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IN VITRO EFFECT OF AMAZON PLANTS SEED EXTRACTS ON STREPTOCOCCUS MUTANS.

ABSTRACT

Dental caries is an oral disease that affects more than 50% of the Brazilian population. Streptococcus mutans is the leading cause of dental caries. The chemical control of dental plaque can be done as a prophylactic or therapeutic treatment, using products of plant origin, with the advantage of having lower rate of side effects, while delivering same quality and efficiency, when compared to synthetics. Seeds of 32 plant species were selected and tested for their ability to inhibit the growth of Streptococcus mutans in culture media, as well as to remove dental plaque by using extracted teeth from humans. The chlorhexidine was used as positive control in all tests. Some seed extracts have presented halos of S. mutans growth inhibition, behaving like chlorhexidine diluted to 0.2%, 0.1% and 0.05%, as Hymenaea courbaril, Zygia cauliflora, Mucuna urens, Parkia pendula and Ricinuscommunis. The seed extracts of the following species have removed 41.6 x 10^7- 39.6 x 10^7 cells, showing significant removal of dental biofilm: Cariniana micrantha, Hymenaea courbaril, Zygia cauliflora, Mucuna urens, Parkia pendula, Ricinus communis, Cucumis sativus and Ormosia excelsa. The results allowed drawing a profile of species, among the 32, for possible future use in dentistry.

Keywords: Antimicrobial activity, Dental plaque, Chlorhexidine, Halo of inhibition, Dental biofilm, Dental carie.

1.0 INTRODUCTION

The mouth cavity of human beings supports a complex microbiota, which reflects the diversity of habitats and ecosystems located therein. It includes a dynamic network of over 500 species of microorganisms, such as bacteria, fungi, protozoan, and mycoplasm (14). Streptococci from the mutans group (particularly Streptococcus mutans and Streptococcus sobrinus) have been strongly identified as the microorganisms that cause dental caries (11). Carie is an oral disease that affects more than 50 percent of the Brazilian population (17).

Removing dental plaque biofilm is an important factor in preventing caries and other periodontal diseases. In view of the limitations of mechanic hygienic methods, antimicrobial agents are being studies as a way of controlling dental biofilm (20). Some plant-based products are used for oral hygiene, with the advantage of having a lower rate of side effects when compared to synthetic products (15, 8, 13). Therefore, with a scientific study of the use of certain plants in oral disease, dentistry could benefit the population by using phytotherapy to resolve ordinary oral problems, by producing plant-based products for daily use (1, 10).

This paper is aimed at assessing the in vitro effect of Amazon plant seed extracts on Streptococcus mutans ATCC 25175.

2.0 MATERIAL AND METHODS

2.1 Selection of plant species

Thirty-two seeds were selected among the species provided by the Germplasm Bank of the National Institute of Amazonian Research. Some of the species used are not native to the Amazon, but were rather introduced in the region, adapting very well to local conditions.

2.2 Extract preparation

A methodology that involved soaking the seeds in water was used to obtain an aqueous extract, based on the first stage of the seed germination, the imbibition, when the seed imbibes water and hydrolytic enzymes are activated, which breaks down stored food resources (starch, proteins or oils) into metabolically useful chemicals. Seed asepsis was carry out by using a 2% sodium hypochlorite solution, for three minutes. Next, the seeds were washed in sterilized distilled water (3). They were then soaked in sterile water for 24 hours, at a ratio of 28 grams seed to 100 mL water, for the purpose of releasing secondary metabolites that could be significant from a scientific perspective (19, 12). Aqueous extracts through soaking the seeds were obtained from the following species: Dinizia excelsa, Parkia multijuga, Leucaena leucocephala, Swartzia laevicarpa, Campsiandra comosa, Artocarpus heterophyllus, Zygiacauliflora, Acosmium nitens, Mucuna urens, Ormosia excelsa, Enterolobium schomburgkii, Buchenavia huberi, Peltogyne paniculata, Hymenaea courbaril, Parkia pendula, Schizolobium amazonicum, Psophocarpus tetragonolobus, Abelmoschus esculentus, Cucumis sativus, Vigna unguiculata, Basella rubra, Vigna unguiculata, Cucumis melo, Cichorium intybus, Morinda citrifolia, Persea Americana, Ricinus communis, Terminalia catappa, and Eugenia stipitata.

