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In vitro Antibacterial Activity of *Maclura tinctoria* and *Azadirachta indica* against *Streptococcus mutans* and *Porphyromonas gingivalis*

Matson Robles Alvaro[1], Herrera Herrera Alejandra[2*] and Díaz Caballero Antonio[3]

[1]"Joven Investigador de Colciencias", "Universidad de Cartagena", Colombia.
[2]"Universidad de Cartagena", Master in Farmacology "Universidad de Cartagena", "Corporación Universitaria Rafael Nuñez" and "Universidad de Cartagena", Colombia.
[3]Universidad de Cartagena, Specialist in Periodontics, "Universidad Javeriana", "Universidad de Cartagena", "Universidad de Cartagena", Director of GITOUC Research Group, Colombia.

Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: *Azadirachta indica* and *Maclura tinctoria* are two plants that have been reported with some ability to inhibit the growth of different types of bacteria so the aim of this project was to evaluate the *in vitro* antibacterial activity of the extract of *Maclura tinctoria* and *Azadirachta indica* on *Streptococcus mutans* and *Porphyromonas gingivalis.*
Methods: Ethanol extracts were obtained from *Azadirachta indica and Maclura tinctoria*. Antibacterial activity was evaluated on *S. mutans* (ATCC25175) and *P. gingivalis* (ATCC33277) determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using broth dilution tests. An analysis of variance (ANOVA) was performed followed by Dunnett's post-test for multiple comparisons, considering the value $P < 0.05$.

**Corresponding author: Email: alejandrah03@gmail.com;*

Results: *Azadirachta indica* showed a MIC of 500 Ppm (µg/mL) for both bacteria, showing bacteriostatic activity. *M. tinctoria* at a concentration of 125 Ppm (µg/mL had a bacteriostatic activity and bactericidal at higher concentrations of 250 Ppm (µg/mL and 500 Ppm (µg/mL for *S. mutans*. Against *P. gingivalis* the extract presented a MIC and a MCB of 500 Ppm µg/mL). **Discussion:** *Azadirahcta's indica* and *Maclura's tinctoria* ethanol extracts showed inhibitory activity against *S. mutans* (ATCC25175) and *P. gingivalis* (ATCC33277) of considerable value. Therefore it is recommended to continue with further studies.

Keywords: Streptococcus mutans; Porphyromonas gingivalis; Maclura tinctoria; Azadirachta indica.

ABBREVIATIONS

S. mutans: Streptococcus mutans; P. gingivalis: Porphyromonas gingivalis; MIC: minimum inhibitory concentration; MBC: minimun bactericidal concentration; DMSO: dimethyl sulfoxide; Ppm: parts per million.

1. INTRODUCTION

Within the framework of the pathologies treated in dentistry, the infectious types are the most common, as they are related to an accumulation of bacteria which found favourable conditions for their development and colony formation causing damage to teeth and periodontium [1,2]. Among the most representative microorganisms in these diseases stands *Streptococcus mutans*, a Gram positive facultative anaerobic bacteria, which predominates as a primary colonizer in the biofilm attached to the teeth surface and represents a greatest danger to the production of dental caries [3-5], as well as *P. gingivalis*, a Gram negative strict anaerobic bacillus which is one of the main pathogens of periodontal disease [6-8].

The use of medicinal plants in dentistry as an adjuvant in the treatment of the above mentioned oral diseases caused by these bacteria has a great impact today because they establish a viable alternative in the treatment plan. *Azadirachta indica* and *Maclura tinctoria* are two plants growing abundantly in tropical and subtropical climates that have been given different medicinal properties, including antifungal and antibacterial activity in species such as *Candida albicans* and *Staphylococcus aureus*. There are few studies that reference them in the field of dentistry [9-12]. Therefore, the objective of this study was to evaluate the in vitro antibacterial activity of the ethanol extracts of these two plants on important dental bacteria as *S. mutans* and *P. gingivalis.*

