Evaluation Of The Antibacterial Activity Of The Extract Of Spilanthes Acmella In Porphyromonas Gingivalis And Streptococcus Mutans

Aims: it was evaluated the antibacterial effect of Spilanthes Acmella plant from the different parts that it possesses: stem, flowers and leaves will be evaluated in bacteria Streptococcus mutans and Porphyromonas gingivalis in order to determine which one is the most effective as bactericidal or bacteriostatic and implement them as a new treatment for these diseases. Study design: Experimental in vitro.

Place and Duration of Study: Department of Dentistry, University of Cartagena between January 2016 and March 2017

Methodology: strains of Streptococcus mutans ATCC 51715 and Porphyromonas gingivalis ATCC 33277 were exposed to this extract to determine the antibacterial effect, the minimum inhibitory concentration (MIC) and also the minimum bactericidal concentration (CBM), which were analyzed by Shapiro Wilk test for normality, and then analysis of one-way variance (ANOVA) with a confidence level of 95% and significance when P <0.05.

Results: the leaves, flowers and stems presented antimicrobial activity in Streptococcus mutans and Porphyromonas gingivalis, as it did not show significant value with respect to the broth; It was found that the three extracts present bactericidal effect with respect to Streptococcus mutans at 500ppm, and that the stems have a bacteriostatic effect at 500ppm, the leaves effect bactericide at 500ppm and the flowers effect bactericide at 250ppm in P. gingivalis. All three extracts have cardiotonic secondary metabolites, coumarins and tannins in abundance.

Conclusion: Spilanthes Acmella natural extract, segmented in stems, leaves and flowers, presented antibacterial effect with respect to the bacteria Streptococcus Mutans and Porphyromonas Gingivalis, specifically bactericidal effect in S. Mutans and bacteriostatic and bactericidal effect in P. Gingivalis, which can prevent the progression of dental caries and periodontal disease.
INTRODUCTION:
In the oral cavity more than 500 species of bacteria inhabit, thus becoming one of the most varied and complex microbial floras (1). Dental caries and periodontal diseases are two of the main dental pathologies that affect humanity that arise from the colonization and accumulation of oral microorganisms especially Streptococcus mutans and Porphyromonas gingivalis, followed by Lactobacillus and Actinomyces. Hence, the control of microorganisms related to dental plaque, as well as the carious process, is of vital importance for the control of the disease and one of the mechanisms is the use of antimicrobials (2). Although it is true, dental caries and periodontal disease are some of the most frequent pathologies in the oral cavity that affect most of the population (3); therefore, it is necessary to carry out a deeper and more detailed approach to its mechanism to understand the reasons for its pathogenesis and extension (4, 5).

It can be observed that both oral pathologies are not only the result of bacterial colonization and propagation, but also are the product of a poor oral hygiene of the patient (6, 7). It is for this reason that most of the research is aimed at solving or finding a highly effective treatment for these and thus avoid further affecting the population and preserve as much as possible, all structures present in the oral cavity (8, 9).

According to recent research, alternative treatments have been found from natural extracts in order to obtain them directly from the environment and take advantage of the demand that exists in the fauna, as well as having various advantages, among which it is easy to access and manage, low cost and above all have few undesirable side effects (5, 10, 11). Specifically the case of the Spilanthes Acmella plant, is known for its therapeutic effect against tooth pain, which also has high medicinal uses, with various bioactive properties in health care, cosmetology and healthy foods (12-14); Therefore, it has an increasing demand throughout the world, due to its important therapeutic value and its high development potential (13).

Several studies report that the use of the extract of Spilantes Acmella is effective for the treatment of oral cavity infections, dental caries and periodontal disease where results were shown indicating that the antibacterial activity of the plant against oral microorganisms in Special with Streptococcus mutans was the most sensitive to plant extracts, while Porphyromonas gingivalis was the most resistant (15, 16). For this reason, this research aims to evaluate the antibacterial effect of the Spilanthes Acmella plant from the different parts it has, that is, the stem, flowers and their leaves, in the bacteria Streptococcus mutans and Porphyromonas gingivalis.

MATERIAL AND METHODS

Type Of Study - Experimental In Vitro.

Plant Material
As a first step, the sample of spilanthes acmella was collected in rural areas of the city of Cartagena, Bolivar; which was sent to the herbarium of the university of Antioquia for taxonomic analysis.

Preparation Of Extract
The plant was separated in each of its parts, that is, stem, flower and leaves; the extract was prepared through the maceration of the leaves, stems and flowers and soaked in a glass container with 95% ethanol for 1 week, in dark conditions. Obtaining three dark extracts of viscous consistency, which were eliminated by rotaevaporation (17). The extract was then filtered because there were many lumps and these could represent a bias in the experiment. Once the extracts were obtained, the solubility test was carried out using different solvents such as Ethanol, Dimethylsulfoxide and Methanol, where it was observed that ethanol was the one with the best properties, so the extract was dissolved in 1% Ethanol obtaining a concentration of 100,000 ppm.

