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Eugenia uniflora Dentifrice for Treating Gingivitis in Children: Antibacterial Assay and Randomized Clinical Trial

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School-age children are frequently at high risk for the onset of biofilm-dependent conditions, including dental caries and periodontal diseases. The objective of this study was to evaluate the clinical efficacy of a dentifrice containing *Eugenia uniflora* Linn. (Surinam cherry) extract versus a triclosan-based comparator in treating gingivitis in children aged 10-12 years. The *in vitro* antibacterial potential of the dentifrice was tested against oral pathogens *(Streptococcus mutans, Streptococcus oralis* and *Lactobacillus casei*). Then a phase-II clinical trial was conducted with 50 subjects aged 10-12 years, with clinical signs of gingivitis. The subjects were randomly assigned to the experimental group (n=25) and control group (n=25), in which participants used the experimental dentifrice and a triclosan-based fluoridated dentifrice (Colgate Total 12®), respectively. Clinical examinations assessed the presence of gingivitis (primary outcome) and biofilm accumulation (secondary outcome) using the Gingival-Bleeding Index (GBI) and Simplified Oral Hygiene Index (OHI-S), respectively, at baseline and after seven days of tooth brushing 3x/day. The data were analyzed using paired and unpaired t-test (GBI) and Wilcoxon and Mann-Whitney (OHI-S), with $p \leq 0.05$. The experimental dentifrice showed efficient antibacterial activity *in vitro*. In the clinical trial, a significant reduction in gingival bleeding was observed in both experimental and control groups ($p<0.0001$), with no statistical difference between them ($p=0.178$), although a small size effect was observed. Biofilm accumulation was only reduced in the control group ($p=0.0039$). In conclusion, *E. uniflora* dentifrice showed anti-gingivitis properties in children aged 10-12 years. Thus, it may be a potentially efficient and safe product to be used alternatively in preventive dental practice

Key Words: gingivitis, dentifrice, biofilm, *Eugenia uniflora*, Surinam cherry.

Introduction

Gingivitis is the most prevalent condition affecting the periodontium in pediatric patients (1,2). This highly prevalent disease among children and young adults results from the interplay between bacterial virulence factors and the host's limited defense capacity (3).

Even developed countries have shown high prevalence and incidence rates of periodontal disease (4). This epidemiological *status quo* requires the adoption of measures to address this public health issue by educational, preventive and therapeutic actions. This background encourages the use of alternative therapies for oral health care, particularly in communities with limited access to health services (4). Natural products have shown to be potential therapeutic resources for treating oral diseases, especially of infectious nature, such as dental caries (5) and candidiasis (6), and of inflammatory origin, such as gingivitis (7).

Based on its common medicinal use, this research group and others have explored the pharmacological potential of *Eugenia uniflora* L. extracts, which is also known as Surinam cherry. Extracts from the leaves, stems and fruits were recognized for their promising antimicrobial and anti-inflammatory activities (8-10). Furthermore, this plant can be found in several countries because it adapts easily to different weather and soil conditions (9).

Previous studies have investigated the toxicity potential of *E. uniflora* L. extract and confirmed that it is safe for clinical use (11). Moreover, the ripe fruit (used in the present study) is edible and there are no reports of adverse effects related to its use.

Therefore, the wide distribution of *E. uniflora* in South America, the proven biological properties and presence of bioactive chemical compounds (mainly sesquiterpenes) (8-10), the negligible toxicity of its fruits (11), and finally the potential development of an effective, low-cost dentifrice for economically disadvantaged communities – at least comparable to the ones available in the market – have encouraged us to develop an *E. uniflora* experimental dentifrice. Herein, this study investigated the *in vitro* antibacterial activity and short-term clinical efficacy of this dentifrice containing *E. uniflora* L. extract versus a triclosan-based comparator in treating gingivitis and biofilm accumulation in children.

Material and Methods

The present study was conducted in two stages: (i) *in vitro* evaluation of the antibacterial activity of *E. uniflora* L. dentifrice against oral pathogens; and (ii) phase II clinical trial of the experimental dentifrice in subjects with gingivitis.