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Extracts

Plants were collected from the botanical garden Guillermo Piñeres located in Sector Matute in the municipality of Turbaco, Bolivar and the municipality of San Estanislao, Bolivar in May and April of 2014 and were sent to the herbarium of the University of Antioquia for taxonomic identification where the species of both plants were confirmed. A total of 444.4 g of leaves was collected of *Azadirachta indica* and 401.1 g of *Maclura tinctoria* leaves. The preparation of the extracts was performed by macerating the leaves of both plants and conserving it in a glass container with 97% ethanol for one week. The solvent was removed by rotoevaporation obtaining two dark and viscous consistency extracts. Later, *Azadirachta indica* extract was dissolved in dimethyl sulfoxide (DMOS) resulting in a solution with a concentration of 100.000 Ppm. *Maclura tinctoria* extract was dissolved in a mixture of 25% DMSO and 75% ethanol obtaining a solution with a concentration of 100.000 Ppm. Ethanol was chosen to obtain the extracts because it is an organic solvent that is commonly used in such studies, and causes fewer adverse effects on bacteria and cells that are used in these methods [13].

2.2 Reconstitution and Culture of Microorganisms

Reconstitution of both bacteria was performed according to the supplier. *S. mutans (ATCC25175)* was grown in trypticase soy agar supplemented with yeast extract, sucrose and bacitracin (TYS20B), at 37ºC, under anaerobic

conditions in an anaerobic jar with AnaeroGenOxoid ® system for 48 hours. *P. gingivalis (ATCC33277)* was cultured on Brucella agar supplemented with hemin (5 ug/mL), vitamin K (1 mg/mL), and 5% human blood at 37ºC, under anaerobic conditions in an anaerobic jar with AnaeroGenOxoid ® system for 7 days.

2.3 Growth Curve

Growth curves were performed by preparing an inoculum of bacteria in their corresponding culture broths which was incubated in anaerobic conditions and performing several measurements along a period of 48-hour.

2.4 Assessment of Bacterial Sensitivity

To evaluate the viability of the extracts, the sensitivity to these two microorganisms was determined. Solutions of each plant extract were prepared at concentrations of 500 Ppm, and by using an inoculum of bacteria in their respective culture broth and a polystyrene 96-well, both bacteria were exposed separately to the extract for an incubation period of 13 hours for *S. mutans* and 22 hours for *P. gingivalis*. As obtained in the growth curves, under anaerobic conditions and at the end of this incubation period, the optical density in each well was measured with the microplate reader Multiskan EX (Thermo ®, UK). Toxicity of solvents and positive growth control with water and negative growth control with Gentamicin 16% were performed.

2.5 Assessment of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Fourteen concentrations of both extracts were evaluated over both bacteria starting from 500 Ppm and decreasing to 0.06 Ppm. Using the broth microdilution method and always keeping the color controls, sterility, and positive and negative growth controls with Gentamicin 16%, a polystyrene plate with 96 wells was used which, after being prepared, was incubated in anaerobic conditions with AnaeroGen® system Oxoid (UK) at 37ºC with an elapsed a time of 13 hours for *S. mutans* and 22 hours to *P. gingivalis.* It was also analysed by the microplate reader Multiskan EX (Thermo®, UK) to determine the optical density in well.

The minimum bactericidal concentration was determined by choosing the concentrations that showed no bacterial growth during the evaluation of the MIC. For this, a sample was taken from the content of the chosen wells using a bacteriological wire loop. A subculture was then placed in Petri dishes with corresponding agar to each bacteria and subsequent incubation at 37ºC for a specified time, depending on the kind of microorganism. Then it was observed whether or not there was bacterial growth and thus determined if there was bactericidal or bacteriostatic activity.

