Phytomedicine 21 (2014) 1043–1047

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

The efficacy of three formulations of *Lippia sidoides* Cham. essential oil in the reduction of salivary *Streptococcus mutans* in children with caries: A randomized, double-blind, controlled study

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ARTICLE INFO

Article history:
Received 24 October 2013
Received in revised form 23 February 2014
Accepted 20 April 2014

Keywords:
Lippia sidoides Cham. essential oil
Dental caries
Streptococcus mutans

ABSTRACT

Essential oils of many plants have been previously tested in the treatment of oral diseases and other infections. This study was a randomized, double-blind, in parallel with an active control study, which aimed to evaluate the efficacy of three formulations of the *Lippia sidoides Cham.* essential oil (LSO) in the reduction of salivary *Streptococcus mutans* in children with caries. 81 volunteers, aged 6–12 years, both genders, with caries, were recruited to participate in this study, and randomly assigned to either one of five different groups. Each group received topical treatment with either 1.4% LSO toothpaste, 1.4% LSO gel, 0.8% LSO mouthwash, 1% chlorhexidine gel, or 0.12% chlorhexidine mouthwash. A 5-ml volume of each gel was placed inside disposable trays, and applied for 1 min, every 24 h, for 5 consecutive days. The mouthwash groups used 5-ml volume of a mouthwash inside disposable syringes. In the toothpaste group, children brushed their teeth for 1 min, once a day for 5 days. Saliva was collected before and after treatment. MS colonies were counted, isolated and confirmed through biochemical tests. Differences in MS levels measured in different days within the same treatment group was only verified with LSO toothpaste, chlorhexidine gel and chlorhexidine mouthwash. Comparison between groups of LSO mouthwash, toothpaste and gel showed that the toothpaste group expressed significantly lower MS levels than the mouthwash and gel groups at day-30. Chlorhexidine significantly reduced MS levels after 5 days of treatment, but these levels returned to baseline in other periods of the study. LSO toothpaste reduced MS levels after 5 days of treatment, and MS levels remained low and did not return to baseline during subsequent analysis. Hence, LSO toothpaste demonstrated the most long-lasting MS reduction in saliva, whereas other LSO formulations did not effectively reduce MS levels in children with dental caries.

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Introduction

The rise of herbal medicine has stirred interest in the effects of plant extracts for the control of plaque and other oral diseases (Buffon et al. 2001). Plaque is considered a primary factor in dental caries, thus justifying the use of measures for its control. Dental caries can progress rapidly resulting in mass destruction of primary dentition, compromising oral function and the child's well-being (Den Besten and Berkowitz 2003). Since early contamination with mutans streptococci (MS) is a major issue in this population

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http://dx.doi.org/10.1016/j.phymed.2014.04.021
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(Kohler et al. 1988) with the potential for significantly increasing the possibility of caries, strategies for treating this disease in children must focus on controlling growth of these pathogenic bacteria (Thibodeau and O'Sullivan 1999).

The essential oils of many different plants have been previously tested in both *in vitro* and *in vivo* studies, as promising agents in the treatment of oral diseases and other infections (Nostro et al. 2007; Pai et al. 2004). *Lippia sidoides* Cham., a plant of the verbenaceae family, popularly known as "Alecrim-Pimenta", is a bush with a brittle stem and odoriferous leaves, typically found in Northeastern Brazil. The chemical composition of *Lippia sidoides* Cham. essential oil (LSO) has been previously described (Botelho et al. 2007; Fontenelle et al. 2007; Sousa et al. 2002). The oil itself has proven to possess significant antifungal activity, and broad antimicrobial action against many different bacteria (Fontenelle et al. 2007). The two major constituents of LSO are thymol (50–59%) and carvacrol (7–16%) (Botelho et al. 2007; Fontenelle et al. 2007; Sousa et al. 2002). Phenolic compounds such as carvacrol and thymol have had their wide spectrum antimicrobial action against yeasts and bacteria established, being also constituents of other essential oils (Nostro et al. 2007). In spite of a limited number of clinical studies demonstrating the antimicrobial efficacy of LSO on dental caries and periodontal disease (Fernandes Filho et al. 1998; Girao et al. 2003; Botelho et al. 2007), no previous work has investigated its effect in children with dental caries.

