The anti-adherence effect of *Lippia sidoides* Cham. Extract against microorganisms of dental biofilm

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RESUMO: Efeito anti-aderente de Lippia sidoides. Extrato contra microorganismos do biofilme dental. É fato comprovado que a maior parte das doenças que acometem a cavidade bucal são de origem infecciosa. Várias categorias de agentes químicos têm sido utilizadas no controle químico do biofilme dental através de estratégias que visam a redução da adesão bacteriana, a inibição do crescimento, e a proliferação dos microrganismos na superfície do dente. A utilização das plantas pela medicina popular, seu uso na Odontologia e a divulgação dos êxitos, conduziram a exploração científica resultando no conhecimento químico-farmacológico de milhares de plantas. A presente pesquisa objetivou avaliar a atividade antiaderente da folha da *Lippia sidoides* Cham. comparando seus resultados com a Clorexidina 0,12%, através de uma simulação, *in vitro*, do biofilme dental. As linhagens bacterianas utilizadas na pesquisa foram o *Streptococcus mutans*, *Streptococcus sanguinis,* e o *Lactobacillus casaram*, principais responsáveis pela aderência do biofilme. O extrato estudado mostrou-se efetivo na inibição de aderência das bactérias ensaiadas até uma concentração de 1:16, sobre o *Streptococcus mutans*, sendo comparável à Clorexidina. O extrato da *Lippia sidoides* Cham. demonstrou efeito antiederente, sobre os principais microrganismos responsáveis pela consolidação do biofilme dental.

Palavras-chave: Biofilme dental, Microrganismos, *Lippia sidoides*.

ABSTRACT: Most illnesses affecting the oral cavity are proven to have infectious origin. Several categories of chemical agents have been used in the chemical control of dental biofilm through strategies that aim at reducing bacterial adhesion and inhibiting the growth and the proliferation of microorganisms on the tooth surface. The use of plants in folk medicine and in Dentistry, as well as the spread of successful cases, has led to scientific exploration, resulting in chemical-pharmacological knowledge of thousands of plants. The present study aimed to evaluate the anti-adherence activity of *Lippia sidoides* Cham., comparing the results with those of 0.12% chlorhexidine by means of an *in vitro* simulation of dental biofilm. The studied bacterial strains were *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus casei,* main responsible for the biofilm adherence. The studied extract was effective in inhibiting the adherence of *Streptococcus mutans* up to a concentration of 1:16, compared to Chlorhexidine. *Lippia sidoides* Cham extract showed anti-adherence effect on the major microorganisms responsible for dental biofilm consolidation.

Key words: Dental biofilm, Microorganisms, *Lippia sidoides.*

INTRODUCTION

Dental biofilm consists of microorganisms, squamous epithelial cells, food remains and macromolecules synthesized by proliferating bacteria, which adhere to the acquired film or directly onto the tooth surface (Lindhe, 1992).

Several categories of chemical agents have been used in the chemical control of dental biofilm by means of strategies to reduce bacterial adhesion, to inhibit microorganism growth and proliferation on the tooth surface, and to inhibit the formation of biofilm

Recebido para publicação em 05/08/2001
Aceito para publicação em 02/07/2012

intercellular matrix, the modification of biochemical activity and biofilm ecology by a less pathogenic microbiota (Ignácio; Peres; Cury, 1999; Moreira et al., 2001).

A large number of studies have evaluated the antimicrobial properties of substances associated with the prevention and the control of biofilm and cavity. Some of these substances, such as chlorhexidine, have been frequently reported in the scientific literature, whereas others have been little explored, as is the case for plant extracts (Almeida et al., 2006).

Chlorhexidine is one of the most effective and safest substances since it is strongly absorbed by the oral surface and is gradually released from the action sites, potentially reducing the growth and the metabolism of dental biofilm and favoring the adherence of microorganisms (Vinholis et al., 1996). Chlorhexidine acts in the general disorganization of the cellular membrane and in the specific inhibition of membrane enzymes. It inhibits glucose uptake by *Streptococcus mutans*. Although it is an excellent antimicrobial, its side effects make its prolonged use not recommended. Thus, there is the need of developing substances equally effective but without those side effects, which include: tooth coloration, reversible squamous mucous membrane, taste alterations and increased calcified supragingival deposits (Towers et al., 2000).

Natural products used in Dentistry, according to Gebara et al. (1996), present good perspectives for the market due to the popular acceptance of phytotherapy and could be introduced since they have been thoroughly supported by specific laboratorial and clinical studies. Several substances have been used as antiseptic agents in folk medicine. Those studies have also demonstrated the actions of a series of chemical products, biological agents and natural antiplaque and anticavity substances which primarily act on the formation of extracellular polysaccharides (Cury, 1998).

