used. The microplates were kept in an incubator, providing a microaerophilic environment at 36°C, for 16 hours and 50 minutes.

On day 2, after 16 hours and 50 minutes, the hydroxyapatite discs were transferred to new 24-well microplates containing 1 mL/well of the extracts, prepared at different dilutions, according to the concentrations described in Figure 2. The hydroxyapatite discs were incubated for 1 minute at room temperature. Saline solution was used as a negative control, and Periogard® and different CHX concentrations (0.12%, 1.0% and 2.0%) were used as positive controls. Next, the hydroxyapatite discs were transferred to new 24-well microplates containing 2 mL of sterile saline solution *per* well for three washes of up to 10 seconds each. Afterward, the hydroxyapatite discs were again placed in 24-well microplates containing sterile BHIB, and the plates were kept in an incubator at 36°C for another 4 hours (20.5 hours from the beginning of the experiment). The treatment-wash step was repeated twice. After the last wash, the hydroxyapatite discs were incubated for another 16 hours. On day 3, the procedures from day 2 were repeated (treatment-wash-incubation in sterile medium). On day 4, 64.5 hours from the beginning of the experiment, the hydroxyapatite discs are individually removed from each well and transferred to 2 mL vials containing saline solution. Each sample was tested in sextuplicates.

Bacterial count

Each support was transferred to 2 mL vials (Costar, Tewksbury, MA, USA) containing 1 mL of sterile saline solution. The vials were vortexed (Quimis, Diadema, SP, Brazil) for 2 minutes at room temperature to remove the biofilm from their surfaces. The bacteria in each suspension were

Extract #	Colector's #	Herbarium registration #	Scientific name	Family	Used organs	Concentration in the assay
71	PS - 252	167	*Cordia* sp.	Boraginaceae	Aerial organs	5 mg/mL
271	AAO – 3330	472	*Casearia spruceana Benth.* Ex Eichler	Salicaceae	Leaves	5 mg/mL
272	AAO – 3330	472	*Casearia spruceana Benth.* Ex Eichler	Salicaceae	Leaves	5 mg/mL
631	AAO - 3299	340	*Zanthoxylum* sp.	Rutaceae	Stem	5 mg/mL
1099	AAO - 3580	2564	*Psychotria* sp.	Rubiaceae	Stem	5 mg/mL
1109	AAO - 3577	2561	*Annona hypogauca* Mart.	Annonaceae	Stem	5 mg/mL
1119	AAO - 3543	2527	*Cordia cf.* exaltata	Boraginaceae	Stem	5 mg/mL
1129	AAO - 3580	2564	*Psychotria* sp.	Rubiaceae	Aerial organs	5 mg/mL
1229	AAO - 3687	4918	*Gnetum leyboldii* Tul.	Gnetaceae	Stem	5 mg/mL
1257	AAO - 3713	4947	*Symphonia globulifera* L.f.	Clusiaceae	Leaves	0.3 mg/mL
1343	IBS – 142	5072	*Moronobea coccinea* Aubl.	Clusiaceae	Leaves	5 mg/mL
1383	IBS – 61	5018	*Cordia nodosa* Lam.	Boraginaceae	Aerial organs	5 mg/mL
1407	IBS – 121	5047	*Solanum* sp.	Solanaceae	Aerial organs	5 mg/mL
1493	AAO - 4031	5165	*Ipomea alba* L.	Convolvulaceae	Aerial organs	0.16 mg/mL
1539	AAO - 4067	5192	*Casearia javitensis* Kunth	Salicaceae	Aerial organs	5 mg/mL
1673	MPB - 768	5875	*Annona hypogauca* Mart.	Annonaceae	Stem	5 mg/mL
1765	IBS – 142	5072	*Moronobea coccinea* Aubl.	Clusiaceae	Flowers	0.3 mg/mL
1779	AAO - 3812	5128	*Dioscorea altissima* Lam.	Dioscoreaceae	Aerial organs	5 mg/mL
1933	AAO - 4005	5140	*Cordia* sp.	Boraginaceae	Aerial organs	5 mg/mL

Figure 1- Botanical data for the plants used to obtain organic (odd numbers) or aqueous (even numbers) extracts and concentration of each extract used in the biofilm assay

counted by the serial dilution technique[18,19,22].

Statistical analysis

One-way ANOVA and Tukey's post-test were used to compare means (GraphPad® Prism 5.0, San Diego, CA, USA) and Bartlett's test for homocedasticity. Significance was considered if $p<0.05$.