2.6 Statistical Analysis

The results obtained in the evaluation of the bacterial growth curves, sensitivity tests and minimum inhibitory concentration were analysed and graphed using the GraphpadPism 5.01 (Graphpad Software Inc®) software, where a one-way analysis of variance (ANOVA) was performed followed by a Dunnet post-test for multiple comparisons, keeping in mind that the difference between the treated groups and the control group were significant when $P < 0.05$. The data were plotted as the mean ± standard deviation of the mean. Before the analysis, a normality test was carried out by performing a Kolmogorov-Smirnov test using the Statistical Package for the Social Sciences (SPSS) software, version 20 (IBM®) and watching a normal distribution of the data.

3. RESULTS

3.1 Obtaining Extracts

After maceration process, soaking in 97% ethanol for 1 week and rotoevaporation, it was obtained from each sample a total of 118.5 g and 125.16 g of *Azadirachta indica* and *Maclura tinctoria* respectively. The extracts had a viscous consistency and dark color and showed no sensitivity to light.

3.2 Culture and Bacterial Growth Curve

The culture on the specific media for bacteria, following the methodology proposed was plentiful and growth curves are shown in Fig. 1. It was determined that the measurement times of the following tests is 13 hours to *S. mutans* and 22 hours to *P. gingivalis*, since these correspond to the peak of the exponential growth phase.

3.3 Bacterial Sensitivity

When assessing the susceptibility of bacteria to the two extracts at 500 Ppm, it was observed that *S. mutans* showed no statistically significant difference between the reference, which was the culture broth, and bacterial growth in the presence of both extracts. Meanwhile, in *P. gingivalis*, the sensitivity test showed a significant difference with reference, which in this case was the overall growth of the inoculum, and bacterial growth exposed to both extracts.

3.4 Assessment of MIC and MBC

The results of the evaluation of the MIC of the ethanol extracts of *Azadirachta indica* and *Maclura tinctoria* on both bacteria are summarized in (see above) Fig. 2 to Fig. 5. *Azadirachta indica* presented a MIC over the two microorganisms at a concentration of 500 Ppm showing bacteriostatic activity and this concentration. This was also observed on *Maclura tinctoria* which obtained a MIC over P. gingivalis at a concentration of 500 Ppm showing bacteriostatic activity. On the other hand, *Maclura tinctoria* which showed inhibitory activity over *S. mutans* at 500 Ppm, 250 Ppm and 125 Ppm, being the last one (125 Ppm) the minimum inhibitory concentration. At 500 Ppm and 250 Ppm *Maclura tinctoria* exhibit bacteriostatic activity but and the MIC concentration, 125 Ppm showed bactericidal activity.

