ACTIVITY OF IN VITRO FORMS OF DENTIFRICES CONTAINING THE HYDROALCOHOLIC EXTRACT OF THE RIPE FRUIT OF EUGENIA UNIFLORA L. (SURINAM CHERRY) ON CARIOGENIC BACTERIA

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ABSTRACT

The aim of this study was to evaluate the in vitro activity of dentifrices containing the hydroalcoholic extract of the ripe fruit of Eugenia uniflora L. (Surinam cherry) on Streptococcus oralis (ATCC 10557) and Lactobacillus casei (ATCC 7469). Five dentifrices were used: D1: containing hydroalcoholic extract of Eugenia uniflora L.; D2: containing fluoride and hydroalcoholic extract of Eugenia uniflora L.; D3: containing triclosan and hydroalcoholic extract of Eugenia uniflora L; D4: containing triclosan, fluoride and hydroalcoholic extract of Eugenia uniflora L.; D5: positive control (Colgate Total 12®). To determine the antibacterial activity, the technique used was the minimum inhibitory concentration by the diffusion method in solid culture medium. At the concentration 0.05 g / mL, the best results were achieved with D1 (18mm) and D4 (24mm) on L.casei, and with D3 (19mm) on S. oralis. The dentifrices D3 and D4 were found to have greater activity on the Streptococcus oralis, while D4 and D1 were found to have greater activity on Lactobaccilus casei. It is concluded that dentifrices with Eugenia uniflora L. have antimicrobial activity, suggesting that clinical trials should be conducted.

Key words: Microbiology, Dentifrices, Plants, Medicinal.

ATIVIDADE IN VITRO DE DENTIFRÍCIOS CONTENDO EXTRATO HIDROALCÓLICO DA FRUTA MADURA DA EUGENIA UNIFLORA L. (PITANGA) SOBRE BACTÉRIAS CARIOGÊNICAS

RESUMO

Este estudo avaliou in vitro a atividade de dentifrícios contend o extrato hidroalcólico da fruta madura da Eugenia uniflora L. (Pitanga) sobre Streptococcus oralis (ATCC 10557) e Lactobacillus casei (ATCC 7469). Foram utilizados 5 dentifrícios: D1: extrato hidroalcólico da Eugenia uniflora L.; D2: flúor e extrato hidroalcólico da Eugenia uniflora L.; D3: triclosan e extrato hidroalcólico da Eugenia uniflora L; D4: triclosan, flúor e extrato hidroalcólico da Eugenia uniflora L.; D5: controle positivo (Colgate Total 12®). Para determinar a atividade antibacteriana, a técnica utilizada foi a da concentração inibitória mínima por meio da difusão em meio sólido. Na concentração de 0.05 g/mL os melhores resultados foram encontrados no D1 (18mm) e D4 (24mm) sobre L.casei. e 19mm sobre S. oralis para o D3. Observou-se que os dentifrícios D3 e D4 mostraram maior atividade sobre Streptococcus oralis, enquanto os dentifrícios D4 e D1 sobre Lactobaccilus casei. A partir dos achados, conclui-se que os dentifrícios com Eugenia uniflora L. possuem atividade antibacteriana, sugerindo a realização de ensaios clínicos.

Palavras-chave: Microbiologia, Dentifrícois, Plantas medicinais.

INTRODUCTION

The methods of prevention and control of dental caries include the use of toothbrush and mouthwash, classified as forms of mechanical and chemical removal of dental biofilm, respectively. Dentifrices are supporting forms for controlling this disease because they contain abrasive substances that remove biofilm mechanically and chemical agents that inhibit the development of pathogenic microorganisms[1].

Among the microorganisms involved in the carious process is *Streptococcus*, which has significant acidogenic and aciduric activities, in addition to the ability to express glycoproteins in their cell mem-

brane, which facilitate the adhesion process on dental tissues, thus resulting in the formation of a pathogenic biofilm[2].

Other bacterial species may be involved in the pathogenesis of dental caries, such as *Lactobacillus*, which acts mainly at the level of pits, fissures and areas of retention in the teeth and is related to the disease progression. The production of acids by these microorganisms from the metabolism of sugar can generate microenvironments with pH below 3.5, resulting in tooth demineralization[3].

Thus, some chemical agents are recommended for the control of microorganisms that make up the oral microbiota, in order to restore the ecological balance of the oral cavity[4]. Among the synthetic substances are chlorhexidine, triclosan, sodium sulfate lauryl and sodium fluoride, which, however, have limitations (microbial resistance, side effects, cost). Therefore, other substances of natural origin have been investigated against a wide range of oral diseases[5-7]. Brazil is said to be emerging as a promising country in the discovery of natural products with pharmacological activity, owing it to the great variety in available flora[8].

