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Original Article

Evaluation of Chewing Gums Containing Natural Products on Salivary Concentrations of *Streptococcus mutans* in Children

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ABSTRACT

Background: *Streptococcus mutans* (*S. mutans*) are present in the bacterial biofilm involved in the onset and progression of childhood caries, favoring acid production and dental demineralization. Sugar consumption favors the high incidence of caries in childhood, from 5 to 12 years old. A chewable lozenge incorporated with natural antimicrobial agents of recognized potency, such as red propolis and xylitol, would be an adjuvant to control caries. **Materials and Methods:** This is a randomized, placebo-controlled, cross-sectional clinical trial. 40 participants were divided into four groups: Placebo (group I), Red Propolis 2.5% (group II); Red propolis 6.4% (group III) and Xylitol 15% (group IV). They had saliva collected at different times of the trial for analysis of antimicrobial action. The study was registered with REBEC. **Results:** In the group I there were variations before and after the administration of the tablets, but no statistical significance, in relation to the group II it was found a significant reduction in the salivary concentration of *S. mutans* in all salivary dilutions (34,25% - 63,65%), fact observed with the group III (38,42% - 45,02%) and group IV (50,72% - 42,92%). **Conclusion:** With encouraging results, further studies are needed for a longitudinal follow-up, being a low-cost alternative with anticariogenic potential.

Keywords: Chewable tablets, Red propolis, Xylitol, Natural bioactive, *Streptococcus mutans*.

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INTRODUCTION

As a multifaceted condition, oral health involves social, biological, psychological and spiritual aspects, being an integrative reflection of systemic health. Caries disease is a chronic, sugar dependent disease, of multifactorial etiology, defined as the process of dental matrix demineralization in the enamel and/or dentin induced by acid its prevalence is determined by biological, dietary, behavioral and socio-economic factors, as well as factors related to access to consumer goods and health services. Socio-economic, biological, behavioral, dietary factors and access to health services are decisive for its prevalence.[1-4] The national oral health policy lists conditions such as dental caries, periodontal disease and oral cancer as risks for systemic diseases that can affect the population.[5]

Poor oral health conditions of the population, often associated with unfavorable socio-economic conditions, difficulty in accessing services and harmful habits, such as high consumption of sugar. It can also indicate limited access to fluoride. Dental caries corresponding to the ICD-10 K02 code.[6]

Dental demineralization occurs in a chronic and multifactorial way, being caused by organic acids, products of the fermentation of carbohydrates from the diet by oral micro-organisms, culminating in caries disease in sugar-dependent mechanism.[7,8]

Streptococcus mutans (*S. mutans*) are Gram-positive cocci, of the Lancefield group A, acidogenic and aciduric, that use sucrose and other fermentable carbohydrates to produce extracellular polysaccharides, which facilitate adhesion to the dental matrix.[9,10] *S. mutans* are the main cariogenic markers related to bacterial biofilm involved in the appearance and progression of childhood

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caries (inadequate diet and eating habits).[11-14] Under conditions of sucrose richness, the cariogenic potential of *S. mutans* is increased, generating a reduction in pH and dental demineralization. Therefore, *S. mutans* is considered an important microorganism within the complex cariogenic biofilm.[4,15-17] Children who are fed formulas sweetened with fermentable carbohydrates are at risk for the development of cariogenic lesions. When this habit extends into adolescence, associated with poor oral health care, there is an increased risk of caries throughout life.[18-20]

The incidence of caries in children aged 5 to 12 years old is related to the consumption of sugars in the form of candies, chewing gum and chocolates and other "empty calories", that is, a consumed diet, with cariogenic micro-organisms present in the saliva and bacterial plaque, causing a localized and progressive loss of mineralized tissue from the teeth. Saliva plays an important role in the control of cariogenic bacteria (biofilm-forming), protecting teeth and mucosa in pH regulation, through salivary buffers, mucin, bicarbonate and monophosphate, preventing injuries produced by acids and bases; and still autoclysis through chewing movements, that increases the power of remineralization and decreasing demineralization.[3,21,22]