We conducted a pilot study, which demonstrated that *Lippia sidoides* (LSO) was safe and had a good acceptance by children. This was a randomized, double-blind, in parallel with active control study, which aimed to evaluate the efficacy of three different formulations of LSO in the reduction of salivary *Streptococcus mutans* in children with caries.

Materials and methods

Extraction and chemical analysis of LSO

Samples of Lippia sidoides Cham. were originally obtained from the main garden of the Laboratory of Natural Products at UFC. Botanical identification of the plant's species was obtained at the Department of Biology. The collected leaves were dried under shadow, ground, kept in vacuum-sealed plastic bags and identified for future use. The essential oil was extracted approximately 9 months later by the steam distillation method in a Clevenger apparatus (Craveiro et al. 1976) and stored in glass containers, under refrigeration until the moment to be used. Chemical constituents were identified by specialists at the Department of Chemistry, in the same university by using a gas chromatographer coupled to a mass spectrometer system (GC–MS, Shimadzu, model QP 5050, Japan). The main components of the Lippia sidoides Cham. essential oil used in the present study were: cycloheptatriene (0.98%), benzene (2.07%), caryophyllene (3.59%), thymol/carvacrol (93.36%). Three different formulations of Lippia sidoides Cham. essential oil (LSO) were prepared for this clinical trial: (1) toothpaste, (2) gel and (3) mouthwash. The toothpaste and gel preparations contained a 1.4% LSO concentration, which rendered a total of 1.3% thymol/carvacrol, whereas the mouthwash formulation consisted of 0.8% LSO, rendering 0.74% of the Thy/Car mixture.

Patients

The study protocol was approved by the Medical School's Ethics Committee of the Federal University of Ceará, Brazil (Protocol #182/07). It complies with the current Brazilian laws. After written informed consent was given by parents or legal guardians, 81 volunteers, aged 6–12 years, from both genders, with at least one carious cavitated or non-cavitated lesion, were recruited to participate in the study. The volunteers were recruited by two graduate and one postgraduate student out of a population of 400 children searching for dental care at the Pediatric Dental Clinic of the Federal University of Ceará. Patients with a history of allergies or allergic diseases, e.g. asthma, urticaria, rhinitis, sinusitis, or intraoral soft tissue lesions, were excluded from the study. None of the participants underwent antibiotic treatment during the course of this clinical trial.

Treatment application

Participants were randomly assigned to either one of five different groups. Each group received topical treatment that was formulated by the Laboratory of Pharmaceutical Science at the Federal University of Ceará, Fortaleza, Brazil, with either a 1.4% LSO toothpaste, or 1.4% LSO gel, or 0.8% LSO mouthwash, or 1% chlorhexidine gel, or 0.12% chlorhexidine mouthwash. The gel and mouthwash of LSO and chlorhexidine were formulated with similar color and taste and the identification of each substance was concealed from the postgraduate student in charge of applying the treatment, and from the study participants, until the clinical trial was concluded. Therefore, this clinical trial with the exception of the toothpaste group consisted of a double-blind, randomized study. Gel and mouthwash treatments were applied in the Pediatric Dental Clinic at the Federal University of Ceará under the supervision of the study's principal investigator and with the assistance of a postgraduate student, whereas the toothpaste group was treated at home by parents, who were previously instructed to brush their child's teeth with a pea-size amount of toothpaste, during 1 min, once a day, for 5 consecutive days.

Before the start of treatment, a clinical examination was performed by only one examiner, using a visual/tactile method to calculate the number of decayed, missing and filled surfaces of these patients. All of the patients received the same toothpaste, toothbrush and recommendations for oral hygiene and diet to be followed throughout the study. The gel groups had the gel placed inside disposable trays, as a 5-ml volume, and applied for 1 min, every 24 h, for 5 consecutive days. The mouthwash groups used mouthwash placed inside disposable syringes, as a 5-ml volume.

Saliva collection and microbiological analysis

During saliva collection patients were asked to chew on a 3 cm × 3 cm piece of Parafilm® during 60 s in order to stimulate salivary secretion and release plaque into the salivary fluid. Saliva was then collected with a disposable plastic cannula and stored in sterile ependorfs® for subsequent analysis. Samples were transported to the laboratory for microbiological analysis in a hermetically sealed case containing ice, and analyzed no longer than 2 h after collection (Lopez et al. 2002).