RESULTS

Nineteen plant extracts were tested in the Zürich biofilm assay. The results were grouped according to the botanical affinities of the plants

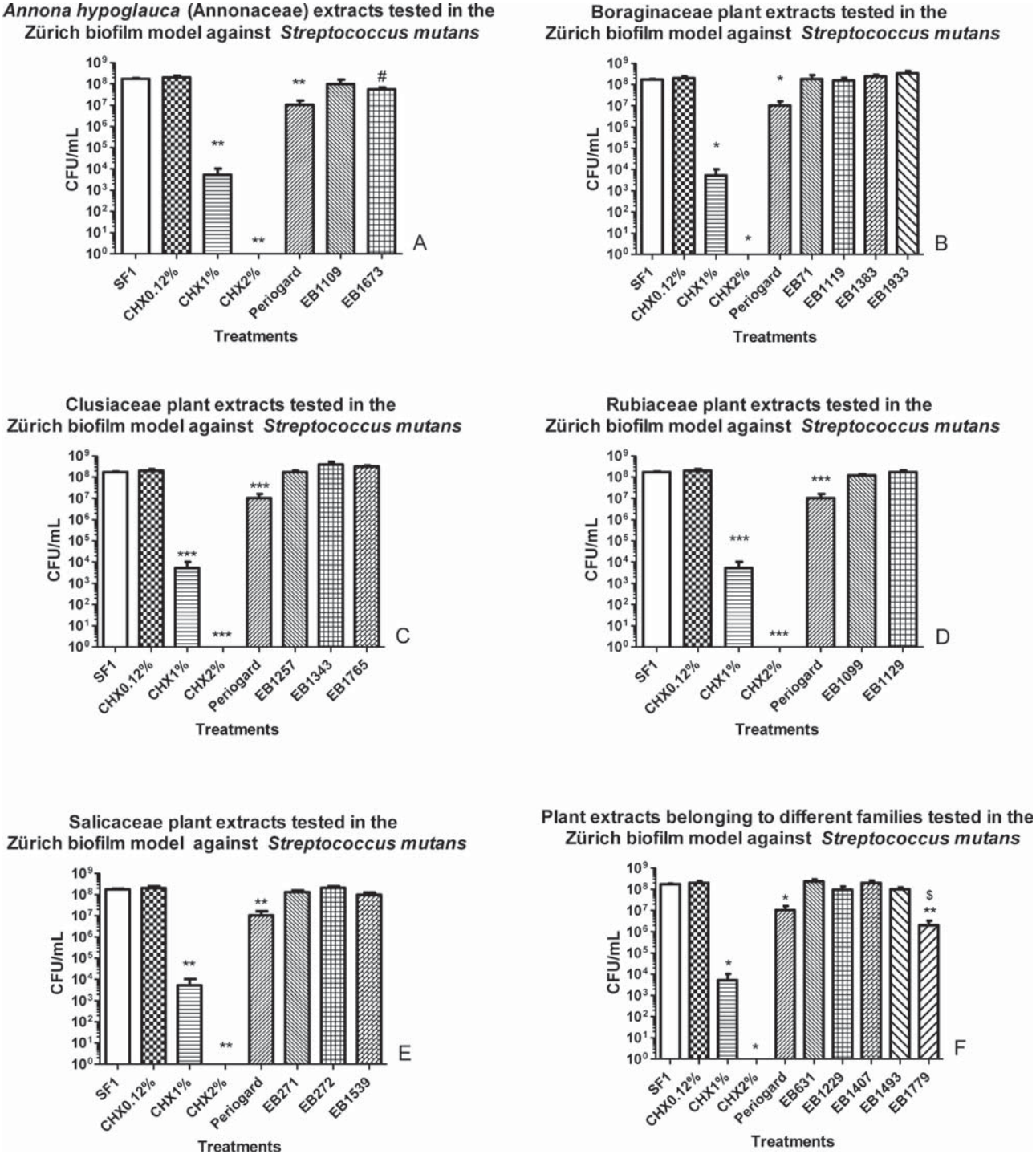

Figure 2- Results obtained from the bacterial count that remained from biofilm treatment with Amazon plant extracts. One-way ANOVA was used, and Tukey multiple comparison test was used to compare means. Significance level of $p<0.05$ (*); or $p<0.01$ (**); or $p<0.001$ (***) when comparison is made with saline solution; (#)=significance when comparison is made with chlorhexidine digluconate 0.12% ($p<0.05$); ($)=significance when comparison is made with saline solution ($p<0.05$)