The use of hydroalcoholic extract of the ripe fruit of Surinam cherry (*Eugenia uniflora* L) thus appears as an option, since it has proven antibacterial action[9]. *Eugenia uniflora* L., a Brazilian native species, is a medicinal plant that has attracted the interest of researchers. Belonging to *Myrtaceae* family, the *Eugenia uniflora* L. is popularly known as common pitanga, true pitanga, ubipitanga, red pitanga, Surinam cherry or pitanga-cuba[10]. The Brazilian Ministry of Health has suggested that the public health system should conduct pharmacological research involving *Eugenia uniflora* L.[11].

The use of extracts originated from this botanical material has been justified by the low toxicity of *Eugenia uniflora* L., which is safe for oral use[12]. Furthermore, the fruit, which is the botanical material used in this research, is eaten as food by the population, reinforcing the idea that it has low levels of toxicity and indicating that its use is safe.

Thus, it was purpose of this study to evaluate the antibacterial activity of a dentifrice containing the hydroalcoholic extract of *Eugenia uniflora* L. (Surinam cherry), as well as to identify a possible synergism or antagonism when it is combined with other chemicals with proven antibacterial activity commonly found in commercially available dentifrices.

MATERIAL AND METHODS

The research was conducted in two stages: 1) production of the hydroalcoholic extract of the ripe fruit of *Eugenia uniflora* L. and preparation of the dentifrice, and 2) microbiological tests for determining antibacterial activity.

Stage 1

Production of the hydroalcoholic extract of the ripe fruit of Eugenia uniflora L. (Surinam cherry)

The procedures for extraction and preparation of dentifrices containing the hydroalcoholic extract of the ripe fruit of Surinam cherry were performed at the Natural Chemicals Laboratory (NCL), Department of Chemistry, Center of Exact Sciences and Nature (CESN), Federal University of Paraiba, Paraíba, Brazil.

Eugenia uniflora L. fruits were collected in the municipality of João Pessoa - Paraíba - Brazil, from December 2007 to February 2008. From this plant material, three exsiccates of flowers and/or fruits were duly made and deposited in the Herbarium Lauro Pires Xavier of the CESN/FUPB for botanical identification.

The hydroalcoholic extracts of the powdered fruit of *E. uniflora* L. were obtained using Soxhlet extractors (for 72 consecutive hours). Parts of the extracts were submitted to systematic motion to remove dyes, greases and apolar substances. The plant material was dried in an incubator at 60^0C with forced air circulation. The extracts, degreased and free of dyes, were evaluated according to their microbiological activity (antibacterial) in the techniques shown below.

The dentifrices were produced from the hydroalcoholic extract of the fruit of *Eugenia uniflora* L.

Formulation of dentifrices containing Eugenia uniflora L.

Dentifrices were prepared at a prescription pharmacy in João Pessoa city – Paraíba, Brazil.

Every 10 mL of dentifrice containing *Eugenia uniflora* L. has the following composition:

Hydroalcoholic extract of the ripe fruit of Eugenia uniflora L 3%

Preservatives (parabens) 0.02

Dentifrice base ..... q.s. (quantum sufficit)

Five dentifrices containing different compositions were made, as shown in Table 1.

Table 1: Composition of dentifrices evaluated according to their active substances.

D1	Dentifrice containing only the dentifrice base and hydroalcoholic extract of *Eugenia uniflora* L.
D2	Dentifrice containing the base plus fluoride at a concentration of 1450 ppm and hydroalcoholic extract of *Eugenia uniflora* L.
D3	Dentifrice containing the base plus triclosan at a concentration of 0.3% and hydroalcoholic extract of *Eugenia uniflora* L.
D4	Dentifrice containing the base plus triclosan, fluoride and hydroalcoholic extract of *Eugenia uniflora* L.
D5	Dentifrice used as positive control (Colgate Total® 12).

Stage 2

Microbiological Tests - Determination of Minimal Inhibitory Concentration

To perform the determination of *in vitro* antimicrobial activity of dentifrices containing *Eugenia uniflora* L, the diffusion in solid culture medium technique was used. Serial dilutions were prepared from the dentifrices of hydroalcoholic extract of ripe fruit of *Eugenia uniflora* L. (Surinam cherry) and of the positive control (Colgate Total® 12) for *in* vitro analysis of the Minimum Inhibitory Concentration (MIC).

