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ANTI-Streptococcus sanguinis ACTIVITY OF PLANT EXTRACTS

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Summary

Objective: To evaluate the anti-bacterial activity of more than 2,000 plant extracts against commensal *Streptococcus sanguinis*, which is related to the first steps of biofilm formation, claimed to be a sign of oral health, but has been associated to the development of infectious endocarditis, artheroschlerosis and ischemia, after reaching blood stream. Also, nature plays an important role as source of new antibacterial substances likely to assist good oral health conditions.

Methods: More than 2,000 Brazilian Amazon plant extracts were tested against *S. sanguinis* using the disk diffusion assay. *Results*: Twenty six out of 2,000 extracts (1.3%) showed activity against the micro-organism when evaluated by their growth inhibition zone. All the extracts showed equal or higher antibacterial activity when compared to chlorhexidine digluconate 0.12, 1% and 2%, such as EB271, obtained from *Casearia spruceana* (growth inhibition zone of 24.65±0.50mm), or EB1129, obtained from *Psychotria* sp. (growth inhibition zone of 21.63±0.88mm).

Conclusions: The screening of Amazon plant extracts showed that *Casearia sprucena* and *Psychotria* sp. are potential sources of preventive agents that can be used in preventive care against *Streptococcus sanguinis*.

Keywords: Dental Caries; *Streptococcus sanguis;* Amazonian Ecosystem; High-Throughput Screening Assays

Introduction

S. sanguinis is an oral commensal bacterium that pioneers bacterial colonization related to oral biofilm (1), which may lead to caries formation (2). The bacterium is considered harmless in healthy individuals' oral cavity, and its frequency is antagonistically related to cariogenic *S. mutans*. *S. sanguinis* may eventually enter into circulating blood and cause severe infective endocarditis (3), and other important diseases as artheroschlerosis in risk-patients (4), specifically after a trauma.

Bacteria that pioneer biofilm formation, such as *S. sanguinis*, show a specific temporal and spatial distribution that is crucial for the development of biofilm and the maintenance of the initial phases of bacterial colonization (5). The development of caries is based on the microbial attachment to enamel and to mechanical forces of detachment in the oral cavity (6). If the biofilm is formed, there could be a progressive development to caries formation, or even to blood infection leading to severe health conditions. If the ecological balance between *S. sanguinis* and *S. mutans* related to a health condition is, for some reason, disturbed, caries may be formed starting from alterations in the bacterial composition of biofilms. Such unbalance may also lead to periodontitis or even to infective diseases as endocarditis, commonly associated to a trauma generated from dental procedures as surgical extractions, restorative procedures, periodontal probing or surgery, scaling, endodontic procedures, orthodontic manipulation, as well as brushing, flossing and gum chewing. Although still controversial, *S. sanguinis* and other oral pathogens were claimed to be platelet activators and may be related to thrombus formation implication in artheroschlerosis or even in acute ischemia(4). The relationship of *S. sanguinis* and diseases such as endocarditis or artheroschlerosis may be mediated by lipoproteins contained in the bacterium (7), which are strictly related to endocarditis virulence.

Although *S. sanguinis* is frequently associated to a "healthy condition" it is becoming clear that there is a strict relationship between *S. sanguinis* and infectious diseases other than those related to oral cavity, which is the natural environment to this bacterium. For that reason, the control of oral biofilm formation by *S. sanguinis* is a matter of general health care. Studies have demonstrated that substances as antiadhesives as chitosan and antiseptics as chlorhexidine and others were tested for their ability to inhibit *S. sanguinis* viability, so as to justify their use as chemotherapeutics in prophylactic procedures, but suggest the introduction of new oral formulations to this purpose (8).

In the face of the foregoing, it is crucial that antibacterial agents involved in the first steps of biofilm formation, particularly those obtained from nature (9), shall be investigated. Our group established a focused search for new natural products (10,11) active against oral bacteria to be used in maintenance of general health balance based on oral health, as the identification of new active compounds or even extracts to be used in dentifrices. For that reason, the present work aims the screening of anti-*Streptococcus sanguinis* agents prospected from nature.