The hydroalcoholic extract of *E. uniflora* ripe fruit and the pharmaceutical formulation of the dentifrice were performed at the Natural Products Chemistry Laboratory of the Federal University of Paraiba. Each 10 mL of *E. uniflora* L. dentifrice comprises the following components as described by Jovito et al. (8): hydroalcoholic extract of the ripe fruit of *E. uniflora* L. (3.0%), preservatives (parabenes, 0.02 g), and dentifrice base (silicon dioxide, sodium lauryl sulfate, white dye, aromatic compounds, sodium saccharin and Gangrez sodium salt; q.s.p.).

In Vitro Phase: Evaluation of Antimicrobial Activity

The *in vitro* minimum inhibitory concentration (MIC) of the dentifrices was determined by the agar diffusion technique described by Bauer (12). Serial dilutions of the experimental and control dentifrice (Colgate Total 12®) were prepared at concentrations ranging from 0.3 g/mL to 0.005 g/mL. The latter was used as a positive control due to its known antibacterial and anti-inflammatory activities. Briefly, 3 g of dentifrice was dissolved in 10 mL of sterile distilled water, yielding a solution that was subsequently centrifuged at 5,000 rpm for 10 min to precipitate the solid particles. After centrifugation, the supernatant obtained from each dentifrice was used for the antibacterial assay. *Streptococcus mutans* (ATCC 25175), *Streptococcus oralis* (ATCC 10557), and *Lactobacillus casei* (ATCC 7469) strains were tested in the present study. The inoculum was adjusted according to the 0.5 tube of McFarland standard, which corresponds to approximately 1.5 x 10^8 CFU/mL. The bacterial suspensions were seeded onto agar plates and well-shaped 6-mm perforations were made in the solid culture medium afterwards. Samples of 50 µL of dentifrice supernatant at different concentrations were inserted in the wells. The plates were incubated at 37 °C and 5% CO_2 for 24 h. The MIC was defined as the lowest concentration of the dentifrice that inhibited visible bacterial growth.

Phase II Clinical Trial

Study Design

After the antibacterial activity of the experimental dentifrice was confirmed *in vitro*, was carried out the phase II: a randomized, controlled, double-blind clinical trial comparing the experimental and control dentifrices. The study had a parallel design and investigated the short-term clinical efficacy of Surinam cherry dentifrice against gingivitis and biofilm accumulation). The CONSORT guidelines were followed to design this investigation.

Study Participants

Eligibility criteria were used to select a homogenous sample to participate in the clinical trial. As inclusion criteria, were selected children of both sexes aged 10 to 12 years, with presence of at least 15 teeth, showing biofilm-induced gingivitis and whose participation was consented by their parents/legal guardians. Excluded subjects were those with systemic conditions that could interfere with periodontal health; wearers of fixed/removable orthodontic appliances; mouthrinse users or subjected to topical fluoride application in the previous three months; and undergoing treatment with antimicrobials or anti-inflammatory drugs. All subjects were recruited at a public school in the city of João Pessoa, PB, Brazil, where their examinations were carried out and one of the three daily tooth-brushings was supervised (directly).

The sample size was estimated using Fleming's single-stage procedure for phase II trials (13). With regard to the primary outcome, a response proportion of at least 50% was defined as being clinically significant. The type I error was set as 0.05, and the type II error was set at 0.1. Based on these values, a sample size of 20 individuals per trial arm would provide 90% power (1-β) to detect any clinically relevant treatment difference of 50% or greater compared to baseline. Taking into consideration an attrition rate of approximately 20%, the final sample size per trial arm comprised 25 individuals.

Randomization

After meeting the eligibility criteria, subjects were number-coded and their allocation into the experimental or control group was determined by drawing lots with replacement. Allocation data were concealed by a single researcher, who did not examine the subjects, in a sealed envelope during the whole performance of the study.

Blinding

The experimental and control dentifrices were kept in similar containers, and had the same color (white), taste (mint) and smell (mint). Thus, neither the participants nor the examiners were aware of the allocated interventions, characterizing the study as double-blinded.

Control

A commercially available dentifrice containing 1500 ppm fluoride and 0.3% triclosan (Colgate Total 12®, Colgate-Palmolive Company) was used as control due to its known effects against gingivitis and plaque formation.

Subjects allocated to the control group followed the same procedures as those of the experimental group.

Intra-Examiner Agreement

The examination of subjects concerning the study outcome indices was performed by a single examiner who was adequately calibrated with a Kappa statistic of 0.8, which is considered to be satisfactory agreement, according to Landis & Koch (14).