2.3 Streptococcus mutans growth

The growth of gamma Streptococcus mutans non-hemolytic, was obtained through Tryptic Soy Agar culture. The culture media was then distributed in Petri dishes and tubes for the purpose of cultivating and maintaining the cultures.

2.4 Microbiological analysis

The microbiological analysis of the samples was made based on a comparative method. The seed extract samples were assessed for their microbial properties and the ability to remove dental plaque biofilm, having Streptococcus mutans as a reference.

2.5 Evaluation of the extracts antimicrobial activity on S. mutans using the disc diffusion method

Antimicrobial properties were analyzed through in vitro bactericidal and bacteriostatic action of the extracts on S. mutans. Culture media was distributed in Petri dishes and chilled. The inoculum (liquid culture media containing the bacteria) was added to each dish at a rate of 0.5mL. Five sterile paper filter discs, measuring 5 mm in diameter, were individually soaked in the extracts and added on top of the dishes, along with one disc in the center, with sterile water. Chlorhexidine at 2% and also diluted in water at 0.4, 0.2, 0.1 and 0.05% were used as treatment (4, 7). The quantitative analysis took into account the size of the halos created by the seed extracts in inhibiting the growth of S. mutans at the culture media on the Petri dish. Therefore, the halos of inhibition were measured with a digital caliper and statistically analyzed.

The values listed were measured in mm of actual inhibition halos, calculated based on the difference between the overall halos and the diameter of the disc, i.e., the measure of bioactive molecule diffusion in the bacteria culture media.

2.6 Evaluation of S. mutans survival in the presence of the extracts

An amount of 1.5mL of inoculated media with S. mutans was used and 0.3mL of seed extract for each test tube. An amount of 1.8 mL of inoculated media with S. mutans was used for negative control. For positive control, 1.5 mL of liquid media with S. mutans was used along with 0.3 mL of chlorhexidine (2%, 0.4%, 0.2%, 0.1% and 0.05%). After 24 hours, a survival test was conducted on the bacteria, exposing it to the seed extracts. A rate was obtained from the tube, which was then streaked out on the Petri dishes. The dishes were kept in an incubator at 36.5°C, and an assessment was conducted after 24 hours. According to the way colonies developed in the four areas of the dish (Figure 1), a score was assigned based on growth in each area ranging from 1 (without and visible growth in the dish) to 4 (maximum growth in all areas) (18). Scores were analyzed from a statistical perspective.

Figure 1. Scale to assess survival of S. mutans in the presence of plant-based extracts.

2.7 Dilution test to evaluate the number of cells/mL equivalent of each value obtained on the Petri dishes

Erlenmeyers with S. mutans culture media were placed on a shaker for 72 hours for culture growth purposes. The diluting solution used was 0.2% sterile magnesium sulfate. The following dilutions were used: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}. The dishes were kept in an incubator at 36.5°C for 24 hours. Scores ranging from 1 to 4 were assigned for growth, based on the way the colonies developed in the four areas of the dish. Three Petri dishes with 10^{-3}, 10^{-5} and 10^{-7} dilution were used to count the colonies.

2.8 Evaluation of dental biofilm removal by the seed extracts

To test biofilm removal, previously sterilized teeth were tied on a string and immersed in Erlenmeyers containing media inoculated with S. mutans, kept in a shaker for a week. The goal was to develop biofilm on the teeth, caused by the bacteria present in the culture. After the biofilm developed, the teeth were immersed for 40 minutes at a time in test tubes, each containing a seed extract. For negative control, sterile water was used and, for positive control, chlorhexidine (2%, 0.4%, 0.2%, 0.1%, and 0.05%). The degradation of bacteria on the teeth was evaluated based on the amount of bacteria found in the suspension present in the tubes. A Neubauer-House hemocytometer was used to count the bacteria. The greater the number of S. mutans cells removed from the teeth, the higher the number of cells present in the solution, and so is the counting in the hemocytometer.