An interesting alternative would be the pharmaceutical and clinical development of a chewable tablet composed by natural antimicrobial agents such as Brazilian red propolis (BRP) and xylitol, as several studies suggest the use of them to inhibit the appearance of carious lesions, by controlling the microbial population, as one of its main biological effects, antimicrobial activity. Several studies prove the therapeutic effect of BRP extract on microbial inhibition, including the cariogenic agents. This activity is related to the flavonoid compounds present in it, especially to vestitol, neovestitol and isoliquiritigenin, which promotes synergism between antimicrobial and antioxidant activity. In addition, it presents itself as a clinical option of low toxicity and high biocompatibility.[3,23-25]

The biological activity of BRP is often associated with the presence of phenolic compounds, mainly flavonoids, such as flavone (rutin, luteolin), isoflavone (formononetine, daidzein), dihydroflavonol (pinobanksin, pinobanksin-3-acetate) and others. The constituents vestilol and neovestilol are the main compounds of BRP, where both have a synergistic effect, decreasing the production of protein and polysaccharide constituents, helping this way in the inflammatory process, thus promoting the micro-organism death and disorder of the biofilm that forms the caries.[23,25-27]

These compounds are responsible for several biological properties of BRP,[28-30] including the antiproliferative effect of cancer cells and therapeutic potential against resistant anaerobes microorganisms found in odontogenic infections and periodontal diseases.[25,27,31-33] In addition, this type of propolis can reduce the colonization of facultative anaerobic micro-organisms such as *S. mutans*, acting in the dental caries prevention.[29] Being generally resistant to fermentation by oral bacteria, xylitol presents itself as a sucrose substitute due to its protective activity.[34] The molecular formula is $C_5H_{12}O_5$, found in several organisms, and it is also an intermediary in the metabolism of human carbohydrates, being an alternative in diabetic patients and inducing the remineralization of dental enamel.[35,36]

Xylitol has well-documented and proven benefits in several studies, and can be used in chewing gums, lozenges, oral antiseptics and dentifrices with anticariogenic action.[37,38] Its use favors the buffering action of saliva, increasing salivary flow, stabilizing oral pH and reducing the accumulation of bacterial biofilm.[36,38,39] Evidences show that xylitol has three anticariogenic mechanisms: increased salivation, sucrose substitute and activity against *S. mutans*.[40,41,42] Antimicrobial action through the action of some proteins and enzymes and neutralization of oral acids through buffer systems, mainly the bicarbonate/carbonate and monophosphate/bisphosphate system. The use of chewable tablets increases salivary flow due to the masticatory and gustatory stimulus, allowing greater control of the oral microbiota, making it difficult for caries lesions to appear and reducing the number of colony forming units of *S. mutans*.[36]

No reports of studies were found in the literature analysis covering this age group of 5 to 12 years old, a period in which the transition from deciduous to permanent (mixed dentition) occurs; as well as the use of chewable tablets based on BRP with concentration 2.5% and 6.4%. Most of the *in vivo* studies with Propolis usage it as an extract in the pharmaceutical form of mouthwash, varnish and not as a chewable tablet, that has good acceptance in children, favoring studies. Xylitol, which has already been researched and used to replace sugar in chewable tablets, serves as an effective adjunct to plaque control, since frequent sucrose intake is one of the main factors that lead to an increase in the quantity and pathogenicity of bacterial plaque, leading to the carious lesions appearance. As a result of the exhibited above, it would have a good use/acceptance in pediatric patients for the purpose of controlling caries disease, since xylitol and BRP are natural substances with antibacterial properties and a viable alternative to conventional formulations. The objective of the study was to evaluate the action of chewable tablets composed by determined concentrations of BRP and xylitol facing the concentrations of *S. mutans* present in the saliva of children aged between 5 and 12 years old who presents caries, by determining variations in the amount of *S. mutans*/mL of saliva of each patient through Colony Forming Units per milliliter (CFU/mL) quantification before and after using the tablets and BRP Propolis (placebo); BRP 2.5% (Group II), containing BRP 6.4% (Group III) and Xylitol 15% (Group IV).