The bacterial strains of *Streptococcus oralis* (ATCC 10557) and *Lactobacillus casei* (ATCC 7469) were reactivated in liquid BHI (Brain Heart Infusion) (DIFCO® São Paulo- SP) medium and cultured in Blood Agar medium (Mueller Hinton Agar - DIFCO® plus 5% human blood) by the flooding technique. Wells measuring 6 mm across were perforated in the culture medium. 50µL of the supernatants from the dentifrices evaluated were placed in the wells. To obtain the supernatant, a solution with 3g of dentifrice in 10mL of sterile distilled water was centrifuged at 5000 rpm for 10 minutes to precipitate the solid particles.

After centrifugation, each dentifrice generated a supernatant from which serial dilutions from 0.3 g/mL to 0.005 g / mL were prepared. The plates were incubated in bacteriological incubator at 37°C for 24 hours in microaerophilic conditions. MIC was considered as the lowest concentration of the product able to inhibit bacterial growth.

Data were analyzed through Kruskal-Wallis statistical test, followed by Dunn's post-test. These tests are available in the statistical program Graphpad Prism 5.0.

Study of interference of chemicals with recognized antibacterial activity (fluoride and/or triclosan) on the effect of hydroalcoholic extract of Eugenia uniflora L.

The effect was considered synergistic when the zone of inhibition of microbial growth formed by the combined application of the hydroalcoholic extract of *Eugenia uniflora* L. plus the chemicals used (fluoride and triclosan) had a diameter that was at least 2 mm greater than the zone of inhibition formed by the action of the hydroalcoholic extract of *Eugenia uniflora* L. alone[13]. The effect was considered antagonistic when the zone of inhibition caused by the combined action of the hydroalcoholic extract of *Eugenia uniflora* L. and the chemical substances was smaller than that developed by the isolated action of hydroalcoholic extract of *Eugenia uniflora* L. The effect was considered indifferent when the zone of inhibition resulting from combined application of hydroalcoholic extract of *Eugenia uniflora* L. more fluorine and/or triclosan had a diameter equal to that resulting from isolated application of the hydroalcoholic extract of *Eugenia uniflora* L.

RESULTS

Tables 2 and 3 show the results of dentifrices against the microorganisms.

All dentifrices evaluated showed considerable antibacterial activity on *S. oralis*, with MIC 0.005 g/mL. When the antibacterial activity on *L. casei* was evaluated, all the dentifrices, except D2, showed MIC 0.05 mg / mL.

In relation to the association study, considering the susceptibility of *S. oralis*, synergistic effects were observed when triclosan and triclosan + fluoride were added to the hydroalcoholic extract of the ripe fruit of *E. uniflora* L., and antagonistic effect when fluoride was added For *L. casei*, an antagonistic effect was observed when fluoride and triclosan were added singly to the hydroalcoholic extract of the ripe fruit of Surinam cherry. Contrarily, a synergistic effect was observed when fluoride and triclosan were added concomitantly to the extract.

Statistical analysis indicated a significant difference ($p<0.05$) only between the values of the diameters

Table 2: Measurements, in mm, of the zones of inhibition of the dentifrices tested on *Streptococcus oralis*.

Dilutions	D1	D2	D3	D4	D5 (Control)	p-value
0.3g/mL	23±1[a]	12±1[a]↓	23±0[a]	23±1[a]	30±1[a]	0.1301
0.15g/mL	19±0[a]	10,5±0[a]↓	23±0[a]↑	20±0[a]	29±0[a]	0.0611
0.075 g/mL	15±0[a]	10±0[a]↓	21,5±0[a]↑	19±0[a]↑	25±0[a]	0.0611
0.0375 g/mL	15±0[a]	10±0[a]↓	21±1[a]↑	18±0[a]↑	24±0[a]	0.0625
0.01875 g/mL	15±0[a]	9±0[a]↓	20±0[a]↑	15±0[a]*	15±0[a]	0.0611
0.009375 g/mL	14±0[a,b,c]	9±0[b]↓	19±0[c]↑	14±0[a,b,c]*	14±0[a,b,c]	0.0611
0.005g/mL	14±0[a,b,c]	9±0[b]↓	19±0[c]↑	14±0[a,b,c]*	13±1[a,b,c]	0.0769

↑: Synergistic effect; ↓: Antagonistic effect *: Indifferent effect. Different lowercase letters on the same line indicate a statistically significant difference (p<0.05).

Table 3: Measurement, in mm, of zones of inhibition of the dentifrices tested on *Lactobacillus casei*.