Microorganisms And Growth Conditions
For the cultivation of Streptococcus mutans (ATCC25175) the broth Tripticase of soy supplemented with yeast extract, sucrose, agar-agar and bacitracin (TYS20B) was used, and for the cultivation of Porphyromonas gingivalis...
(ATCC33277) it was used as culture medium Brucella agar supplemented with hemin (5 ug/ml), vitamin K (1 mg/ml) and 5% lacquered blood. Bacteria preserved at a temperature of -20 °C, were removed from the freezer and placed on Petri dishes with the aforementioned nutrient broth and then taken to a jar of anaerobiosis using the Anaerogen bags (Oxoid® UK) to reach this condition. The culture was carried out for a period of 48 hours for Streptococcus mutans and 5 days for Porphyromonas gingivalis at a temperature of 37 °C. An abundant growth of the bacteria was obtained.

For Streptococcus mutans and Porphyromonas gingivalis colonies were taken from the culture media and inoculated into tubes with their respective nutrient broths to make the growth curves of each strain (18); for which it was verified that it remained at a concentration of 0.08 to 0.1 according to the McFarland scale using a Multiskan EX microplate spectrophotometer (Thermo®, UK). Streptococcus mutans reached its stationary phase at 13 hours and for Porphyromonas gingivalis it was at 22 hours.

**Analysis Of Antibacterial Activity**
The antibacterial activity was determined by the microdilution test in broth using a 96-well polystyrene plate. (18) Using this technique, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of each extract respectively with S. Mutans and P. Gingivalis.

**Quantification Of The Minimum Inhibitory Concentration (MIC)**
The ethanolic extracts of Spilanthes Acmella were diluted in 14 serial concentrations starting from 500ppm (this being the lowest concentration that presented antibacterial activity in the bacterial sensitivity test) until reaching a concentration of 0.0610ppm. To make these concentrations, from the 500ppm solution, 500µL of extract was taken, diluted with another 500µL of water to reduce its concentration by half. Once prepared the plates were taken to the incubator at 37 °C under anaerobic conditions using the Anaerogen bag system (Oxoid® UK) for 13 hours for Streptococcus mutans and 22 hours for Porphyromonas gingivalis. In the same way that the sensitivity was evaluated, the plates were taken to the Multiskan EX (Thermo®, UK) for reading, MIC was interpreted as the minimum concentration of the antimicrobial agent that inhibits the multiplication and growth production of a given bacterial strain in the test system. (18)

**Quantification Of The Minimum Bactericidal Concentration (MBC)**
For the minimum bactericidal concentration (CMB) of the SA extracts, it was determined that the selected samples were of the concentrations that did not demonstrate bacterial growth in the MIC (no significant difference between the optical density of the samples and the broth). We proceeded to take a roast of the wells that contain them, and we performed a subculture in Petri dishes with the corresponding agar for each bacterium (positive and negative control wells were also performed in the same operation). The inoculated petri dishes were incubated in anaerobiosis depending on the bacteria at 37 °C. After this period, the existence or not of growth of bacterial colonies was observed and the extracts were classified as bacteriostatic or bactericidal. (18)

**Preliminary Phytochemical Screening Of Spilanthes Acmella Extract**
A preliminary phytochemical analysis of the ethanolic extract of leaves, stems and flowers of Spilanthes Acmella was carried out. The methodology followed for this analysis was previously standardized by Sanabria (19) and contemplates the detection of the following secondary metabolites generally related to biological activities: alkaloids, reducing sugars, cardiotonic, phenolic compounds, coumarins, flavonoids, leucoanthocyanidin, saponins, tannins and triterpenes / steroid.

**Statistical Analysis**
The results obtained in the evaluation of the bacterial growth curves, sensitivity tests and minimum inhibitory concentration were analyzed and plotted using the Graphpad Prism 5.01 software, where a one-way analysis of variance (ANOVA) was performed taking a degree of confidence considering that the
difference between the treated groups and the control group is significant when P <0.05 (95% confidence). Prior to the analysis, Shapiro Wilk's normality test was performed, using the Statistical Package for the Social Sciences (SPSS) version 20 software (IBM®), observing a normal distribution of the data.

RESULTS
Antibacterial Analysis
The ethanolic extract of stems, leaves and flowers of Spilanthes Acmella showed to have antimicrobial effect against Streptococcus mutans and Porphyromonas gingivalis. These bacteria were sensitive to the extract at 500ppm and 1000ppm because they have a statistically significant difference with respect to the culture broth (inoculum), that is, if there was bacterial growth inhibition since it was compared with the total growth of the inoculum, and the bacterial growth exposed.