A volume of 0.1 ml of each sample was aseptically drawn and transferred into one sterile test tube containing 0.9 ml of saline. Procedure was repeated twice, establishing dilutions of 1:10 and 1:100. A corresponding volume of 10 μl of each dilution was plated onto Mitis Salivarius-Bacitracin (MSB) agar medium (18) in triplicates. The plates were then incubated at 37 °C, during 48 h, in jars under microaerofilic conditions. Representative colonies with morphological characteristics of MS were counted, isolated and biochemically confirmed to be MS utilizing mannitol, sorbitol, lactose, raffinose, melibiose and esculin. Bacterial counts were expressed as colony forming units (CFU)/ml of saliva.

Statistical analysis

Data on the number of CFU were initially processed in order to homogenize the variances and make the distribution closer to

normality. To this end, we used a logarithmic transformation as the following equation: $y = \log 10(x) = \log(x)$. Because there were zero values, it was added 1 to x, since the logarithmic function is defined only for $x > 0$. Quantitative variables, continuous and discrete, were initially analyzed by the Kolmogorov–Smirnov test, in order to verify the normality of the distribution. For descriptive statistics, we calculated the mean and standard deviation (parametric data) or median, interquartile range and minimum and maximum values (nonparametric data). Comparisons between two treatment groups at each time were made by using the unpaired t test (parametric data) or Mann–Whitney test (nonparametric variables). To compare three or more groups (between groups analysis), we used analysis of variance (ANOVA) associated with Tukey's multiple comparisons test, to check differences between every two groups (parametric data), or Kruskal–Wallis test complemented by Dunn's multiple comparison test (nonparametric variables). Comparisons between different times in the same group (within group analysis) were carried out by repeated measures analysis of variance associated with the Tukey's multiple comparison test (parametric data) or by Friedman test complemented by Dunn's multiple comparisons test (nonparametric variables). In all cases, the significance level was set at 0.05 (5%), being considered statistically significant a p value less than 0.05. The data were analyzed using the software GraphPad Prism 5.00 (GraphPad Software, San Diego, California, USA, 2007).

Results

No important side effects were described by children, parents or guardians. Parents and guardians of children who used toothpaste LSO, reported an improvement in the breath of the children after 5 days of treatment.

Within group analysis

A difference in salivary MS levels measured in different days within the same treatment group was only verified with LSO toothpaste, chlorhexidine gel and chlorhexidine mouthwash. Chlorhexidine gel and mouthwash treatment demonstrated a significantly higher efficacy in MS reduction after 5 days of treatment ($p < 0.001$), but the amount of *Streptococcus mutans* returned to baseline on day 30 and remained so until the end of the study (Fig. 1). The temporal progression of the amount of salivary SM is showed in Fig. 2. The use of LSO gel did not cause a statistically significant variation in SM levels throughout the duration of the study. However, in relation to baseline, the treatment with LSO toothpaste significantly reduced the amount of salivary SM on days 5 ($p < 0.05$), 180 ($p < 0.01$) and 365 ($p < 0.001$), while the LSO mouthwash caused a significant decrease only in the second half of the study, on days 180 ($p < 0.05$) and 365 ($p < 0.001$).

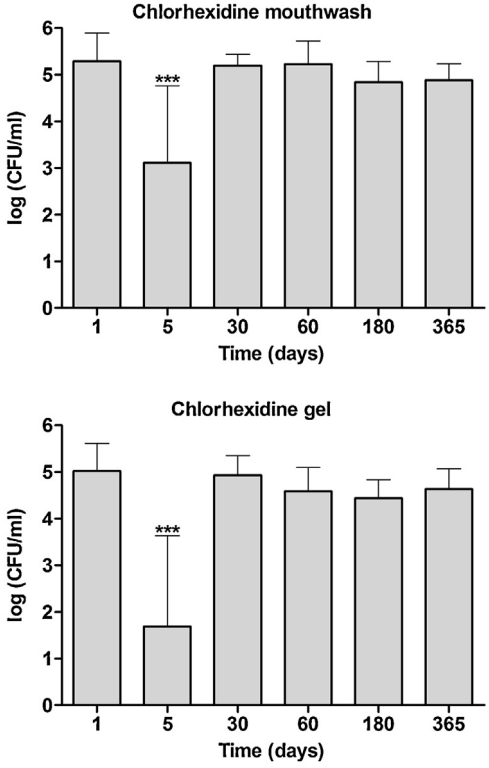

Fig. 1. *Streptococcus mutans* levels measured at days 1, 5, 30, 60, 180 and 365, expressed in log (CFU/ml), after a 5-day treatment with (A) chlorhexidine mouthwash or (B) gel formulations. Friedman and Dunn's tests used for comparisons ($p < 0.05$).