Intervention Protocol

The experimental group comprised 25 subjects who used the dentifrice with *E. uniflora* L. ripe fruit hydroalcoholic extract, three times per day, for seven consecutive days. The control group comprised 25 subjects who used the control dentifrice (fluoride, 1500 ppm and triclosan, 0.3%), three times per day, for seven consecutive days. The participants (and their parents) were instructed as to the number of three daily brushings and one of them was supervised at school by the researchers. They were also instructed concerning the pea-sized amount of dentifrice to be used. An active personal and telephonic contact was established with the parents in order to assure protocol compliance.

Primary and Secondary Outcomes of Interest

Clinical examinations were performed at baseline and after seven days of dentifrice use. The examination data were recorded in a specific clinical chart. The diagnosis of gingivitis (primary outcome) was established by bleeding on probing using the Gingival Bleeding Index (GBI) proposed by Ainamo and Bay (15). The accumulation of biofilm (secondary outcome) was assessed with the Simplified Oral Hygiene Index (OHI-S) described by Greene and Vermilion (16). Prior to the study, all subjects were instructed on correct tooth brushing techniques and received a kit that contained a toothbrush and one of the dentifrices (without the identification of its contents).

Table 1. Evaluation of the Minimum Inhibitory Concentration (MIC) of the experimental and control dentifrices against *Streptococcus mutans* ATCC 25175, *Streptococcus oralis* ATCC 10557 and *Lactobacillus casei* ATCC 7469. The zones of inhibition are expressed in millimeters (mm)

Sample concentration	Inhibition zone (mm)					
	S. mutans		*S. oralis*		*L. casei*	
	SC	Ctrl	SC	Ctrl	SC	Ctrl
0.3 g/mL	14.0	18.0	23	30	26	20
0.15 g/mL	12.0	14.0	19	29	25	19
0.075 g/mL	11.0	12.0	15	25	19	15
0.0375 g/mL	10.0	11.0	15	24	18	13
0.01875 g/mL	7.0	10.0	15	15	18	12
0.009375 g/mL	7.0	9.5	14	14	18	12
0.005 g/mL	0.0	9.0	14	13	18	11

Note: SC, Surinam cherry experimental dentifrice; Ctrl, control dentifrice (Colgate Total 12®).

Ethical and Regulatory Aspects

The present study was approved by the Ethics Research Committee of the João Pessoa University Center (Centro Universitário de João Pessoa, UNIPÊ), João Pessoa, PB, Brazil. The parents/legal guardians of each of the participating children signed a detailed informed consent form to authorize their participation. The study followed the guidelines of the 466/12 Resolution of the Brazilian National Health Council, which encompasses the Helsinki Declaration. Moreover, the clinical trial was registered at ClinicalTrials.gov under protocol NCT02648139.

Statistical Analysis

The data were analyzed on GraphPad Prism version 5.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). Intra- and intergroup comparisons at baseline and post-interventions were performed using paired t and unpaired t tests (for GBI data) and Wilcoxon signed-rank test and Mann-Whitney test (for S-OHI data), with a significance level of 5%.

Results

Table 1 shows the results of the *in vitro* antibacterial activity of the dentifrices against oral microorganisms. The MIC of both the experimental and control dentifrices was 5 mg/mL against all bacterial strains, except for *E. uniflora* dentifrice against *S. mutans*, which was 9.3 mg/mL.

All participants completed the phase II clinical trial, therefore with no loss of follow-up. Regarding the experimental and control arms, 32% and 52% of the sample were males, respectively, and 68% and 48% females. The median number of teeth was 24 in the experimental group and 26 in the control group. The baseline levels of gingival inflammation and biofilm accumulation were not different between groups ($p>0.05$), thus characterizing a clinically homogenous sample.

As seen in Table 2, the study findings demonstrated that both the experimental and control dentifrices significantly reduced the levels of gingival bleeding as compared to baseline ($p<0.0001$). The means of differences between baseline and post-intervention data showed no significant difference in the effectiveness of Surinam cherry dentifrice and the triclosan-based comparator ($p=0.178$), although a small size effect was observed. Table 3 shows the effect of the dentifrices in reducing biofilm accumulation after seven days of dentifrice use. It may be clearly seen that

only the triclosan-based dentifrice was able to reduce the accumulation of dental biofilm during this period (p=0.0039) (Table 3). No adverse effects were reported or clinically identified during examinations.