Methods

Plant collection and extraction

Plants were randomly collected in the Amazon and Atlantic rain forests from 1997 up to 2002 (license for plant collection and access to biodiversity MMA 012A/2010). Different organs of the plant were collected, such as leaves, stem, flowers, fruits, barks, according to their biomass availability. Once biomass was not available to be collected alone, combined organs were collected together and turned out to be called "aerial parts". Each plant material was dried in air-circulating incubator (Fanem, Diadema, Brazil) at 40 °C and was ground in a hammer mill (Holmes, United States) (12). Ground plant material was put into a glass percolator (Kontes, New Jersey, United States) and solvent system composed of a 1:1 mixture of dichloromethane: methanol (Synth, Diadema, Brazil) was used,

in order to obtain a 24h-maceration. After that, the extract was drained and the solvents rotavaporated (Buchi, Switzerland). Milli-Q-grade water (Millipore, São Paulo, Brazil) was added to the remaining plant ground material that remained in the percolator and a second 24h-maceration was done. Aqueous extracts were drained from the percolator and were lyophilized. Organic and aqueous extracts were kept at -20 °C until use (13).

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.[12] Screening tests in disk diffusion assay were done with extracts prepared at 200 mg/mL Chlorhexidine digluconate (CHX; Biodinâmica®, Paraná, Brazil) was used as standard drug at concentrations of 0.12%, 1% and 2%, in the assays. The procedure resulted in more than 2,000 plant extracts, obtained from more than 660 plant species. Organic extracts received odd numbers, while aqueous extracts received even numbers.

Bacteria

All procedures were done in sterile conditions. *Streptococcus sanguis* (ATCC® 10556™, Microbiologics®, St. Cloud, Minnesota United States) were used in the 4th passages in all experiments. Bacteria were re-suspended in saline solution at a concentration of 0.5 MacFarland (corresponding to $1.5x10^8$ CFU/mL) to the disk diffusion assay (DDA) (14).

Culture medium

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

Quality control for all BHIAB Petri dishes batch was done as follows: two BHIAB Petri dishes recently prepared were first kept in an incubator for 48 hours and then left on a bench for more 48h. BHIAB Petri dishes were used if no bacteria growth was observed in control dishes.

Disk diffusion assay

Disk diffusion assay (DDA) with changes that are described below, adapted from CLSI (8th edition, Brasília, Brazil), was chosen as the screening test. BHIAB was used in the DDA. Firstly, bacteria in suspension were superficially inoculated on Petri dishes containing BHIABwith a sterile swab (Deltalab, Beijing, China). After that, sterile paper disks measuring 6 mm diameter and 1 mm high were equally distributed over the inoculated agar-blood medium. Ten µL of treatments were added to the paper disks. Petri dishes were kept in microaerophyllic-environment chambers, which were maintained in incubators (Fanem, Diadema, Brazil) at 36 °C for 48 hours. The presence of any degree of inhibition-growth zone was considered as a positive result, independent of what is determined for pure antimicrobial compounds. Extracts that inhibited bacteria growth in this method were retested in triplicate, and false positive results could be eliminated. Means and standard deviations were assessed and compared.

Statistical analysis

One-way ANOVA and Tukey post-test were used to compare means (GraphPad® Prism 5.0, La Jolla, United States). Significance was considered if $p<0.05$.

Results

High-throughput screening using DDA technique was applied on more than 2,000 plant extracts against *S. sanguis* and resulted in 26 active aqueous and organic plant extracts, which are displayed in Table 1. Botanical names, as well as date of collection, collect number, extract number and the organs of the plants used to obtain the extracts are given.