Dilutions	D1	D2	D3	D4	D5 (Control)	p-value
0.3g/mL	26±1[a]	0↓	15±0[a]↓	29±1[a]↑	20±0[a]	0.0639
0.15 g/mL	25±0[a]	0↓	14±0[a]↓	26±0[a]↑	19±0[a]	0.0611
0.075 g/mL	19±0[a]	0↓	12±0[a]↓	25±0[a]↑	15±1[a]	0.0625
0.0375 g/mL	18±1[a]	0↓	10±1[a]↓	25±0[a]↑	13±0[a]	0.0639
0.01875 g/mL	18±0[a]	0↓	10±0[a]↓	24±1[a]↑	12±0[a]	0.0625
0.009375 g/mL	18±0[a]	0↓	10±0[a]↓	24±0[a]↑	12±0[a]	0.0611
0.005g/mL	18±0[a]	0↓	10±0[a]↓	24±0[a]↑	11±0[a]	0,0611

↑: Synergistic effect; ↓: Antagonistic effect *: Indifferent effect. Different lowercase letters on the same line indicate a statistically significant difference (p<0.05).

of the growth inhibition zones promoted by D2 and D3 at concentrations of 0.009375 g / mL and 0.005 g / mL against *S. oralis* strains.

DISCUSSION

The use of phytotherapy has been increasing within the dental practice, since it has been recognized that a large variety of plant species have potential against the development and growth of microorganisms involved in the formation of dental biofilm, thus contributing to the reduction of dental caries and periodontal disease. It has also been pointed out that the primary indication of such natural products is often supported by popular knowledge and use of them[14-16]. *Eugenia uniflora* L., also known as Surinam cherry, is one of these plants that has been studied and has shown its antimicrobial potential[12]. The fruit, which is the botanical part used in this study, shows promise in pharmacological research, in view of the fact that people eat it, proving its low toxicity, and that development of the plant is not affected, allowing its preservation.

One of the pioneering studies that portrayed the antibacterial potential of *Eugenia uniflora* L. on microorganisms from the oral cavity showed that the essential oil, which is obtained from the leaves, was able to inhibit growth of the following microorganisms: *Streptococcus mitis, Streptococcus mutans, Streptococcus sanguis, Streptococcus sobrinus* and *Lactobacillus casei*[17]. Data obtained from our study confirm the potentiality of this product to act on bacteria species involved in the process of dental caries. The microorganisms used in this study have effective participation in pathological processes of the oral cavity. *S. oralis*, as some authors report [18,19], can be commonly found in saliva of individuals with high caries activity and also has high capacity to adhere to oral surfaces, with its colonization

being influenced by factors related to the oral environment, especially the standard of oral hygiene. In addition to participating in dental caries, *S. oralis* is involved in other infections, such as endocarditis, alveolar-dental infections and septicemia[20-22].

In this study it was observed that the D1 dentifrice also showed antimicrobial activity against *Streptococcus oralis* and *Lactobacillus casei* in all dilutions. The results of our research show the need to evaluate the interactions of chemical substances present in the dentifrice, especially those of natural origin, in order to ascertain possible synergism or antagonism, since other chemical components, such as fluoride, are major agents in the prevention of dental caries.

Several studies have been conducted in order to identify the antimicrobial potential of dentifrices[23,24]. However, few studies assess the pharmacological activity of the associated chemical substances, whether natural or not, which are clinically indicated in dentistry.

Among the synthetic substances found in dentifrices are triclosan and fluoride, which have recognized antimicrobial activity. Thus, the results found for the standard dentifrice, Colgate Total 12, confirm the findings observed in the literature[23,25].

The addition of fluoride to the dentifrice containing hydroalcoholic extract of the ripe fruit of Surinam cherry promoted pharmacological antagonism, when antibacterial activity on *S. oralis* and *L. casei* was evaluated. However, the addition of triclosan to the dentifrices containing fluoride and the extract evaluated promoted pharmacological synergism at lower concentrations used clinically, suggesting the possibility of application for preventive or therapeutic purposes.

Further studies could evaluate the clinical activity of a dentifrice containing *Eugenia uniflora* L. associated to other chemical substances, seeking to confirm its effectiveness in preventing diseases of the oral cavity.

CONCLUSION

The dentifrices containing hydroalcoholic extract of *Eugenia uniflora* L. have antimicrobial activity. When triclosan and fluoride are added to the dentifrice containing *Eugenia uniflora* L., synergistic activity against the microorganisms tested is observed. The formulation of the dentifrice with *Eugenia uniflora* L. without fluoride and triclosan (D1) and the dentifrice with fluoride and triclosan (D4) had better results than the control group.

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