Discussion

In dentistry, there has been an increase in the use of medicinal plants and molecules extracted from natural products for the preparation of products with pharmacological properties. Nevertheless, many gaps remain in the experimental design and analytical methodology used in most studies, which makes it difficult to translate *in vitro* findings into efficient and safe clinical use (5,6). The present study aimed to contribute to the development of an effective and low cost formulation that improves oral health by promoting the ecological and biochemical balance of the oral cavity.

The Brazilian Ministry of Health recently released a list of plants with the aim of guiding research to evaluate whether they may be used by the public health system. *E. uniflora* L. (Surinam cherry), which has antimicrobial activity against bacteria present in oral biofilm that cause caries and periodontal diseases, was one of the listed species (8,9). These data support the findings of this study, in which the experimental dentifrice exhibited *in vitro* antibacterial activity against streptococci and lactobacilli. As expected, the conducted study also confirmed the antimicrobial potential of the control dentifrice containing triclosan, which is a chemical agent recognized in literature as having antimicrobial properties (17).

The choice to include *S. mutans*, *S. oralis*, and *L. casei* strains was because they are part of the normal oral biofilm microbiota and participate in either the initial (*S. oralis* and *S. mutans*) or advanced (*L. casei*) colonization process of the microbial community (18-20). Because gingival inflammation results from the initial colonization of the dental surface by these microorganisms, it is necessary to disrupt microbial colonization to avoid microbial growth and establishment of periodontal pathogenic bacteria responsible for advanced periodontal disease (20,21).

The observed reduction in gingival bleeding produced by the dentifrice containing the hydroalcoholic extract from *E. uniflora* ripe fruit suggests that the product has the potential to prevent and/or treat gingivitis. In a previous study conducted by this research group, this dentifrice also reduced gingivitis in a sample of 40 undergraduate students between 21 and 24 years of age, following 22 consecutive days of dentifrice use (8).

There was no significant reduction in OHI-S post-intervention scores, which measure tooth surface biofilm accumulation, as compared to baseline in the experimental group. This finding may be due to the following reasons:

Table 2. Mean Gingival Bleeding Index (GBI) values (±SD) for the experimental (Surinam cherry dentifrice) and control (Triclosan-based dentifrice) groups at baseline and after 7-day treatment. 95% confidence interval values are expressed in brackets

Gingival Bleeding Index	Baseline (B)	Post-intervention (PI)	Mean of differences between (B) and (PI)	Effect size*	Intra-group difference†	Inter-group difference#
Surinam cherry dentifrice	3.45 ± 1.80 [2.704 to 4.192]	1.13 ± 1.48 [0.522 to 1.749]	2.32 ± 1.11 [1.856 to 2.769]	0.396 [-0.163 to 0.956]	*P*<0.0001	*P*= 0.178
Triclosan-based dentifrice	2.974 ± 1.15 [2.499 to 3.450]	1.10 ± 0.91 [0.724 to 1.479]	1.87 ± 1.16 [1.393 to 2.354]		*P*<0.0001	

*The effect size for GBI was calculated based on the mean of differences between (B) and (PI) and standard deviation. †Intra-group comparisons before and after intervention (paired-t test, p≤0.05). #Comparisons of the mean of differences (4th column) between the groups Surinam cherry vs. Triclosan-based dentifrice (unpaired-t test, p≤0.05).

Table 3. Median Simplified Oral Hygiene Index (S-OHI) values (plus inter-quartile range) for the experimental (Surinam cherry dentifrice) and control (triclosan-based dentifrice) groups at baseline and after 7-day treatment. Minimum and maximum interquartile range is presented in parenthesis and 95% confidence interval values are expressed in brackets

Simplified Oral Hygiene Index	Baseline (B)	Post-intervention (PI)	Sum of ranks of differences between (B) and (PI) ¥	Effect size*	Intra-group difference†	Inter-group difference#
Surinam cherry dentifrice	1.16 (0.75) [1.075 to 1.607]	1.16 (0.33) [0.982 to 1.411]	714	0.386 [-0.173 to 0.945]	*P*=0.1006	*P*=0.139
Triclosan-based dentifrice	1.33 (0.34) [1.206 to 1.513]	1.16 (0.58) [0.922 to 1.190]	561		*P*=0.0039	