MATERIALS AND METHODS

It is a randomized, placebo-controlled, cross-sectional clinical trial, registered and approved by the ReBEC (Brazilian Registry of Clinical Trials) and The Universal Trial Number (UTN), under number U1111-1221-8758. This study was approved on October 4, 2017 by the Human Research Ethics Committee of the Quixadá Catholic University Center (UNICATÓLICA) under the Consubstantiated Opinion CEP number 2,314,179 and Certificate of Presentation for Ethical Appreciation (CAAE) of n° 68705517.4.0000.5046, and its elaboration was guided by Resolution CNS / MS 466/12 and by the Declaration of Helsinki in its last update in 2013. The same was carried out at Unicatólica's Pediatric Dentistry Clinic (Complex São João Calábria) in August to October 2018, where it determined the action of chewable tablets based on: Placebo (GROUP I), BRP 2.5% (GROUP II); BRP 6.4% (GROUP III) and Xylitol 15% (GROUP IV).

Study population

A total of 40 healthy volunteers, with caries, of both genders, aged between 5 and 12 years old, who sought care at the Unicatólica Odontopediatrics Clinic (São João Calábria Complex) from July to October 2018 each of them was selected, strictly obeying the inclusion and exclusion criteria of this study.

Inclusion criteria

The following criteria for the volunteers' aptitude in the study were used:

- Children of both sexes;
- No history of allergic reactions;
- Aged between 5 and 12 years old;
- With normal growth and development pattern;
- Patients who have not undergone dental treatment during the past six months;
- Children with caries (presence of at least one active carious lesion, cavitated or non-cavitated).

Exclusion criteria

Participants who presented at least one of the following criteria were excluded from the study:

- Patients with a history of allergies (asthma, hives, rhinitis, sinusitis);
- Patients with a history of allergies to medications, foods or other factors;
- Patients with a history of chronic, congenital or any systemic changes;
- Patients with a history of gastrointestinal, liver or kidney disease;
- Patients who have undergone antibiotic treatment (s) up to six months before the research;
- Patients with soft tissue lesions in the oral cavity;
- Patients whose parents or legal guardians refuse to sign the informed consent form;
- Patients who refused to sign the Informed Consent Form;
- Children free from carious injuries;
- Use of an anti-infectious antibiotic or chemotherapy, three months before the beginning of the research.

Brazilian Red Propolis extract

The hydroalcoholic extract of BRP (20%) was obtained from the company Natural Pharma and handled at the Irmã Dulce University Pharmacy of the UNICATÓLICA in a standardized way aiming at the elimination of all moisture present in the extract, due to the water incompatibility, ending in the production of an absolute alcoholic extract. Then, the sample of the absolute alcoholic extract 20% was submitted to chemical identification of its constituents in the Department of Organic and Inorganic Chemistry, of the Federal University of Ceará. For proper chemical characterization and guarantee of the quality of the extract in the reproducibility of the study.

Data collection process and study steps

The volunteers were randomly assigned to one of the four defined groups, with a total of 10 participants in each group. The type of chewable tablet that each group used (Groups I, II, III and IV) was kept confidential, both to professionals and students involved in the clinical trial, as well as to participants, parents or guardians. The tablets were produced in q.s.p 50g (m/v) in commercial forms to obtain gummy tablets. The average weight of 3g was standardized, seeking to obtain similarity in variation in weight, color, odor and flavor.

The tablets production followed the Brazilian Pharmacopeia (Brazil, 2010, 2019) rules with limits of variation in weight of ± 5%, meeting the criteria for evaluating the weight determination for solid dosage forms in unit dose. After the agreement and signature of the terms, Informed Consent Form and Assent (ICF and TALE), by both the legal guardian and the participant, both were instructed about the cooperation and use of the chewable tablet in this study. This orientation was carried out at the Odontopediatrics clinic of the São João Calábria Complex of UNICATÓLICA.