Phytotherapic substances have been used in Dentistry and thus should be compatible with live tissues; subsequently, there is the need for *in vitro* studies. Dental biofilm is considered the main etiological factor of cavity and periodontal illness. The search for alternative resources is already a reality in view of the exposed advantages, justifying, therefore, the need of studying the action of medicinal plants on the microorganisms that form the dental biofilm, such as *Lippia sidoides* Cham. (Rosemary-pepper), a plant broadly found in the Brazilian Northeast region and commonly used by the population.

To determine the effect of green tea infusion at 10% on bacterial plaque formation by *Streptococcus mutans*, successive cultures were prepared at every 24 hours, for 7 days, in 5% sucrose broth. Results showed that *S. mutans* cultures not added of green tea showed formation of strongly adhered bacterial plaque, while cultures added of green tea infusion, when residues were formed, had little adherence, with rapid detachment (Nakata; Cadillo, 2006).

This study evaluated the antimicrobial activity of the stem of cashew tree (*Anacardium occidentale* Linn.) and lemon (*Citrus limon* Linn.) on strains of *Streptococcus mutans*, *Streptococcus mitis, Streptococcus sanguinis, Streptococcus sobrinus* and *Lactobacillus casei*. The bark of cashew tree showed antimicrobial and anti-adherence activity, while lemon bark did not present antimicrobial activity *in vitro* but inhibited the synthesis of glucan (Araújo, 2005).

Lippia sidoides Cham. belongs to the family Verbenaceae. It is a large tree or a bush, largely disseminated and measuring up to three meters high; both its fresh and dry leaves have strong smell and spicy aromatic flavor, and it is commonly known as "*estrepa cavalo*" or "*alecrim do nordeste*" (Matos et al.,1998; Lorenzi, Matos, 2002).

It is a plant from the northeast of Brazil. Its essential oil contains thymol and carvacrol, providing this plant with a strong antiseptic action against fungi and bacteria (Randüz et al., 2001; Costa et al., 2002; Leal et al., 2003).

Nunes (2005a) characterized it as raw matter for pharmaceutical products; that author carried out loss tests based on dissection, essential oil content and chromatographic profile to verify the variety of its use and characterize its identity. He also evaluated the antimicrobial activity, which was proven against *Streptococcus mutans* (Nunes et al. 2005b).

In 1996, Lacoste et al. studied the antibacterial and antifungal properties of *Lippia sidoides* Cham. on the cutaneous microflora and noted that its main active principles (thymol and carvacrol) make it strongly antiseptic.

This study evaluated the effectiveness of essential oil from *Lippia sidoides* Cham. in inhibiting the growth of *Staphylococcus aureus* strains with different antibiotic resistance profiles and isolated from clinical material. Results showed prominent anti-*Staphylococcus aureus* activity by essential oils from *Lippia sidoides,* evidenced by great halos that inhibited the bacterial growth (15-21 mm), supporting the possibility of its rational use as alternative antimicrobial agent (Oliveira et al., 2006).

Couto et al. (2000) verified the biological compatibility of the hydroalcoholic extract from *Lippia sidoides* Cham. with subcutaneous tissue of mice. At the end of the experiments, the extract was shown to be well tolerated by the subcutaneous tissue of

animals. *Lippia sidoides* Cham. also had its effects proven in reducing the presence of marginal gingivitis in dogs (Girão et al. 2003) and its effectiveness on dental biofilm was already evaluated in humans (Fernandes-Filho,1998).

Lippia sidoides is widely used in a social project for pharmaceutical assistance entitled "Living pharmacies", which uses phytotherapy, especially local plants, which are cheap and very effective, to help people from the needy communities. This plant is mainly used as antiseptic due to its strong action on a large number of microorganisms. To evaluate the action spectrum of this plant, pharmacological studies were undertaken and indicated that *Lippia* did not present toxicity at the studied concentrations and was shown as a gastroprotector and anti-inflammatory agent (Monteiro et al., 2007).

Santiago (2007) compared the effectiveness of *Lippia* with that of a rosemary-pepper oral antiseptic without alcohol in preventing cavities.

The aim of this study was to verify the anti-adherence effect of bacteria in order to generate, in future investigation, new possibilities of phytotherapy against the dental biofilm, minimizing the epidemiological problems of cavities.