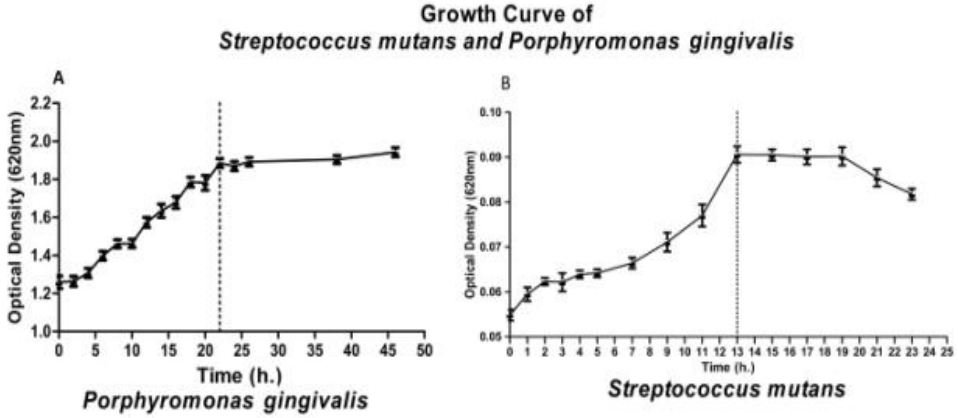

Fig. 1. A: growth curve of *S. mutans*. B: growth curve of *P. gingivalis*

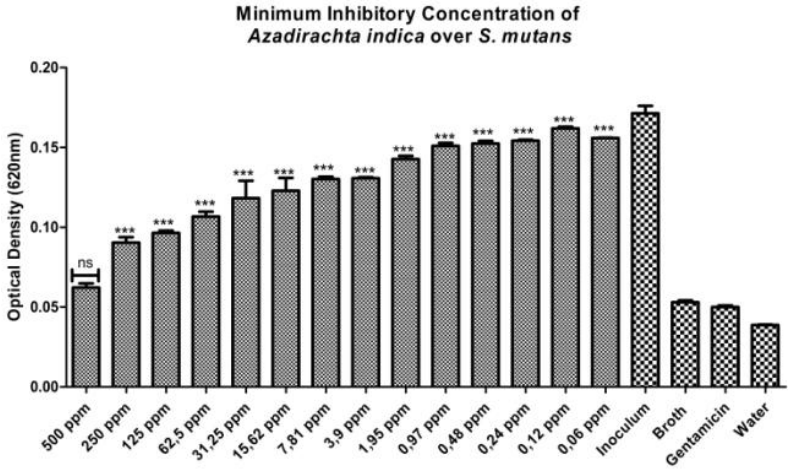

Fig. 2. Minimum inhibitory concentration of *Azadirachta indica* over *S. mutans*. gentamicin 16%

*ppm= parts per million, ns: no statistically significant difference, *: little significance difference, *** high significance difference. In all results a value of p<0.05 was obtained*

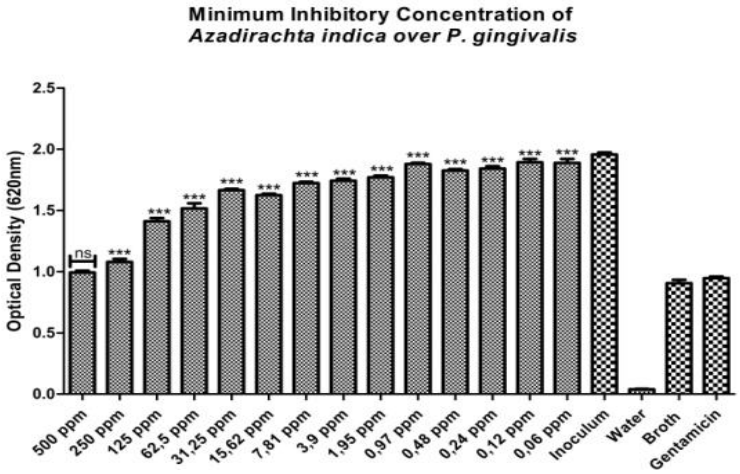

Fig. 3. Minimum inhibitory concentration of *Azadirachta indica* over *P. gingivalis*. gentamicin 16%

*Ppm= parts per million, ns: no statistically significant difference, *: little significance difference, *** high significance difference. In all results a value of p<0.05 was obtained*

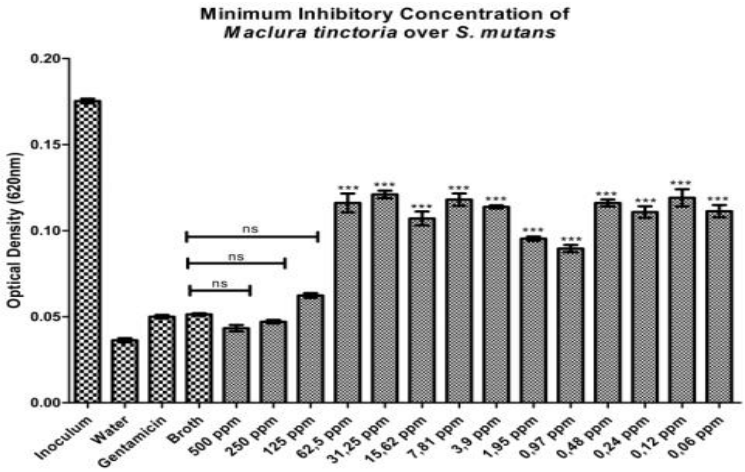

Fig. 4. Minimum inhibitory concentration of *Maclura tinctoria* over *S. mutans*. *gentamicin* 16%