Twenty six out of 2,000 plant extracts (1.85%) inhibited bacteria growth (table 2, figure 1). CHX

prepared at 0.12%, 1% and 2% were used as standard drug. Statistical analysis assumed a normal distribution for all media ($F_{(29,179)}$=275.7; r^2=0.9816; p<0.01). According to one way ANOVA and Tukey post test, EB 271, obtained from the leaves of *Casearia spruceana* (Salicaceae, table 1) showed significant antibacterial activity when compared to CHX 2% (inhibition growth zone diameter of 24.65 mm, p<0.001). EB 1129, obtained from the aerial organs of *Psychotria* sp. (Rubiaceae, table 1), has also shown activity in DDA (inhibition-growth zone diameter of 21.63 mm, p<0.001). Results are displayed in figure 1. CHX 2% showed a significant antibacterial activity against *S. sanguinis* (p<0.001), when compared to that observed to other extracts (table 2, figure 1).

Discussion

S. sanguinis is a commensal bacterium related to the initial steps of biofilm formation, frequently associated to a health oral condition. Recent findings show that the development of some diseases as infectious endocarditis, artherosclerosis and ischemia can be associated to *S. sanguinis* that invaded blood stream, particularly in individuals with particular and fragile health conditions. For that reason, the identification of antibacterial substances to be used as a preventive agent in biofilm formation is crucial for a general health condition. The establishment of a high-throughput screening program aiming the track of active natural products from plants using classical antimicrobial assays, such as DDA was performed to more than 2,000 Amazon plant extracts, based on the literature (15, 16).

The extracts that were selected as the most active ones in DDA were EB 271, obtained from the leaves of *Casearia spruceana*, and EB 1129, obtained from the aerial organs of *Psychotria* sp. (Rubiaceae). No pharmacological studies have been done with *C. spruceana*, but species belonging to the genus *Casearia* were well documented as *C. sylvestris*. Clerodane diterpenoids named casearins A-F were isolated from *C. sylvestris* (17), and since then many authors verified different pharmacological or biological properties related to the group of compounds, such as antitumor, antiulcer (18), anti-snake and anti-bee (19) venoms, antiplasmodial (20), anti-inflammatory activity (21) and anti-hyperlipidemic (22).

Plants have been a source of new antimicrobials. In Dentistry, plants are being tested as antimicrobial agents in irrigation solutions (23), in biofilm control (24) and against oral cancers as squamous cell carcinoma (25). In the present work, the active extracts showed to have a potential pharmacological use in the maintenance of a general improved health condition, to be incorporated in dentifrices, in the near future.

Conclusions

Twenty six out of more than 2,000 Brazilian Amazon plant extracts have been identified as anti-*Streptococci* potential agents. Extracts EB 271 and EB 1129 showed significant antibacterial activity against *S. sanguinis*. The recent findings open a wide range of concerns to be further taken into account, and highlights the importance of the vast Amazon rain forest biodiversity as a source of new oral products to be used in Dentistry and consequently in general health care.

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see Table 1.

see Table 2.