3.0 STATISTICAL ANALYSIS

A statistical analysis was conducted using the ESTAT 2.0 program. The level of statistical significance adopted was 5%, conducted through a Tukey test.

4.0 RESULTS AND DISCUSSION

4.1 Evaluation of the extracts antimicrobial activity on S. mutans using disc diffusion method

It was possible to observe halos of inhibition and halos of stimulation of S. mutans growth with seed extracts from several species that were tested. Figure 2 demonstrates this result using extracts from seeds of Zygia cauliflora. Halos of stimulation of Streptococcus mutans growth were always found outside the halos of inhibition. The position of the growth stimulation halo after the inhibition halo is likely due to the higher molecular weight of the inhibiting substances, which go through a smaller distance from the paper disc in the media, and the lower molecular weight of stimulating substances, which go through a greater distance.

Figure 2. Halos of inhibition and halos of stimulation of Zygia cauliflora on S. mutans, as a result of the properties of the aqueous extract obtained through soaking the seeds.

In the statistical analysis of halos of inhibition, 2% chlorhexidine followed by 0.4% chlorhexidine presented the greatest inhibition halos. The statistical analysis also demonstrated that the chlorhexidines (0.2%, 0.1% and 0.05%) had similar results, not only when compared among themselves, but also with the species Hymenaea courbaril, Zygia cauliflora, Mucuna urens, Parkia pendula, and Ricinus communis, with halos of inhibition measuring from 3.9 to 5.9mm. The Cucumis sativus, Ormosia excelsa, Eugenia stipitata, Vigna unguiculata, Buchenavia huberi, Dinizia excelsa, Morinda citrifolia, Peltogyne paniculata, and Swartzia laevicarpa species had halos of inhibition measuring between 2.5 and 3.7mm.

4.2 Evaluation of S. mutans survival in the presence of the extracts

Three 10^{-7} dilution dishes were used to count the colonies, which had on average $1x10^{10}$ cells/mL. Based on the data, a graph was created showing the relation between the number of cells/mL and the scores achieved (Figure 3). This test demonstrated the amount of cells/mL as a function of the score that each extract obtained. Each score associated with the survival of S. mutans in the seed extracts were represented on y axis (Log_{10} of the estimated population of S. mutans).

Figure 3. Number of cells/mL (Log10) and scores obtained in the tests on Petri dishes.

The data on the survival rate of S. mutans immersed in the seed extracts demonstrated that 2%, 0.4% and 0.2% chlorhexidine have shown similar behavior, in that there was no bacterial growth in the dishes. Next, the 0.1% and 0.05% chlorhexidine and the Hymenaea courbaril, Parkia pendula, Mucuna urens, Ricinus communis, and Zygia cauliflora were the most effective against S. mutans, significantly inhibiting its growth on the Petri dishes. The final estimated population of S. mutans was 1.8×10^5 cells/mL, a 50-fold reduction, which could potentially reduce the action of the bacteria on the teeth, if they were used one day as dental products.

Seed extracts of Cucumis sativus and Morinda citrifolia, reduced the S. mutans population by 10 fold, when compared with the control (water). Acosmium nitens, Artocarpus heterophyllus, Campsiandra comosa, and Schizolobium amazonicum, reduced the population 4 fold. The other species had similar statistical results compared to the negative control (water).

4.3 Evaluation of S. mutans survival in the presence of the extracts

According to the results obtained and the statistical analysis of the number of cells that were removed by the extract (Table 1), the species that were the most effective in removing biofilm were Hymenaea courbaril, Mucuna urens, and Zygia cauliflora, with removal results higher than 41.6×10^7 cells. Parkia pendula, Ricinus communis, Cucumis sativus, and Ormosia excelsa removed from 37.6 to 39.6×10^7 cells, followed by Eugenia stipitata, Vigna unguiculata, Peltogyne paniculata, Swartzia laevicarpa, Campsiandra comosa, Dinizia excelsa, Morinda citrifolia, and Buchenavia huberi, which removed from 29.6 to 33.6×10^7 cells. They were followed by Acosmium nitens, Artocarpus heterophyllus, Leucaena leucocephala, Schizolobium amazonicum, Psophocarpus tetragonolobus, and Parkia multijuga, which removed from 21.6 to 26.8×10^7 cells. Enterolobium schomburgkii, Persea americana, Basella rubra, Cichorium intybus, and Cucumis melo, Abelmoschus esculentus, Vigna unguiculata, and Terminalia catappa removed from 10 to 2×10^7 cells.