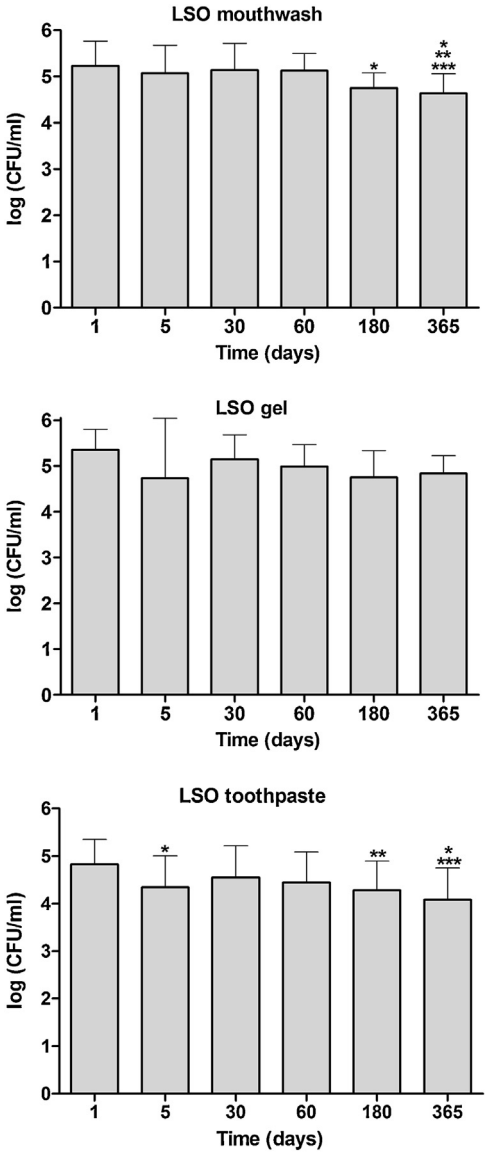

Fig. 2. *Streptococcus mutans* levels measured at days 1, 5, 30, 60, 180 and 365, expressed in log (CFU/ml), after a 5-day treatment with (A) Lippia sidoides Cham. (LSO) mouthwash, (B) gel, or (C) toothpaste formulations. Friedman and Dunn's tests used for comparisons ($p < 0.05$).

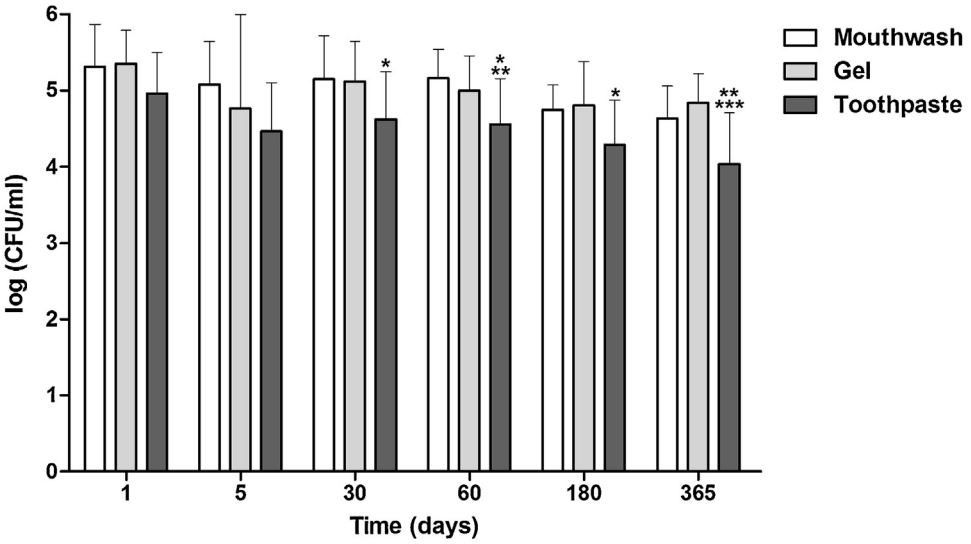

Fig. 3. Comparison of *Streptococcus mutans* (log CFU/ml) levels between groups treated with Lippia sidoides Cham. (LSO) mouthwash, gel, or toothpaste formulations, at days 1, 5, 30, 60, 180 and 365. Kruskal–Wallis and Dunn's tests used for comparisons ($p < 0.05$).