from which the extracts originated to achieve a better understanding of the findings. H0 was that treatments with the extracts are identical to treatments with the positive controls, i.e., CHX, and our hypothesis was that the treatments with the extracts exhibit better antibacterial activity than the treatments with the positive controls. We admit that H0 is also a good result because the extracts can be purified, and better antibacterial activity may appear in isolated substances. Figure 2A shows that both extracts obtained from the stem of *Annona hypoglauca* did not show significant differences from the controls, although differences were evident among the controls ($F_{(6,31)}$=7.29; R^2=0.5854; p<0.05). Nonetheless, EB1673 slightly diminished the bacterial count when compared with CHX0.12% (p<0.05). Consistent with our hypothesis, both *Annona* extracts can be considered for potential use against biofilm formation, but a more conclusive affirmation can only be made after the chemical analysis and purification of certain groups of phytochemicals in each extract, despite the lack of differences found among the two extracts and the saline group. Here, it is important to distinguish the Annonaceae group from the next four groups to be analyzed. Despite certain statistical similarities observed in the results of the biological assay, the Annonaceae group differed from the next four groups because EB1673 showed a slight diminishment in the bacterial count when compared with CHX0.12%.

Figure 2B shows results related to four extracts obtained from plants belonging to Boraginaceae ($F_{(8,49)}$=3.790; R^2=0.4251; p<0.05). The results were not significant in this group of extracts. The same observations were performed for the families of extracts made from plants belonging to Clusiaceae ($F_{(7,43)}$=6.336; R^2=0.5520; p<0.05; Figure 2C), to Rubiaceae ($F_{(6,37)}$=16.60; R^2=0.7626; p<0.05; Figure 2D) and Salicaceae ($F_{(7,41)}$=11.76; R^2=0.7078; p<0.05; Figure 2E).

The last group of extracts was analyzed and reunited in graph (Figure 2F) and did not form a botanical group. This group consisted of extracts obtained from species belonging to different botanical families. Thus, a significant difference ($F_{(9,55)}$=7.365; R^2=0.5903; p<0.05) was found between EB1779 and saline (p<0.05) or CHX0.12% (p<0.01), and no significant difference was observed in the biological activity of EB1779 when compared with that of CHX1%, CHX2% or Periogard®, corroborating our hypothesis.

DISCUSSION

Although Zürich biofilm was designed to be developed from saliva and its complex microflora, we decided to use only one bacterial species. Our argument was that although plant extracts are complex mixtures of distinct compounds and although such variation might be the only variability in the experiments, the unpredictable amount of each bacterium composing a biofilm formed from saliva could have unpredictable effects when crude plant extracts are being evaluated. As *S. mutans* was previously used in the identification of antibacterial extracts in microdilution assays[1,19], the biofilm used in the current study was exclusively composed of the same bacteria. If one or more extracts showed activity against the single-species biofilm, then it was selected to be evaluated against a biofilm composed of more than one bacterial species.

It is known that the mechanism of action of CHX can be explained by the fact that its cationic molecule is readily attracted to the negative charge of the bacterial surface and stays adsorbed to the cell membrane through electrostatic interactions, likely through hydrophobic interactions or hydrogen bonds, in a concentration-dependent manner. Thus, at high concentrations, CHX may cause cytoplasmic protein precipitation or coagulation or bacterial death, and at lower concentrations, membrane integrity can be altered, resulting in the release of low-molecular-weight bacterial components[9,10,17].

According to our results, treatments with saline and CHX0.12% did not differ significantly (p>0.05). Although CHX0.12% is an effective agent that is largely used in bacterial burden reduction as a mouthwash, we could not confirm the prevention of planktonic biofilm formation under the conditions of the present experiment. CHX1% was more effective than saline or CHX0.12% (p<0.001), and CHX2% showed complete effectiveness, as no bacteria could be counted. Periogard® showed activity similar to that observed for CHX1% and CHX2% (p>0.05). The evaluation of the preventative potential of CHX in the development of root caries were previously done[11] and results suggested that the minimum concentration of CHX required to kill bacteria in a biofilm is considerably higher (10-100 times) than the amount required to kill bacteria in suspension. In addition, their results showed that CHX prevents bacteria from forming a biofilm and leads to a selective long-term suppression of *S. mutans*.