Table 1. *Streptococcus mutans* removal by seed extracts from dental surface.

The 2% chlorhexidine and its dilutions produced a milky reaction at the time when the teeth with dental biofilm got in contact with the chlorhexidine, and findings regarding the amounts of cells left demonstrate that it failed to adequately remove the biofilm that was on the teeth (Table 1). Zaura-Arite et al (18), Auschill et al (1) and Zanatta et al (17) demonstrate that chlorhexidine is ineffective in removing the most internal layers of biofilm with more than 24 hours of formation. In this study, the teeth were kept in the shaker for one week, for the purpose of developing biofilm. It is recommended that biofilm developed longer than 24 hours, should be removed with the help of mechanic processes, such as brushing and the use of dental floss, to complement the chemical properties of the chlorhexidine.

Halos of inhibition of S. mutans were also observed in Soares et al (16), in which a 20% alcoholic extract of Schinus terebinthifolius was used, proving that it is effective in removing contamination from tooth brushes. Seed extracts from the following species present halos of inhibition of S. mutans, in much the same way as 0.2, 0.1, and 0.05% chlorhexidine: Hymenaea courbaril, Zygia cauliflora, Mucuna urens, Parkia pendula, and Ricinus communis.

The Agar diffusion technique that was used in this study was also used in a paper by Mussi et al (13), which evaluated the effect of a Copaifera officinalis solution on Streptococcus mutans. Results indicate that a 10% Copaifera officinalis solution may be a possible alternative to combat S. mutans, and inhibit bacterial growth.

The property to inhibit the development of dental plaque and oral microbiota, such as Streptococcus mutans and Staphylococcus aureus, was tested using an Aster lanceolatus ethanol extract. For positive control, 0.2% chlorhexidine was used. An Aster lanceolatus ethanol extract inhibited the growth of all microorganisms tested, exceeding the results obtained with positive control, similar to the report by Dias et al (6).

The antimicrobial potential of the Zingiber officinalle extract was evaluated in the study of Grégio et al (9) and Daud et al (5), and presented significant antibacterial and antifungal action for dentistry, with the possibility of being able to contribute to the treatment of oral diseases. According to our study, the seed extracts of the following species also contributed with significant effectiveness in removing dental plaque biofilm: Hymenaea courbaril, Zygia cauliflora and Mucuna urens, removing more than 41.6 x 10^7 cells, and Parkia pendula, Ricinus communis, Cucumis sativus, and Ormosia excelsa, with 37.6 to 39.6 x 10^7 cells removed.

5.0 FIGURES

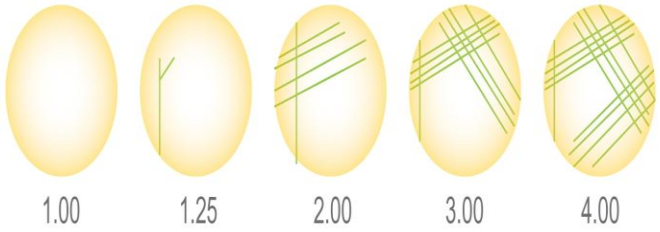

Figure 1 - Scale to assess survival of *S. mutans* in the presence of plant-based extracts.

