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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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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.

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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(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