¥Mann-Whitney U: 236. *The effect size for S-OHI was calculated based on median values and interquartile range. †Intra-group comparisons before and after intervention (Wilcoxon signed-rank test, p≤0.05); #Comparisons of the sum of ranks of differences (4th column) between the groups Surinam cherry vs. triclosan-based dentifrice (Mann-Whitney test, p≤0.05).

low abrasive potential of the dentifrice, resulting in poor mechanical removal of biofilm; or a low *in vivo* concentration, which may have not been enough to produce the same effects observed *in vitro*, due to salivary clearance, substantivity and other factors inherent to the human body. In contrast, the control dentifrice was efficient in reducing biofilm accumulation, which corroborates previous studies that evaluated the potential of triclosan-based dentifrices (17,22-24).

The ripe fruit of *E. uniflora* contains flavonoids, which are chemical markers of anti-inflammatory activity. The activity of the experimental dentifrice active component may be directly associated with certain pathways of the inflammatory process because there was a reduction in gingival bleeding but not in biofilm accumulation (25).

Similarly, Soares (10) conducted an *in vivo* evaluation of a mouthwash containing the hydroalcoholic extract of *E. uniflora* ripe fruit in individuals wearing orthodontic braces. The author observed a statistically significant reduction in gingival bleeding based on the GBI ($p<0.05$) and in bacterial counts for *S. mutans* ($p<0.01$), but not in biofilm accumulation based on the OHI-S ($p>0.05$) before (baseline) and after (post-intervention, 15 days) dentifrice use. These findings could also be explained by the anti-inflammatory activities of *E. uniflora*.

The results of the present study suggest the need for additional clinical trials focusing on the anti-inflammatory potential of *E. uniflora*. Although no adverse effects were reported by research subjects, it is important to investigate the long-term clinical and toxicological effects of the experimental dentifrice.

Furthermore, the need for pharmaceutical technological support should be stressed for formulating a dentifrice with satisfactory physical and chemical properties. In particular, certain aspects of the dentifrice: color, taste and abrasiveness require improvement.

This study demonstrated that the dentifrice containing the hydroalcoholic ripe fruit extract of *E. uniflora* L. (Surinam cherry) showed anti-gingivitis properties in children aged 10 to 12 years. *E. uniflora* dentifrice may be a potentially efficient and safe product to be used alternatively in preventive dental practice.

Resumo

Crianças em idade escolar apresentam, com frequência, alto risco para o desenvolvimento de doenças biofilme-dependentes, incluindo cárie e doenças periodontais. Este estudo investigou a eficácia clínica de um dentifrício contendo o extrato de Eugenia uniflora Linn. (pitanga) comparado a um dentifrício com triclosan no combate à gengivite em crianças de 10 a 12 anos. Foi avaliado o potencial antibacteriano *in vitro* do dentifrício sobre microorganismos da cavidade oral (S. mutans *S. oralis* and *L. casei*) e realizado um estudo clínico fase II incluindo 50 sujeitos, com sinais clínicos de gengivite, divididos aleatoriamente em dois grupos: Experimental – 25 sujeitos usaram o dentifrício contendo extrato de pitanga; e Controle – 25 sujeitos que usaram um dentifrício fluoretado contendo triclosan (Colgate Total 12®). Na baseline e após sete dias consecutivos de escovação, foi realizado o exame clínico para diagnóstico de gengivite (desfecho primário) e acúmulo de biofilme (desfecho secundário), utilizando o Índice de Sangramento Gengival (ISG) e Índice de Higiene Oral Simplificado (IHO-S). Os dados foram analisados utilizando o teste t pareado e não pareado (ISG) e teste de Wilcoxon e Mann-Whitney (IHO-S), com nível de significância de $p\leq0,05$. Observou-se uma efetiva atividade antibacteriana do dentifrício experimental. No estudo clínico, observou-se redução de sangramento gengival em ambos os grupos experimental e controle ($p<0,0001$), não havendo diferença entre eles (p 0,178), embora com uma pequena magnitude de efeito. Apenas o grupo controle reduziu significantemente o acúmulo de biofilme ($p=0,0039$). Concluiu-se que o dentifrício experimental de E. uniflora mostrou-se eficaz na redução de gengivite em crianças de 10 a 12 anos. Assim, este dentifrício apresenta potencial para ser utilizado de forma eficaz e segura em odontologia preventiva.

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