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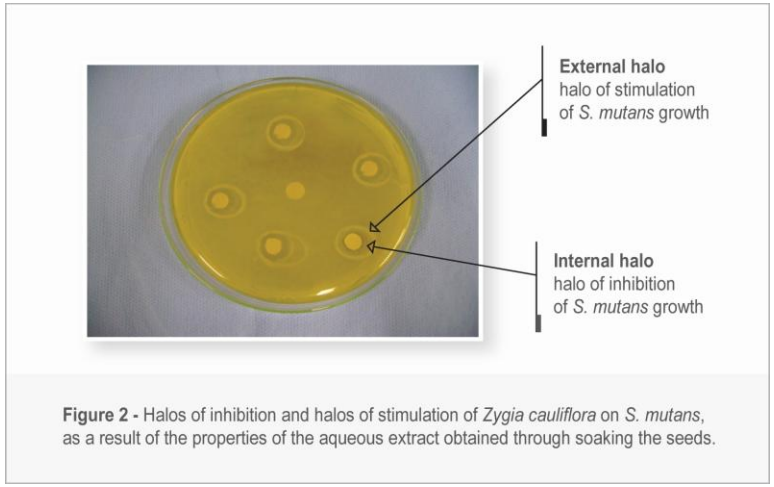

Figure 2 - Halos of inhibition and halos of stimulation of *Zygia cauliflora* on *S. mutans*, as a result of the properties of the aqueous extract obtained through soaking the seeds.

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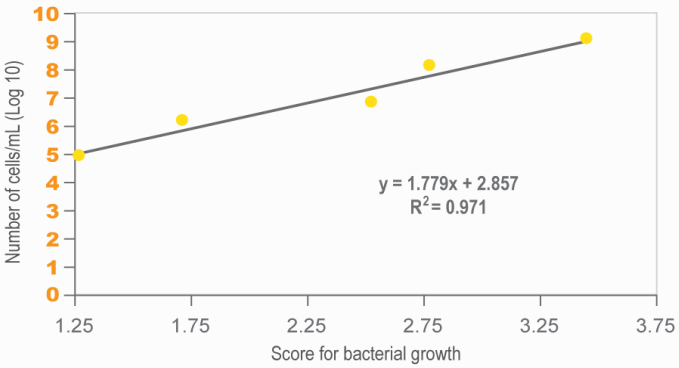

Figure 3. Number of cells/mL (Log10) and scores obtained in the tests on Petri dishes.

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6.0 TABLE

Treatments	Number of cells removed x10^7	
	per mL	per tooth
Hymenaea courbaril	10.7ab	42.8
Mucuna urens	10.5ab	42.0
Zygia cauliflora	10.4ab	41.6
Parkia pendula	9.9cd	39.6
Ricinus communis	9.9cd	39.6
Ricinus communis	9.7d	38.8
Cucumis sativus	9.5d	38.0
Ormosia excelsa	9.4d	37.6
Eugenia stipitata	8.4e	33.6
Vigna unguiculata	8.1ef	32.4
Peltogyne paniculata	7.9ef	31.6
Swartzia laevicarpa	7.6f	30.4
Campsiandra comosa	7.5fg	30.0
Dinizia excelsa	7.5fg	30.0
Morinda citrifolia	7.5fg	30.0
Buchenavia huberi	7.4ef	29.6
Acosmium nitens	6.7h	26.8
Artocarpus heterophyllus	6.4hi	25.6
Leucaena leucocephala	6.4hi	25.6

Schizolobium amazonicum	6.4hi	25.6
Psophocarpus tetragonolobus	5.7ij	22.8
Parkia multijuga	5.4j	21.6
Enterolobium schomburgkii	2.5k	10.0
Persea americana	2.5k	10.0
Basella rubra	2.4k	9.6
Cichorium intybus	2.4k	9.6
Cucumis melo	2.2kl	8.8
Abelmoschus esculentus	1.62mn	6.5
Vigna unguiculata	0.87no	3.5
Terminalia catappa	0.5o	2.0
Water	0.0p	0.0
Chlorhexidine 2%	0.0p	0.0
Chlorhexidine 0.4%	0.0p	0.0
Chlorhexidine 0.2%	0.0p	0.0
Chlorhexidine 0.1%	0.0p	0.0
Chlorhexidine 0.05%	0.0p	0.0

Table 1: *Streptococcus mutans* removal by seed extracts from dental surface.

7.0 CONCLUSION

In conclusion, seed extracts from the species that presented the best results in the present study, after further research about the elucidation of the dental plaque removal substances, biological assays and toxicity, may be effective components in mouth washes and tooth pastes.

8.0 ACKNOWLEDGMENTS

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