see Fig. 1

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Collect #	Extract #	Organ	Family	Genus	Species	Author
PSC252	71	Aerial organs	Boraginaceae	*Cordia*	sp.	
AAO3330	271	Leaves	Salicaceae	*Caseria*	*spruceana*	Benth. Ex Eichler
AAO3330	272	Leaves	Salicaceae	*Caseria*	*spruceana*	Benth. Ex Eichler
AAO3292	309	Wood	Rabdodendraceae	*Rabdodendron*	sp.	
PS91	327	Aerial organs	Fabaceae	*Swartzia*	sp.	
PS265	601	Wood	Fabaceae	*Swartzia*	sp.	
AAO3362	607	Stem	Ebenaceae	*Diospyros*	sp.	
AAO3299	631	Stem	Rutaceae	*Zanthoxylum*	*compactum*	(Huber ex Albuq.) P.G.Waterman
AAO3491	869	Stem	Ebenaceae	*Diospyros*	*guianensis*	(Aubl.) Gurke
AAO3494	921	Roots	Rubiaceae	*Psychotria*	sp.	
AAO3470	935	Stem	Flacourtiaceae	*Xylosma*	*pseudosalzmanii*	
AAO3561	1059	Wood	Fabaceae	*Swartzia*	sp.	
AAO3586	1063	Wood	Bignoniaceae	*Memora*	*longilinea*	
AAO3580	1099	Stem	Rubiaceae	*Psychotria*	sp.	
AAO3579	1115	Wood	Myrtaceae	-	-	
AAO3543	1119	Stem	Boraginaceae	*Cordia*	cf. *exaltata*	Lam.
AAO3580	1129	Aerial organs	Rubiaceae	*Psychotria*	sp.	
AAO3688	1227	Wood	Fabaceae	*Swartzia*	sp.	
AAO3717	1303	Wood	Rubiaceae	*Palicourea*	sp.	
IBS95	1355	Stem and leaves	Rubiaceae	*Palicourea*	sp.	
IBS61	1383	Aerial organs	Boraginaceae	*Cordia*	*nodosa*	Lam.
IBS100	1401	Aerial organs	Rubiaceae	*Palicourea*	sp.	
IBS113	1403	Wood	Ebenaceae	*Diospyros*	sp.	
AAO4035	1489	Aerial organs	Convolvulaceae	*Ipomoea*	sp.	
AAO4067	1539	Aerial organs	Salicaceae	*Casearia*	*javitensis*	H.B.K.
AAO3812	1779	Aerial organs	Dioscoreaceae	*Dioscorea*	sp.	

Table 1. Botanical data related to the plants used to obtain the active anti-*Streptococcus sanguis* extracts.

Extract number	Disc 1 (V)	Disc 1 (H)	Disc 2 (V)	Disc 2 (H)	Disc 3 (V)	Disc 3 (H)	Mean	S.E.
N271	24.95	24.95	24.00	24.00	25.00	25.00	24.65	0.50
N1129	20.20	21.00	22.00	21.95	22.65	22.00	21.63	0.88
N1779	17.85	17.95	17.30	17.95	20.00	19.00	18.34	0.98
N1099	18.95	18.95	18.00	17.00	17.50	17.00	17.90	0.89
N1539	16.80	16.95	18.40	18.00	18.70	17.95	17.80	0.77
N1063	14.00	15.00	15.00	14.95	14.80	14.95	14.78	0.39
N1303	16.00	16.00	18.80	17.95	19.00	18.00	17.63	1.33
N869	17.40	17.80	17.80	18.00	16.70	17.10	17.47	0.50
N1119	17.00	16.60	17.95	18.00	16.00	17.20	17.13	0.78
N1489	16.00	16.00	17.00	17.20	16.00	17.00	16.53	0.59
N1059	16.00	16.00	16.00	16.35	17.00	17.00	16.39	0.49
N1403	15.00	16.00	15.55	16.80	16.00	15.00	15.73	0.69
N1383	16.80	15.95	16.00	17.00	14.00	14.00	15.63	1.33
N309	15.00	16.00	16.00	16.00	15.00	15.00	15.50	0.55
N272	15.00	15.00	16.00	16.00	15.80	15.00	15.47	0.52
N935	14.80	15.00	14.00	14.60	15.00	14.00	14.57	0.46
N607	14.65	14.00	13.00	12.50	15.00	15.00	14.03	1.07
N601	13.00	12.95	13.55	13.80	15.95	13.90	13.86	1.10
N1115	12.00	13.00	12.45	12.60	12.95	12.00	12.50	0.44
N1227	12.00	12.60	12.45	11.00	11.00	11.00	11.68	0.77
N631	11.60	11.00	11.60	11.00	11.00	11.70	11.32	0.35
N327	9.00	10.00	11.60	10.40	12.00	13.00	11.00	1.46
N71	10.00	9.85	12.00	11.00	11.00	11.70	10.93	0.87
N921	11.00	11.00	11.00	10.00	10.65	11.00	10.78	0.41
N1355	10.00	10.40	10.35	11.00	11.00	10.40	10.53	0.40
N1401	11.00	11.00	10.00	10.10	10.00	10.85	10.49	0.51
CHX 2%	14.15	14.00	15.05	13.85	15.40	14.00	14.41	0.65
CHX1%	13.90	14.45	15.35	14.90	14.35	14.65	14.60	0.50
CHX0.12%	10.10	9.50	10.55	10.40	10.45	10.90	10.32	0.48
DMSO 50%	-	-	-	-	-	-		
DMSO 100%	-	-	-	-	-	-		