Between group analysis

During comparison between groups of LSO mouthwash, toothpaste and gel it was found that the amount of MS matter measured in the toothpaste group was significantly lower than that of the mouthwash and gel groups on days 30 ($p < 0.05$ compared to groups mouthwash and gel), 60 ($p < 0.01$ compared to mouthwash and $p < 0.05$ compared to the gel), 180 ($p < 0.05$ compared to groups mouthwash and gel) and 365 ($p < 0.01$ compared to gel and $p < 0.01$ compared to mouthwash) (Fig. 3).

In the comparison between groups of chlorhexidine mouthwash, chlorhexidine gel and LSO toothpaste at the end of a 5 day period, MS matter measured in the chlorhexidine gel group was significantly lower (***$p < 0.001$) than that of the groups chlorhexidine mouthwash and LSO toothpaste. Further, chlorhexidine mouthwash was significantly lower (*$p < 0.05$) than LSO-dentifrice. On day 30, the amount of MS matter measured in the LSO-toothpaste group was significantly lower (**$p < 0.01$) than that observed in group-Chlorhexidine mouthwash. Moreover, in 60 days, the quantities of salivary SM measured in the groups LSO-dentifrice (**$p < 0.01$) and chlorhexidine gel (**$p < 0.01$) were significantly lower than that observed in group-Chlorhexidine mouthwash. It was further observed that in 180 days, the SM quantities measured in groups LSO-dentifrice (**$p < 0.01$) and chlorhexidine gel (*$p < 0.05$) were significantly lower than that observed in the chlorhexidine mouthwash group. Finally, it was found that in 365 days, the amount of MS matter measured in the LSO-toothpaste group was significantly lower than that observed in the groups chlorhexidine mouthwash (***$p < 0.001$) and chlorhexidine gel (**$p < 0.01$) (Fig. 4). It was also observed a tendency of decreasing in the amount of salivary SM in the LSO toothpaste group over time.

Discussion

Studies have shown the success of antimicrobial treatment against dental caries (Lopez et al. 2002; Zhan et al. 2006). Chlorhexidine as an antiplaque and antigingivitis agent remains a gold standard, but in dental caries its effectiveness has been

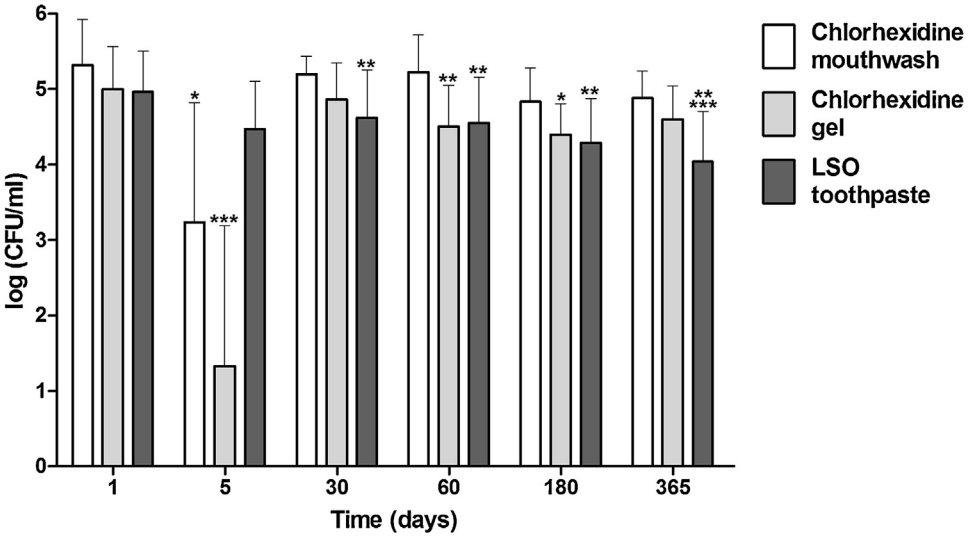

Fig. 4. Comparison of *Streptococcus mutans* (log CFU/ml) levels between groups treated with Chlorhexidine mouthwash, gel, or Lippia sidoides Cham. (LSO) toothpaste formulations, at days 1, 5, 30, 60, 180 and 365. Kruskal–Wallis and Dunn's tests used for comparisons ($p < 0.05$).

