

Full Length Research Paper

Antibacterial activity and phytochemical screening of extracts of *Lippia alba* (Mill). NE Brown

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Received 22 March, 2014; Accepted 27 June, 2014

The use of natural products as an alternative for treatment and prevention of diseases has been increasing daily. *Lippia alba* is a shrub widely distributed throughout Brazil and its aqueous extracts, as infusion and decoction made from its leaves, are currently being used in popular medicine. Thus, the objective of the study was to do a phytochemical screening of these solutions as well as evaluate *in vitro* antimicrobial activity of these solutions in the reduction of: *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Staphylococcus aureus* (ATCC 12600) and *Lactobacillus casei* (ATCC 27216). The negative and positive controls were respectively, BHI and Chlorhexidine. Statistical analysis was performed using the Mann-Whitney and Kruskal-Wallis test with Bonferroni's penalization, showing significant difference between the products tested ($p < 0.001$). We found some phenolic compounds in the solutions. In all the microorganisms, the decoction and the infusion were more effective as compared to other products, having significant difference ($p < 0.05$). Therefore, the aqueous extracts of *L. alba* as well as being natural and easy to handle, were more effective against oral bacterias than chlorhexidine, which is considered a gold standard bactericidal agent.

Key words: Phytotherapy, dentistry, antimicrobial activity.

INTRODUCTION

The knowledge on plants has always followed the evolution of human civilizations throughout the ages. The use of medicinal plants dates from ancient times, when they were used by ancient Egyptian and Chinese civilizations in approximately 3000 BC (Jeon et al., 2011). Currently, it is seen as a favored alternative due to the lower risk of toxicity and ease of preparation.

Phytotherapy has been recommended by the World

Health Organization (WHO) since 1970. In Brazil, the use of teas in popular medicine is regulated by the Resolution Board 267/05, considered relevant by the National Medicinal Plants and Herbal Medicine Board and secured by the National Policy on Integrative and Complementary Practices in public health (Brazil, 2010).

In this context, *Lippia alba* (Mill.) NE Brown is one of the many plants that can be used as herbal medicine. It

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belongs to the Verbenaceae family and it is commonly found in the Americas, from southern United States to Argentina, being of Asian origin. The flowers have colors ranging from blue to purple (Henao et al., 2011), this being an important tool in the differentiation from *Melissa officinallis*.

The decoction and infusion of the *L. Alba* leaves are currently used in popular medicine to combat various diseases. The extracts are used in the treatment of gastric diseases, diarrhea, fever, cough, asthma and as a tranquilizing drug (Sena-Filho et al., 2006). Furthermore, many authors have confirmed the antibacterial activity of *L. alba* against various bacteria such as *Staphylococcus aureus*, *Klebsiella pneumonia* (Sena-Filho et al., 2006), *Escherichia coli*, *Helicobacter pylori* (Henao et al., 2011) and *Vibrio parahaemolyticus* (Ara et al., 2009). Aguiar et al. (2008) demonstrated antimicrobial activity in the *L. alba* leaves against *Micrococcus luteus*, *Bacillus subtilis*, *Mycobacterium smegmatis* and *Monilia sitophila*.

Due to its popularity and the scarcity of data on the antibacterial activity of *L. alba* against oral bacteria, this study aimed to analyze the inhibitory activity against bacterial strains colonizing the oral environment, *in vitro* studies have been realized with oral monocultures to evaluate the effects of various substances (Moon et al., 2011). Thus, we sought to contribute to the body of research on herbal medicines against oral microbiota, as there are no reports on the literature of activity from the decoction and infusion of *L. alba* against oral microorganisms.

MATERIALS AND METHODS

Microorganisms

Four strains of bacteria commonly found in the mouth were used: *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (ATCC 49456), *Staphylococcus aureus* (ATCC 12600) and *Lactobacillus casei* (ATCC 27216), they were obtained by request from the Oswaldo Cruz Foundation (Rio de Janeiro/RJ) and then reactivated in the Microbiology Department of Odontology/CCS/UFRN. These bacteria were selected because they are directly responsible for the colonization of tooth surfaces and consequently the formation of biofilm, thus being responsible for caries. The *S. aureus* was used because it is a microorganism that co-exists in the mouth, having high pathogenic potential.

Plant material

The plant specimen was collected from the city of Ceará-Mirim, Rio Grande do Norte, Brazil, in November 2010. The pharmacogenes used were part of the plant, specifically the leaves. The plant was botanically identified by the Federal University of Rio Grande do Norte herbarium with the reference number 16583.