All 19 extracts were evaluated against planktonic biofilm. Only EB1779 showed a degree of anti-biofilm activity, and EB1673 showed a slight tendency toward inhibition. EB1779 was obtained from the aerial organs of *Dioscorea altissima* (Dioscoreaceae). Dioscoreaceae, the yam family, contains approximately 750 species distributed among eleven genera and 900 species. *Dioscorea* sp. occurs in all Brazilian tropical regions[16]. There is a lack of studies on the chemical composition of Brazilian *Dioscorea* sp. However, yam species are

widely used in Asian cultures, and their chemical composition may include saponins[24,25] or clerodane diterpenoids[11]. Extract EB1673 was obtained from the stem of *Annona hypoglauca* (Annonaceae, or the custard-apple family), a previously studied plant[20] that showed activity against the breast cancer cell line MCF-7 (-32.77% lethality index). *Annona* sp. are well characterized in terms of their chemical composition, and acetogenins[3], flavonoids[6], alkaloids[4] and sesquiterpenes[2] have already been isolated and revealed to have diverse biological activities. It is possible to observe that although extracts EB1109 and EB1673 were obtained from the stem of *Annona hypoglauca*, sistematicaly using the same extraction technique, they were collected in different years, 02/25/2000 and 01/27/2003 respectively, which may have led to chemical composition disparities due to seasonal changes.

The limitations of the study included the analysis of extracts instead of isolated natural products, the extracts being tested in only one anti-biofilm method despite the large number of samples tested and the use of chlorhexidine as standard drug being unequally compared to plant extracts, which are complex mixtures of different compounds. Future studies may include the development of a technique which is more practical to test samples in the high-throughput standard, as well as to proportionate the analysis of isolated compounds, in order to study the real influence over biofilm net formation, including microscopic analysis. In the meantime, we have established a method to look over the Brazilian biodiversity to track for natural products active against biofilm. Thus, more than 2,000 plant extracts were evaluated against *S. mutans*[18] in a DDA. From that screening, 18 extracts were considered to be active and were submitted to a microdilution broth assay to obtain corresponding minimal inhibitory and minimal bactericidal concentrations. From that screening, extracts EB271, EB1129, EB272 and EB1779 showed larger inhibition growth zones, and extracts EB1493, EB631, EB1099, EB1407, EB1933 and EB1779 showed the best minimal inhibitory and minimal bactericidal concentrations (results not shown). This previous work supported the present project, in which all 18 extracts, plus an extra extract, were submitted to a biofilm assay. We decided to test all of the extracts because any of them could have been a biofilm-formation inhibitor. In this assay, we observed that the biological responses differed from our first findings. The present results not only have encouraged us to seek more information about both biofilm-active extracts but also motivated us to introduce new techniques to track anti-biofilm activity in the other 2,000 extracts that compose our extract collection.

Only two of 19 extracts showed a degree of interference with biofilm formation. The results motivate us to continue the search for natural products to be used in dentistry.

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REFERENCES

1- Castilho AL, Saraceni CH, Díaz IE, Paciencia ML, Suffredini IB. New trends in dentistry: plant extracts against *Enteroroccus faecalis*. The efficacy compared to chlorhexidine. Braz Oral Res. 2013;27(2):109-15.

2- Chavan MJ, Wakte PS, Shinde DB. Analgesic and anti-inflammatory activities of the sesquiterpene fraction from *Annona reticulata* L. bark. Nat Prod Res. 2012;26(16):1515-8.

3- Chen Y, Chen JW, Wang Y, Xu SS, Li X. Six cytotoxic annonaceous acetogenins from *Annona squamosa* seeds. Food Chem. 2012;135(3):960-6.

4- Costa EV, Pinheiro ML, Souza AD, Barison A, Campos FR, Valdez RH, et al. Trypanocidal activity of oxoaporphine and pyrimidine-β-carboline alkaloids from the branches of *Annona foetida* Mart. (Annonaceae). Molecules. 2011;16(11):9714-20.

5- Cragg GM, Newman DJ. Natural products: a continuing source of novel drug leads. Biochim Biophys Acta. 2013;1830(6):3670-95.

6- Formagio AS, Kassuya CA, Neto FF, Volobuff CR, Iriguchi EK, Vieira MC, et al. The flavonoid content and antiproliferative, hypoglycaemic, anti-inflammatory and free radical scavenging activities of *Annona dioica* St. Hill. BMC Complement Altern Med. 2013;13:14. doi: 10.1186/1472-6882-13-14.

7- Guggenheim B, Giertsen E, Schüpbach P, Shapiro S. Validation of an *in vitro* biofilm model of supragingival plaque. J Dent Res. 2001;80(1):363-70.