controversial (Twetman 2004). Chlorhexidine results in the present study confirmed those from previous studies reporting that it strongly reduces MS but it still allows recolonization (Emilson 1981, 1994; Lobo et al. 2008). The action of chlorhexidine is perhaps due its favorable chemical properties. In addition to binding to the negatively charged bacterial cell wall, exerting a bacteriostatic or bactericidal effect, chlorhexidine binds to the oral surfaces, dental pellicle and saliva (Oliveira et al. 2007), but recolonization remains a problem.

In this study, LSO toothpaste demonstrated a reduction of salivary MS after 5 days of treatment, and low MS levels were maintained throughout the study and these levels did not return to baseline during subsequent analysis (days 30, 60, 180 and 365). All patients received the same brush and toothpaste throughout the study, and new toothbrushes were provided every 3 months. The use of LSO was based on previous work by Nunes et al. (2006) that has standardized *Lippia sidoides* raw material and its vegetal extract, and has determined its antimicrobial activity against *Streptococcus mutans* in an *in vitro* study. The authors concluded that *Lippia sidoides* raw material and extract contained volatile constituents, which in plants relate to an intrinsic biological action, and thus could be employed in the production of clinical formulations for dental use, since its effectiveness against *Streptococcus mutans* was demonstrated. In 1990, Lemos et al. analyzed the antimicrobial action of different essential oils, and verified activity of LSO against *E. coli, S. aureus, P. aeruginosa, B. subtilis* and *M. smegmatis*. Limited antimicrobial activity was observed against gram negative bacteria, such as *P. aeruginosa* and *E. coli*. A later study of the antibacterial effects of essential oils of plants from Northeastern Brazil, demonstrated sensitivity of *S. aureus* and *E. coli* to LSO; however, *P. aeruginosa* showed resistance to the action of this essential oil (Bertini et al. 2005). These results have confirmed LSO as having mainly a gram positive antibacterial spectrum, in addition to a limited action against gram negative bacteria. However, to our knowledge, only two clinical trials testing the effectiveness of LSO formulations have been reported in the literature and none have tested LSO in a gel or toothpaste formulation. In addition, this study is the first to provide clinical data on the efficacy of LSO in reducing salivary MS in children with dental caries.

LSO mouthwash and gel did not demonstrate significant MS reductions, probably due to the lack of mechanical brushing. Furthermore the combination of natural products with chemicals, like fluoride, might improve the performance of antimicrobial agents, as a synergism between fluoride and LSO may improve MS reduction and therefore the control of caries. These findings were observed previously (Ditterich et al. 2007; Oliveira et al. 2007; Rosell et al. 2004). Some studies have thwarted the antimicrobial activity of natural substances in toothpastes. Ditterich et al. (2007) evaluated the *in vitro* antimicrobial action of 7 toothpastes composed of natural substances where the formation of inhibition zones were observed. The authors concluded that the addition of natural antimicrobial agents to dentifrices can act as an adjuvant in the mechanical control of the dental biofilm. Another *in vitro* study evaluated the antimicrobial activity of 9 toothpastes against MS in individuals with high bacterial levels in saliva, and observed the inhibition of MS, concluding that the combination of natural ingredients can help maximize the performance of toothpastes against the main bacterium that causes tooth decay (Rosell et al. 2004).

In the present study, all different formulations of chlorhexidine significantly reduced MS levels in the saliva of children with dental caries after 5 days of treatment, but MS levels returned to baseline on day 30 and remained so until the end of the study. LSO mouthwash and gel formulations did not significantly reduce salivary MS levels in children with dental caries. However, the presently tested 5-day LSO toothpaste treatment successfully reduced MS levels, and most importantly, MS levels remained low and did not return to baseline during subsequent analysis, suggesting a synergist effect between fluoride and the antibacterial constituents present in *Lippia sidoides Cham.* essential oil, and emphasizing the importance of the mechanical removal of the dental biofilm in long-term antibacterial effect against *Streptococcus mutans*.

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