MATERIAL AND METHODS

Botanical material and extract preparation

Raw matter (leaves) of *Lippia sidoides* Cham., at the beginning of flowering, was collected on the same day, at 10 a.m., when the essential oil concentration was higher, from the garden of medicinal plants (plant nursery) of the Laboratory of Pharmaceutical Technology (LTF), Univ Federal da Paraíba (UFPB); voucher specimens were deposited in the collection of the Herbarium "Lauro Pires Xavier" of the Department of Systematic and Ecology of UFPB, Voucher number JPB 37176, identified by Maria Regina de Vasconcellos Barbosa.

Extracts were obtained in the Laboratory of Pharmaceutical Technology at the Department of Pharmacy of Univ Federal de Pernambuco. For extraction, the leaching method in continuous flow at room temperature was adopted, using the extraction solution (80% v/v) constantly renewed for a 12-h period.

Bacterial strains

In the present study, standardized bacterial strains were used: *S. mutans* (ATCC 25175), *S. sanguinis* (ATCC 10557) and *Lactobacillus casei* (ATCC 7469); they were obtained in the Tropical Foundation for Technological Research "André Tozello" (Campinas, São Paulo State) and Adolfo Lutz Institute – São Paulo State; they were sent in Blood Agar slants and later reactivated at the Laboratory of Microorganism Genetics - Department of Molecular Biology/CCEN/UFPB.

Determination of minimum inhibitory concentration of adherence for the hydroalcoholic extract from *Lippia sidoides* Cham.

The minimum inhibitory concentration of adherence (MICA) of the bacteria *Streptococcus mutans*, *Streptococcus sanguinis* and *Lactobacillus casei* (main responsible for the adherence) to the glass was determined in the presence of sucrose at 5%, used to grow and double the concentrations of the diluted solution of the isolate and in association extracts, varying from 1:1 to 1:1024. The overnight growth of the strains was subcultured at 37ºC in Müeller-Hinton broth (DIFCO), obtaining a10^6 CFU/ml inoculum. The culture (1.8 mm) was distributed in hemolysis tubes and 0.2 ml of the solution was added, corresponding to the extract scale. Incubation was conducted at 37º C for 24 hours in microaerophilia, with tubes bent to 30º. Reading was based on the visual observation of bacterial adherence to the walls of the tube after agitation. MICA was defined as the smallest concentration of the agent by means of the sucrose that prevented adherence to the glass tube.

Data were collected, organized and presented as tables with their respective percentiles.

RESULTS

Minimum inhibitory concentration of adherence (MICA) for the plant extract.

Results regarding the minimum inhibitory concentration of adherence (MICA) for the extract from *Lippia sidoides* Cham. against *S. mutans, S. sanguinis* and *L. casei* are shown in Table 1.

According to the results shown in Table 1, the hydroalcoholic extract presents anti-adherence activity, which is represented by the absence of bacterial adherence to the wall of the glass tube, demonstrating the extract capacity to inhibit glucan

TABLE 1. Comparative data of minimum inhibitory concentration of adherence for extracts from *Lippia sidoides* Cham., compared with 0.12% chlorhexidine gluconate, against *Streptococcus mutans, Streptococcus sanguinis* and *Lactobacillus casei*.

Strains	*Lippia sidoides*	Chlorhexidine
S.mutans	1:16	1:16
S.sanguinis	1:4	1:16
L.casei	1:2	1:16

synthesis by glucosiltransferase. *Lippia sidoides* presented anti-adherence effect on *S. mutans* which was similar to that of chlorhexidine.

The MICA for *Lippia* on *Streptococcus mutans* is represented in Figure 1. There was bacterial adherence to the wall of the glass tube after staining with fuchsine. From a concentration of 1:32, in the superior tube, and absence of adherence to the wall of the glass tube when in the form of pure extract, represented by the inferior tube.

DISCUSSION

Phytotherapy is the field of knowledge that searches for the cure of illnesses by using medicinal plants and, although associated with empirical common knowledge, it has been gradually recognized and incorporated into scientific knowledge (Dantas, 2006).

Filho (1998) evaluated the antimicrobial activity of several essential oils from Northeastern plants on bacteria of the genus *Streptococcus*, isolated from the oral microbiota; rosemary-pepper was the most effective and was thus chosen for the preparation of the used oral antiseptic, reducing by 6% the dental biofilm and inhibiting by 12% the microorganisms.

Every infectious process presents an initial phase that consists in the implantation, i.e., in the adherence and colonization of the pathogen to some tissues of the host. Such adherence does not occur at random and the microorganisms do not randomly adhere to tissues. For bacteria to adhere to the host tissue, their adhesins must be recognized by specific receptors that are on the tissue surface. Some bacterial species, such as *Streptococcus mutans*, consolidate their adherence to the tissues by producing extracellular polysaccharides such as glucan. Adherence is very important in the beginning of the infectious process and currently the fringes, fibrils and their adhesins are considered virulence factors. Based on that fact, researchers have been trying to develop means of preventing this first phase of infection.