*Ppm= parts per million, ns: no statistically significant difference, *: little significance difference, *** high significance difference. In all results a value of p<0.05 was obtained*

4. DISCUSSION

The results obtained in this study show antibacterial activity, expressed as minimum inhibitory concentration and minimum bactericidal concentration of two plants: *Azadirachta indica* and *Maclura tinctoria*, which showed values of 500 Ppm and 125 Ppm for *S. mutans* respectively and 500 Ppm for *P. gingivalis*. When comparing these two plants it can be concluded that both show inhibitory activity at concentrations below 1000 Ppm. This can be considered experimentally important, however *Maclura tinctoria* showed better results for both bacteria.

This can be corroborated when making comparisons with other studies on the same plants as performed by Wolinsky et al. [14], where extract obtained from the branches of *Azadirachta indica* showed that the minimum inhibitory concentration of this was above 320 Ppm on various bacteria of the *Streptococcus* spice, which is similar to the results obtained in the present study expressed in value of 500 Ppm for both bacteria.

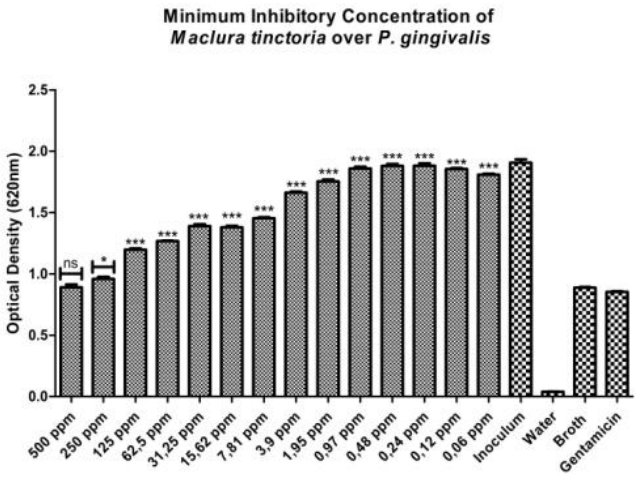

Fig. 5. Minimum inhibitory concentration of *Maclura tinctoria* over *P. gingivalis*. gentamicin 16%

*Ppm= parts per million, ns: no statistically significant difference, *: little significance difference, *** high significance difference. In all results a value of p<0.05 was obtained*

Fabry et al. [15], assessed the minimum inhibitory concentration and minimum bactericidal concentration of *Azadirachta indica* on different bacteria such as *S. aureus, enterococci, P. aeruginosa, E. coli, Salmonella* and *Klebsiella*, where the concentrations ranging between 250 Ppm and 2000 Ppm inhibit the 50% or the 90% of the population of bacteria. Bactericidal concentrations were shown to be the lowest at 1000 Ppm and highest above 8000 Ppm. Although it was not the same bacteria, it can be assumed that the extract prepared in this essay in a concentration of 500 Ppm is in a similar range of inhibitory bacteriostatic activity.

It is important to highlight that this trial was not previously reported on important Periodontopathogenic bacteria such as *P. gingivalis*, but the results derived from this study can be compared with those obtained from similar studies with other plants. For example, the study carried out by Bakri IM in 2005, where the extract of *Allium sativun* on various oral bacteria was evaluated. Among the bacteria were *P. gingivalis*, obtaining a MIC and MBC using the same methodology, of 4.4 mg/mL and 8.9 mg/mL respectively, which when confronted with the values thrown by *Azadirachta indica* of 500 Ppm that equates to 500 µg/mL, are significantly above these, indicating that in the same volume, less *Azadirachta indica* extract is needed to produce inhibition of growth of *P. gingivalis* compared to the amount of *Allium sativum* extract to produce the same effect. The same happens when comparing the values obtained on *S. mutans* being lower the inhibitory and bactericidal concentrations reported for *Azadirachta indica* [16].