Preparation of extracts

The extracts (decoction and infusion) were prepared following the Brazilian regulations recognized by ANVISA – National Agency of

Sanitary Supervision. These regulations aim to standardize the extracts and will be used by SUS– the national public health system. Accordingly, both extracts were prepared following ANVISA's decree 519/98 (Brazil, 1998), at a ratio of 1:10 (mass/volume) and filtered vacuum. Thus, for the decoction, 50 g of pulverized dried leaves were mixed with 500 ml of water and boiled for 5 min. For the infusion, 500 ml of water was boiled at 100°C for 5 min and then poured over 50 g of pulverized dried leaves, after the mixture was left to rest for 5 min. Since they are extractions (infusion and decoction) that have been prepared following the popular mode of preparation and intended for home use, the shelf life is limited to 24 h when left at ambient temperature, this is because both have water as vehicle extractor which favors the proliferation of fungal extracts and becomes more unstable with microbial growth leading to changes in color, odor and flavor. However, if stored in the refrigerator or freezer at -20°C (203.15 K) the period of consumption can be up to 90 days, provided that the preparation has been successful as advocated by specific legislation (Brazil, 2010).

Phytochemical analysis

The phytochemical screening was based on the Wagner and Bladst (2001) protocol. The thin layer chromatography (TLC) followed the protocol proposed by Julião et al. (2003) and Lionço et al. (2001), using Ferric Chloride at 5% and A natural was revealed.

Evaluation of antimicrobial activity

In dentistry, there are already many studies evaluating the minimum inhibitory concentration (MIC) of herbal products and this proposal was to present a new methodology adapted from the study of Christensen et al. (1985) and Stepanovic et al. (2000) which also evaluated the antimicrobial activity of medicinal plants but mimicked the mouth environment. There was no initial intention to evaluate the bactericidal kinetics or time of death of such microorganisms. The *in vitro* antimicrobial evaluation in similar research is usually carried out by dilutions of the extract and the same tests carried out on disks or wells (holes) made in Petri dishes of isolated bacteria. However, despite promising results in bacterial suspension monoculture our intention was to use *in vitro* sterile polystyrene microplates with inert flat bottom, containing 96 wells (Nunclon; InterMed) to simulate an environment closer to mouth reality. For this purpose, modifications were carried out to streamline the protocol. The culture medium used was BHI (DIFCO®, Michigan, USA) broth with 5% sucrose and the culture was diluted 1:100. The negative control that was used was 10 µL of bacteria + 990 µL of BHI. To evaluate the antimicrobial activity of *L. alba*, we used 10 µL culture + 90 µL of the extract + 900 µL of BHI. The positive control was 0.12% chlorhexidine (Periogard®, Colgate-Palmolive Company, New York, USA). This solution was applied to 200 µL in microtiter plate wells. The plate was incubated for 20 h at 37°C. The optical density (OD) growth was measured at 570 nm. The experiment was done eight times for greater certainty in the results.

Statistical analysis and percentage

Statistical analysis was carried out with STATA (10.0) software using the Kruskal-Wallis and Mann-Whitney test with Bonferroni penalty. The quantitative results obtained after statistical analysis were converted into quality for better viewing of the antimicrobial activity from the products tested. Following the logic of the percentage inhibition (PI), a PI>90% was considered as very strong (+++); 90%>PI>1% was considered strong (+), PI < 1% inhibition very low (---).

Table 1. Phytochemical screening of the infusion and decoction of *L. alba*.

Test	Infusion	Decoction
Saponins	+	++
Phenols	+++	+++
Tannins	+	+++
Gum	+++	+++
Flavonoids	++	++
Heterosides	S	+++
Alkaloids	+ (Dragendorff e Bouchard)	+++ (Bertrand)
Resins	++	+++

Strong presence (+++); presence moderate (++), trace (+), reaction suspected (S).

Table 2. Antimicrobial activity of infusion and decoction of *L. alba*.

Product Bacteria	Product			
	BHI	Infusion	Decoction	Chlorhexidine
<i>Streptococcus mutans</i> (ATCC 25175)	---	+++	+++	+
<i>Streptococcus mitis</i> (ATCC 903)	---	+++	+++	+
<i>Staphylococcus aureus</i> (ATCC 25923)	---	+++	+++	+
<i>Lactobacillus casei</i> (ATCC 9595)	---	+++	+++	+

Intensity of inhibition of bacterial growth: very weak (---); strong (+); very strong (+++).

Table 3. Median (quartile 25/quartile 75) and statistical significance of the tested products.