Each patient in each group chewed the chewable tablet only once, for 3 to 5 min (swallowing), with subsequent normal brushing of the teeth. Therefore, after group I (chewable tablet without Xylitol

and without BRP), group II (chewable tablet containing BRP 2.5%) were compared with group III (chewable tablet containing BRP 6.4%), with the group IV (chewable tablet containing 15% Xylitol), comparing groups two by two and then groups between them.

Saliva collection

Two saliva samples were collected. Sample collection was encouraged, where the child / participant chewed a piece of Parafilm® (3cm x 3cm) attached to a piece of long dental floss to avoid swallowing the material. After a period of 60 (sixty) seconds, the saliva was removed with the aid of a Pasteur pipette (3mL) from the child's mouth and placed in a sterile flask (Eppendorf®) marked by an identification number for further analysis. The second collection was performed after using / swallowing the chewable tablet, where all participants were instructed not to drink, eat or perform chemical or mechanical methods of biofilm control for at least one hour before collections.

For microbiological analysis, the saliva was transported to the microbiology laboratory in sterile flasks, containing ice (2 - 8°C) that allows analysis in up to 2 hr. In return exams (clinical records), the appearance of possible adverse effects, such as staining of teeth, burning sensation, and peeling of the oral mucosa, were not reported.

Microbiological analysis

Serial dilutions (10^{-1}, 10^{-2} and 10^{-3}) of saliva in sterile 0.9% saline solution, homogenized on a magnetic stirrer, were used to isolate and count CFU / mL of *S. mutans*. A volume of 0.1 ml of each sample was transferred to a sterile test tube, containing 0.9 ml of saline. This process was repeated twice and the volume corresponding to 10 µL of each saliva sample (dilution) was seeded quantitatively on the MSA Agar (Mitis Salivarius Agar) supplemented (temperature ≤50°C) with Bacitracin (InLab®) 50mg / mL and 1% potassium tellurite (InLab®), both in triplicates. The plates were incubated at 37°C in anaerobiosis for 48 hr. Anaerobiosis was generated using Anaerogen® (Oxoid) generators in acrylic jars.

After incubation, the estimated S. *mutans* colonies were counted by multiplying the number of colonies in a standardized area of 1 cm^2 by the respective dilution factor. After this period, colonies with morphological characteristics of S. *mutans* were counted. The bacteria were expressed as CFU / mL of saliva. The analysis regarding the bacteriological risk for the development of caries will be based on the counting of the number of colonies (CFU / mL) of the *S. mutans* group in the saliva. Two to three colonies with characteristic morphology of *S. mutans* were transferred to specific means for biochemical tests, in order to complete the identification, and stained by Gram for the analysis of their micromorphology. After counting, biochemical tests were performed to confirm the bacterium and differentiate some species of streptococci from the *mutans* group.

Statistical methods

After collecting data through the form, all the information's were inserted, analyzed and organized through tables and graphs, in the Microsoft® Excel 2016 database and Statistical Package for the Social Sciences (SPSS®) version 24.0, for Windows 2017. The logarithmic transformation related to the number of CFU/mL of the dilutions was used, in order to homogenize the variances and make the distribution close to normal, where all analyzes showed normality. Quantitative variables were initially analyzed by the Kolmogorov-Smirnov test to verify the normality of the distribution, where normality was found in all pre-test distributions. For descriptive statistics, the mean and standard deviation (parametric data) were calculated. Intergroup (independent) comparisons at each stage of the study were performed using the analysis of variance (ANOVA) and Student's *t*-test was applied, setting the probability α of the type I error at 0.05 (5%) (level of significance), being considered as statistically significant a *p* value less than 0.05 ($p < 0.05$) for related samples in each phase, that is, pre-tablet tests (log UFC/mL), dilution at 10, 100 and 100 and post pellet tests (log UFC/mL).

RESULTS

The age groups of the 40 participants who were submitted the research ranged from 5 to 12 years old, with an average age of 8.9 years old (standard deviation ± 2.11). Volunteers between five and eight years old corresponded to 37.5% ($n = 15$) and patients between nine and 12 years old accounted 62.5% ($n = 25$).

