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

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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 bacteria 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
oficinalis.

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, Klebsiela 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 monocolures to eva-
luate 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
decocion and infusion of L. alba against oral micro-
organisms.

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
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
deccoion) 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 consump-
tion 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 Juliaño 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 bactericidial 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 mono-
culture 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 & Bouchard) | +++ (Bertrand) +++ (Bertrand) |
| Resins        | ++       | +++       |

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

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

| Product | Bacteria                         | BHI | Infusion | Decoction | Chlorhexidine |
|---------|----------------------------------|-----|----------|-----------|--------------|
|         | *Streptococcus mutans* (ATCC 25175) | --- | +++      | +++       | +            |
|         | *Streptococcus mitis* (ATCC 903)    | --- | +++      | +++       | +            |
|         | *Staphylococcus aureus* (ATCC 25923) | --- | +++      | +++       | +            |
|         | *Lactobacillus casei* (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.

| Product | Infusion | Decoction | CHX | p     |
|---------|----------|-----------|-----|------|
| S. mutans | -0.38(-0.39/-0.23) | -0.39(-0.57/-0.26) | 0.044(0.001/0.19) | <0.001 |
| S. mitis | -0.15(-0.17/-0.14) | -0.48(-0.57/-0.39) | 0.022(0.009/0.03) | <0.001 |
| S. aureus | -0.19(-0.28/-0.08) | -0.17(-0.47/-0.08) | 0.369(0.33/0.45) | <0.001 |
| L. casei | -0.27(-0.31/-0.21) | -0.52(-0.62/-0.41) | 0.308(0.27/0.34) | <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:

\[
PI = \left[1 - \frac{PTMed}{NCMed}\right] \times 100
\]

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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