	Infusion	Decoction	CHX	p
<i>S. mutans</i>	-0.308(-0.392/-0.238) ^a	-0.398(-0.574/-0.262) ^a	0.044(0.001/0.192) ^b	<0.001
<i>S. mitis</i>	-0.152(-0.172/-0.142) ^c	-0.478(-0.578/-0.399) ^d	0.022(0.009/0.036) ^e	<0.001
<i>S. aureus</i>	-0.190(-0.280/-0.088) ^f	-0.177(-0.470/-0.084) ^f	0.369(0.334/0.456) ^g	<0.001
<i>L. casei</i>	-0.274(-0.313/-0.218) ^h	-0.522(-0.620/-0.411) ⁱ	0.308(0.270/0.340) ^j	<0.001

Same letters indicate no statistically significant difference, different letters indicate statistically significant difference.

Thus, the PI of the products tested were found using the following formula:

$$PI = [1 - (PTMed/NCMed)] \times 100$$

Where, PTMed = Product tested median; NCMed = negative control median

RESULTS

The infusion and decoction, in general, showed very similar chromatographic profiles in all solvent systems used (Table 1). A chromatographic thin layer indicated

the presence of ellagic acid in the infusion, which was not observed in the decoction.

As shown in Table 2, the infusion and decoction showed highly effective antibacterial activity against the bacteria whose percentages of inhibition were higher than the substance considered the gold standard in dentistry, chlorhexidine, which showed an inhibition of between 87-62%, indicating bacterial growth. In Table 3, the statistical significance of the results can be seen.

DISCUSSION

Given the strong presence of phenols, including tannins

and flavonoids in the chromatographic analysis, we decided to research these specific compounds, because many of these compounds have antimicrobial activity which is well known and has been studied since Duarte et al. (2006) found an inhibitory effect of phenols in the formation of *S. mutans* biofilms. Given the potential antimicrobial activity of these compounds, especially tannins, this finding is important as a possible justification for the pharmacological activity of these extracts.

Although both are aqueous and present similar chromatographic profiles, the process of preparation is different, which can influence the antimicrobial activity. The presence of ellagic acid in the infusion shows antimicrobial activity not present in the decoction (absence of ellagic acid), tannin-infusion can be seen as one of the justifications for this statement. Some studies have shown that the extraction temperature influences the chemical distribution of secondary metabolites (Velloso et al., 2009; Mossi et al., 2004). However, the difference between boiling the plant and adding hot water to it should be considered as a factor that influences the withdrawal of the plant's secondary metabolites.

The mechanism of antimicrobial action of tannins can be explained by three assumptions. The first assumes that tannins inhibit bacterial and fungal enzymes and/or complexes with substrates of these enzymes; the second includes the action of tannins on the cell membranes of microorganisms by changing their metabolism; and the third is based on the complexation of tannins with metal ions, decreasing the availability of ions essential for microbial metabolism (Loguercio et al., 2005; Scalbert, 1991). Simões (2003) confirms that the antimicrobial activity present in some plants is directly related to the presence of the metabolite (tannin), which also possesses a bactericidal characteristic.

The flavonoids are composed of hydrosoluble phenolics and are important defense agents against phytopathogenic microorganisms like viruses, bacteria and fungus, acting as a natural defense in plants in the form of a chemical response against an invasion of pathogens. They, thus, possess numerous biological activities, of which we can highlight the antimicrobial and anti carcinogenic activity, which have a very low toxicity in human cells (Capasso et al., 2003; Zuanazzi, 2000).

The strong antimicrobial activity against *S. aureus* is consistent with Soares (2001) study. However, Caceres et al. (1991) has already studied the power of antibacterial ethanolic extracts from *L. alba* against this bacterium. It is important to note that there is no literature to date referring to the antimicrobial activity of aqueous extracts of *L. alba* against *S. mutans*, *S. mitis*, *L. casei* and *S. aureus*, and, therefore, this is a pioneering study in this regard. On the other hand, it is common to find streptococcal species implicated in endocarditis and it is capable of inducing cell death (Okahashi et al., 2011), showing the importance of mouth health to systemic health.

Conclusion

The antimicrobial activity observed in the infusion and decoction of the leaves of *L. alba* encourages new research on single substances in order to establish constituent chemicals that are responsible for this activity. However, more research is necessary, especially *in vivo* and *in situ*, to establish conclusive evidence for the efficiency and clinical applications of these compounds in the prevention of dental caries.

Conflict of Interests

The authors have not declared any conflict of interests.

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