Meanwhile Lamounier [17] in 2012, evaluated the MIC of extracts of *Maclura tinctoria* from tree bark and wood, using different solvents on oral bacteria such as *S. mutans* and *P. gingivalis*, among others, reporting that minimum inhibitory concentration ranged particularly for *S. mutans* between 400 Ppm and 80 Ppm being the highest levels from the wood and the lower value for the extract from the bark. For *P. gingivalis* MIC values dropped to 60 Ppm for bark extract. Comparing these data with those cast in the study, the levels found were lower by Lamounier for both bacteria. These variations may be due to several factors such as the source of the extract. In the present study results were obtained from the leaves while Lamounier used wood and bark. This may influence the results because in the same work it was concluded that the cortex showed a more variable composition of compounds with potential biological activity than the wood. It can therefore be assumed that the same is also true when compared with the leaves. Another important aspect to compare would be solvents and extraction methods since these were considerably different. Despite this, levels of minimum inhibitory concentration found in this study, 125 Ppm on *S. mutans* and 500 Ppm on *P. gingivalis*, with extracts derived from the leaves, are still of major importance and value. Obtaining the extract from leaves has less negative environmental impact than using bark or wood.

In the same manner, these results can be compared with other plants that are already used in dentistry, such as *Calendula officinalis. S*tudies such as those carried out by Parente LM et al. [18] and Iauk [19] reported antibacterial activity at concentrations between 109 Ppm to 4000 Ppm on Gram positive bacteria, and inhibitory concentrations of 2048 Ppm on periodontopathogenic bacteria respectively, thus demonstrating that the MIC of *Azadirachta indica* and *Maclura tinctoria* compete with the products being used in the market. Inhibitory and bactericidal results from both plants can be explained by the phytochemical components, as reported in the study by Siddiqui BS [20] in 2006. The *Azadirachta indica* contains several flavonoids as azharone the azadirone and isoazadironolide, which may explain its antibacterial activity because it is well known that the flavones possess this type of activity as well as being precursors of various phenolic compounds. In the case of *Maclura tinctoria,* Lamounier [17] reported that extracts of this plant is rich in phenolic compounds and proanthocyanidins, also called condensed tannins, which vary in concentration depending on the part of the plant being processed to produce the extract. This may explain the different activities that they show in the trial when compared with other plants and each other.

Finally, in comparison to previous investigations performed by Herrera Herrera A et al. [21] where the extracts of *Mammea americana* was evaluated, the results showed by *Azadirachta indica* and *Maclura tinctoria* are significantly similar specially for *P. gingivalis* because in the results of the three plants where below of 500 Ppm.

5. CONCLUSION

From the foregoing it can be concluded that the ethanol extracts of leaves of *Azadirachta indica* and *Maclura tinctoria* have good inhibitory activity against *S. mutans* and *P. gingivalis*, being that the extract of *Maclura tinctoria* more effective than the first bacteria. This therefore allows one to consider them as experimentally important and viable for use as antibacterial agents for dental use. Finally it is clarified that this is a purely experimental report so it cannot be considered as proper for clinical implementation yet because there are more factors such as cytotoxicity, In vitro activity on wild type bacteria, organoleptic characteristics and capability of prevention of the formation or disruption of oral biofilm, among other factors that have not been evaluated. According to the anterior reasons it is recommended to perform more studies to cover the factors mentioned before clinical use of the extracts of *Azadirachta indica* and *Maclura tinctoria* could be carried out.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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