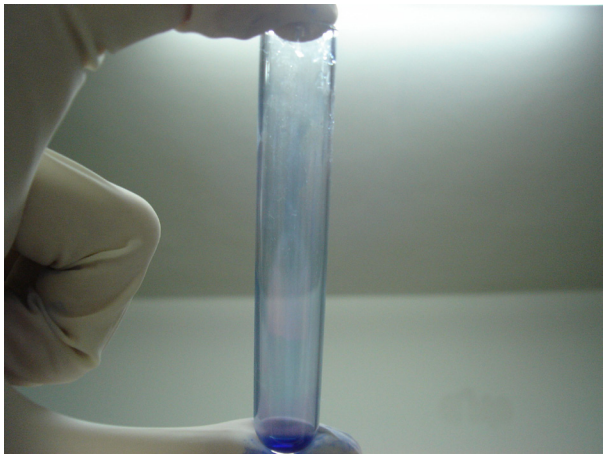

FIGURE 1. Photographic representation of the minimum inhibitory concentration of adherence for *Lippia sidoides* Cham. against *Streptococcus mutans*, at the concentration of 1:32.

This study aimed to inhibit the formation of insoluble glucans from sucrose by using extract from *Lippia sidoides* Cham. for inhibiting the adherence to the glass.

The analyzed extract was effective in inhibiting the adherence of the three tested strains, represented by the absence of adherence to the glass in the presence of sucrose.

Lippia sidoides Cham. inhibited *S. mutans* adherence to the glass tube wall up to the dilution of 1:16, followed by *S. sanguinis* up to 1:4. These results are quite promising once those microorganisms are largely responsible for dental cavity formation.

Landucci (2003) evaluated the effects of different coffee (*Coffea arabica*) solutions on the adherence of *Streptococcus mutans* strains to the glass surface. Coffee solutions were prepared with distilled water with 1ppm fluoride: coffee solution at 8% and coffee solution at 16% both prepared with boiled water drained through the powder (simple coffee solution); coffee solution at 8% and coffee solution at 16% both prepared with water that was boiled together with the powder and then filtered (oiled coffee solution); results indicated that *Coffea arabica* solution at 16% (boiled coffee solution) was more effective in inhibiting *Streptococcus mutans* adherence.

Another study evaluated the extract from pomegranate fruit peel (*Punica granatum* Linn.) for antimicrobial activity and capability of inhibiting glucan synthesis *in vitro* on strains of *Streptococcus mitis, Streptococcus mutans, Streptococcus sanguinis, Streptococcus sobrinus* and *Lactobacillus casei*. Considering MICAs, *Punica granatum* extract showed better results compared to chlorhexidine, except for *S. mutans* which yielded similar findings at the dilution of 1:256 (Pereira et al., 2006).

The antimicrobial activity of cashew (*Anacardium occidentale* L.) stem bark extract was evaluated, showing to be effective in inhibiting the adherence of the three tested strains, represented by the absence of adherence to the glass in the presence of sucrose; the most effective extract was observed for *S. sanguinis*, which is considered the most important agent in the initial phase of bacterial biofilm deposition (Melo et al., 2006).

The effect of extracts from “aroeira-do-sertão” (*Myracrodruon urundeuva* All.), guava tree (*Psidium guajava* Linn.) and mallow (*Malva sylvestris*) was studied on strains of *Streptococcus mutans,*

Streptococcus mitis, Streptococcus sanguinis, Streptococcus sobrinus and *Lactobacillus casei.* The extracts demonstrated excellent effectiveness in inhibiting glucan synthesis, represented by the adherence to the glass (MICA) and results similar to those found for chlorhexidine (Alves, 2005).

The positive control of this experiment (chlorhexidine gluconate at 0.12%) inhibited the adherence of all tested strains at the dilution of 1:16. Comparison of the obtained data indicates that, in this experiment, the three analyzed extracts presented results inferior to those of chlorhexidine, compromising the anti-adherence action.

CONCLUSIONS

According to the used methodology and based on the obtained results, it can be concluded that:

Extract from *Lippia sidoides* Cham. showed effective anti-adherence action on the microorganisms most responsible for the dental biofilm consolidation;

The presented results are quite promising once they demonstrate potential anti-adherence activity for the tested plant extract, which generates future perspectives for continuing this study by means of clinical-toxicological tests, including *in vivo* tests.

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