8- Guggenheim B, Guggenheim M, Gmür R, Giertsen E, Thurnheer T. Application of the Zürich biofilm model to problems of cariology. Caries Res. 2004;38(3):212-22.

9- Hjeljord LG, Rolla G, Bonesvoll P. Chlorhexidine-protein interactions. J Periodontal Res Suppl. 1973;8(S12):11-6.

10- Hugo WB, Longworth AR. Some aspects of the mode of action of chlorhexidine. J Pharm Pharmacol. 1964;16(10):655-62.

11- Kidyu K, Thaisuchat H, Meepowpan P, Sukdee S, Nuntasaen N, Punyanitya S, et al. New clerodane diterpenoid from the bulbis of *Dioscorea bulbifera*. Nat Prod Commun. 2011;6(8):1069-72.

12- Krzyściak W, Jurczak A, Kościelniak D, Bystrowska B, Skalniak A. The virulence of *Streptococcus mutans* and the ability to form biofilms. Eur J Clin Microbiol Infect Dis. 2014;33(4):499-515.

13- McNicol A, Israels SJ. Mechanisms of oral bacteria-induced platelet activation. Can J Physiol Pharmacol. 2010;88(5):510-24.

14- Mei LM, Chu CH, Lo EC, Samaranayake LP. Preventing root caries development under oral biofilm challenge in an artificial mouth. Med Oral Patol Oral Cir Bucal. 2013;18:e557-63.

15- Okahashi N, Nakata M, Sakurai A, Terao Y, Hoshino T, Yamaguchi M, et al. Pili of oral *Streptococcus sanguinis* bind to fibronectin and contribute to cell adhesion. Biochem Biophys Res Commun. 2010;391(2):1192-6.

16- Ribeiro JES, Hopkins MJG, Vicentini A, Sothers CA, Costa MAS, Brito JM, et al. Flora da Reserva Ducke: guia de identificação das plantas vasculares de uma floresta de terra-firme na Amazônia Central. Manaus: INPA; 1999. 799 p.

17- Rölla G, Melsen B. On the mechanism of the plaque inhibition by chlorhexidine. J Dent Res. 1975;54:B57-62.
18- Silva JP, Castilho AL, Saraceni CH, Díaz IE, Paciencia ML, Suffredini IB. Anti-Streptococcal activity of Brazilian Amazon Rain Forest plant extracts presents potential for preventive strategies against dental caries. J Appl Oral Sci. 2014;22(2):91-7.
19- Silva JP, Castilho AL, Saraceni CH, Díaz IE, Suffredini IB. Anti-*Streptococcus sanguinis* activity of plant extracts. Pharmacologyonline. 2012;3:42-9.
20- Suffredini IB, Paciencia ML, Frana SA, Varella AD, Younes RN. *In vitro* breast cancer cell lethality of Brazilian plant extracts. Pharmazie. 2007;62(10):798-800.
21- Suffredini IB, Paciencia ML, Varella AD, Younes RN. *In vitro* prostate cancer cell growth inhibition by Brazilian plant extracts. Pharmazie. 2006;61(8):722-4.
22- Suffredini IB, Sader HS, Gonçalves AG, Reis AO, Gales AC, Varella AD, et al. Screening of antibacterial extracts from plants native to Brazilian Amazon Rain Forest and Atlantic Forest. Braz J Med Biol Res. 2004;37(3):379-84.
23- Trentin DS, Giordani RB, Zimmer KR, Silva AG, Silva MV, Correia MT, et al. Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against *Staphylococcus epidermidis* planktonic and biofilm lifestyles. J Ethnopharmacol. 2011;137(2):327-35.
24- Wang T, Choi RC, Li J, Bi CW, Ran W, Chen X, et al. Trillin, a steroidal saponin isolated from the rhizomes of *Dioscorea nipponica*, exerts protective effects against hyperlipidemia and oxidative stress. J Ethnopharmacol. 2012;139(1):214-20.
25- Wang W, Li P, Wang X, Jing W, Chen L, Liu A. Quantification of saponins in *Dioscorea panthaica* Prain et Burk rhizomes with monolithic column using rapid resolution liquid chromatography coupled with a triple quadruple electrospray tandem mass spectrometry. J Pharm Biomed Anal. 2012;71:152-6.
26- Younes RN, Varella AD, Suffredini IB. Discovery of new antitumoral and antibacterial drugs from Brazilian plant extracts using high throughput screening. Clinics (Sao Paulo). 2007;62(6):763-8.