The CPO-D ranged from 0 to 12 decayed teeth, where found a mean of 4.05 (standard deviation ± 3.09), considered moderate according to the classification of the Ministry of Health.[6] In addition, it was found that 45% of the patients ($n = 18$) had high to very high D-CPO, with a number of decayed teeth greater than ≥4.5.

Regarding to the gender, it was found that 60% were male and 40% female. According to gender, the average DMFT was 4.4 in female patients and 3.7 in male patients, both classified as moderate. The CPO-D ranged from 0 to 12 decayed teeth, where a mean of 4.05 (standard deviation ± 3.09) was found, considered moderate according to the classification of the Ministry of Health.[6]

Table 1 shows the variation in the salivary concentration of *S. mutans* before and after the treatment of the volunteers in the placebo group. Although there was a reduction in all salivary dilutions, this did not show statistical significance.

In relation to the group treated with the BRP 2.5% tablet, it was found that there was a significant reduction in the salivary concentration of *S. mutans* in all salivary dilutions (Table 2).

Table 1: Comparison Pre and Post-treatment among patients in the Placebo Group.

Dilution	Pre-treatment Media±DP	Post-treatment Media±DP	Reduction Variation ±DP	Significancy Test *t* Student
10^{-1}	3.257±1.309	3,248±1,303	0.09±0.006 (-0.27%)	p= 0.134
10^{-2}	2.095±1.082	2,093±1,081	0.02±0.001 (-0.09%)	p= 0.104
10^{-3}	1.143±0.825	1,137±0,819	0.06±0.004 (-0.52%)	p= 0.111

Table 2: Comparison before and after treatment among patients in Group II - Propolis 2.5%.

Dilution	Pre-treatment Media±DP	Post-treatment Media±DP	Reduction Variation ±DP	Significancy Test *t* Student
10^{-1}	4.102±0.755	2.919±0.704	1.183±0.837 (-34.25%)	p<0.001
10^{-2}	2.856±0.790	1.707±0.508	1.149±0.812 (-40.23%)	p<0.001
10^{-3}	1.505±0.739	0.547±0.420	0.958±0.677 (-63.65%)	p<0.001

Table 3: Comparison before and after treatment among patients in Group III - Propolis 6.4%.

Dilution	Pre-treatment Media±DP	Post-treatment Media±DP	Reduction Variation ±DP	Significancy Test *t* Student
10^{-1}	3.971±0.850	2.445±1.105	1.526±1.079 (-38,42%)	p<0.001
10^{-2}	2.577±0.837	1.242±0.820	1.335±0.944 (-52,20%)	p<0.001
10^{-3}	0.935±0.661	0.514±0.262	0.421±0.298 (-45,02%)	p= 0.027

Table 4: Comparison before and after treatment among patients in Group IV - Xylitol 15%.

Dilution	Pre-treatment Media±DP	Post-treatment Media±DP	Reduction Variation ±DP	Significancy Test *t* Student
10^{-1}	3.399±0.878	1.675±0.298	1.724± 1.219 (-50.72%)	p<0.001
10^{-2}	2.069±1.017	1.197±0.533	0.872±0.617 (-42.14%)	p<0.001
10^{-3}	0.897±0.635	0.512±0.328	0.385±0.272 (-42.92%)	p= 0.024

As in the previous group, it was observed that the volunteers treated with the BRP 6.4% tablet also showed a significant reduction in the salivary concentration of *S. mutans* in all salivary dilutions (Table 3).

In Table 4, it was observed that there was a significant reduction in the salivary concentration of *S. mutans* in all salivary dilutions of the volunteers in the group treated with the chewable tablet with 15% xylitol.

During the study, no adverse reactions were reported related to the use of chewable tablets by volunteers.