Table 2. Disk diffusion assay assessment of different antibacterial plant extracts against *Streptococcus sanguinis*.

Results are displayed as the diameter of growth inhibition zones [mm], taken both diagonally (D) and horizontally (H) in each of the three disks tested for each plant extract.
Extract's odd numbers correspond to organic extract; extract's even numbers correspond to aqueous extracts.

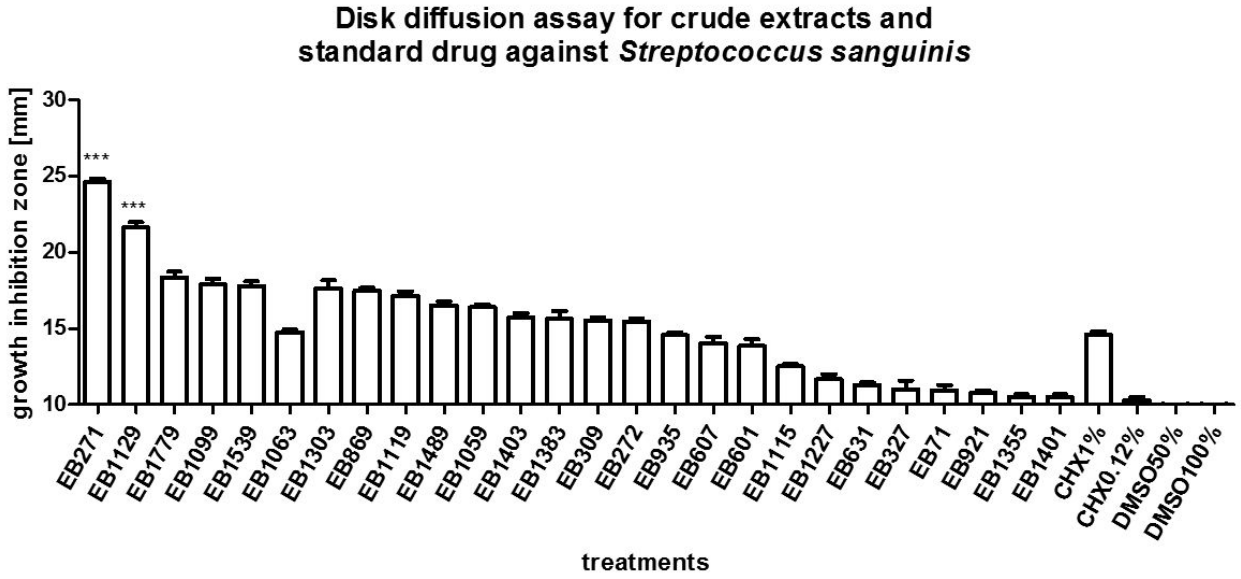

Figure 1. Results of the anti-*Streptococcus sanguinis* activity of crude extracts obtained from the Amazon rain forest.

Growth inhibition zones obtained from disk diffusion assay are given (n=6; n_{total}=246; 30 groups).
One-way ANOVA statistics test was applied, followed by Tukey post test, significance if $p<0.05$. Chlorhexidine digluconate (CHX) at 0.12% and 1% and dimethylsulfoxide 50% and 100%, were used as standard drug and vehicle control, respectively.. One-way ANOVA ($p<0.05$; n=6; n_{total}=252).