DISCUSSION

The CPO-d index found in the study reflects a moderate result (4.05), being above 2.1 (national average) according to the SB Brasil 2010 survey.[6] This data may demonstrate a deficiency in oral hygiene on the part of the participants, in addition to insufficient education in oral health or even, difficulty in accessing health Serbs in an appropriate way. Thus, the strategy of introducing a chewable tablet based on natural products that has a low cost would be a possibility to improve the oral health of this population.[3,25,43]

BRP has, in a well-documented way, substantial antimicrobial activity against several oral micro-organisms such as Gram-negative bacteria, *Lactobacillus* spp., *Candida* spp. and *Streptococcus* spp. Thus, the incorporation of BRP in a chewable tablet could favor the control of oral microorganisms that participate in the acidogenic pathway that leads to the imbalance of the re-demineralization process, thus favoring the appearance of the carious lesion.[3,4,29]

The use of BRP has already been shown to be safe in a previous study in which the perception of volunteers and the appearance of adverse effects were verified after the use of a fluoride toothpaste incorporated with BRP. In this work, there was a good acceptance of the toothpaste by the volunteers, as well as the absence of adverse effects to its use.[27] These data corroborate the data found in this study, in which no adverse effects related to the use of chewable tablets containing BRP at 2.5% and 6.4% were reported. In addition, no adverse effects were reported by the volunteers to the use of the chewable tablet containing 15% xylitol, which was even expected because it is a widely consumed food.

The concentration of 2.5% BRP in pharmaceutical forms of release into the oral cavity had already been validated in a previous studies, in which the reduction in the salivary concentration of *S. mutans* was verified after the application of a dental varnish in this concentration in children. As well, the data from this study also converge in the same direction, since the reduction in salivary concentrations of *S. mutans* was also verified with the use of the tablet in this concentration.[3,24] This reduction was also verified in a study that used a propolis tablet from France at a concentration of 6.4%, corroborating with the data from the present study that also used this concentration in the experimental chewable tablet incorporated with BRP.[44]

Another study evaluated the effectiveness of a dentifrice that incorporated BRP against salivary concentrations of *S. mutans* and Gram-negative micro-organisms in hebiatric orthodontic patients. Their results demonstrated appreciable activity against the tested microorganisms and also the absence of adverse effects reported by the volunteers. Thus, there is a report in the literature of a pharmaceutical product intended for the oral cavity that proved to be safe and effective, as well as the object of this study.[25]

A clinical trial evaluated the antimicrobial activity against *S. mutans*, the accumulation of biofilm and the quantification of active white patches of a propolis dental gel and a propolis dental gel with sodium fluoride. Both gels were found to have beneficial effects in reducing the microbial load of *S. mutans*. These data, in addition to supporting the results of the present, also open perspective for a future association between these assets in the form of a chewable tablet.[45]

An *in vitro* study revealed that the extract of BRP has the strong ability to generate a stress in the metabolism of *S. mutans* by decreasing the activities of several enzymes, including glycosyltransferases, which assists in the biofilm formation, and also other metabolic routes involved in the production of acid by micro-organisms, this long-term effect seems to be beneficial during chronic use of chewable tablets with active, since the increased difficulty in establishing a stable biofilm would be beneficial in the process of carious formation.[29]

Xylitol is a functional food that presents itself as an ally in maintaining oral health by presenting an antimicrobial and anticariogenic effect for acting positively in the tooth de-remineralization process.[36] This asset was tested in a clinical trial with chewable tablets against the salivary concentrations of *S. mutans* and demonstrated an appreciable reduction, these data support the results of the present study that obtained similar reductions. As well as the possible association with fluoride, there can be a good expectation of the association of BRP with xylitol conveyed in a chewable tablet.[44]

CONCLUSION

From what was exposed throughout the research and evinced by the analysis of the data collected in each of the experiments, it is possible to conclude that the formulations tested in this study have safety and a positive effect in the reduction of salivary concentrations of *S. mutans* (UFC/mL) which was evidenced by the comparative analysis with the placebo. Thus, the prospect of new studies for the longitudinal evaluation of these pharmaceutical formulations arises, composed of natural bioactive agents, demonstrating an excellent anticariogenic potential and low cost.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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