Therefore, the antibacterial activity was interpreted with the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MCB). The result obtained for the MIC of each of the extracts of Spilanthes Acmella on Porphyromonas Gingivalis and Streptococcus mutans was 500 ppm, except for the flowers on Porphyromonas Gingivalis in which the minimum inhibitory concentration was observed at 500ppm and 250ppm, because there was no statistically significant difference with respect to the broth. In Figures 1, 3 and 4 it is observed that the stems in P. Gingivalis and the stems and flowers in S. Mutans have a MIC at 500ppm, as do the leaves in the two bacteria; however, in figure 4, it is observed that the flowers present a MIC at 250ppm with respect to P. Gingivalis, because they did not show a statistically significant difference with respect to the broth (p>0.05).

The MCB was determined as found in the MIC, then from 500ppm for all the extracts and 250ppm in the case of the flowers in P. Gingivalis. In the case of stems, leaves and flowers in Streptococcus mutans it is evident that they have a bactericidal effect, because at 500ppm there is no bacterial growth in the MCB. With respect to Porphyromonas gingivalis, the leaves obtained bactericidal effect at 500pm, had a bacteriostatic effect at 500ppm due to bacterial growth, and the flowers also obtained a bactericidal effect from 250ppm, not finding bacterial growth.
Figure 2. Minimum inhibitory concentration of flowers of *Spilanthes acmella* (*P. Gingivalis*).

![Graph showing minimum inhibitory concentration for flowers of *Spilanthes acmella* (*P. Gingivalis*).](image)

*Note:* It is observed that, of the 14 concentrations from the extract of flowers, 250 ppm is the minimum inhibitory concentration on *Porphyromonas Gingivalis*.

Figure 3. Minimum inhibitory concentration of stems of *Spilanthes acmella* (*S. mutans*).

![Graph showing minimum inhibitory concentration for stems of *Spilanthes acmella* (*S. mutans*).](image)

*Note:* It is observed that, of the 14 concentrations from the extract of stems, 500 ppm is the minimum inhibitory concentration on *Streptococcus Mutans*.
Preliminary Phytochemical Screening Of Spilanthes Acmella Extracts

It is observed that the stems have abundant secondary metabolites of reducing sugars, cardiotonic, phenolic compounds, coumarins, flavonoids, saponins and tannins. With regard to flowers, the abundant presence of cardiotonic secondary metabolites, phenolic compounds, coumarins, flavonoids, saponins and tannins is evident. On the other hand, there is abundant presence of cardiotonic secondary metabolites, coumarins and tannins in the leaves; slight presence of flavonoids and the presence of phenolic compounds and saponins could not be determined due to the interference of color. (See table 1.)

![Graph showing minimum inhibitory concentration of flowers of Spilanthes acmella (S. Mutans)](image)

Note: It is observed that, of the 14 concentrations from the extract of flowers, 500ppm is the minimum inhibitory concentration on Streptococcus Mutans.

Table 1. Results of preliminary phytochemical screening of the extracts of stems, flowers and leaves of Spilanthes Acmella.

<table>
<thead>
<tr>
<th>N°</th>
<th>Extract</th>
<th>Organ</th>
<th>AL</th>
<th>RS</th>
<th>CA</th>
<th>PC</th>
<th>CU</th>
<th>FL</th>
<th>LE</th>
<th>SA</th>
<th>TA</th>
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<td>+++</td>
<td>+++</td>
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<td>-</td>
<td>+++</td>
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</table>

Note: abundant presence [+++], moderate presence [++], slight presence [+], absent [-].

AL: alcaloids; RS: Reducing sugars; CA: Cardiotonics; PC: Phenolic compounds; CU: coumarins; FL: flavonoids; LE: Leucoantocianidinas; SA: saponins; TA: tannins; TE: triterpens/esteroides. ND: not determined, due to color interference.
It is shown that the three extracts, stems, leaves and flowers, have abundant secondary metabolites cardiotonics, coumarins and tannins; as well as the stems are those that present a greater number of secondary metabolites both in quantity and abundance.

DISCUSSION
Current scientific studies show that the Spilanthes Acmella plant has various effects among which we find anesthetic, antipyretic, antifungal, antioxidant, analgesic and antimicrobial effect; its anesthetic effect evidently predominating. It also shows good insecticidal effect (20-22). However, there is very little current scientific literature about the antibacterial effect of the Spilanthes Acmella plant, and even more so, on bacteria in the oral cavity such as Streptococcus mutans and Porphyromonas gingivalis.

In the present study, the results show that the flowers could be the component of the plant that has the best antimicrobial activity with respect to Streptococcus Mutans and Porphyromonas Gingivalis, due to the diversity and quantity of secondary metabolites that it has since it was the extract that had concentration Minimum inhibitory inhibition, this being 250ppm, which would represent a great advance for the academic community and society since this could mean that a product could be generated from the Spilanthes Acmella that acts as an anesthetic effect and in turn has an effect antimicrobial, which would be a good product to use in patients.

The studies carried out in scientific evidence go much deeper into what type of active metabolite the plant possesses that makes it have these diverse properties; They found that Spilanthes Acmella has a metabolite called Spilanthol, which is a chemical N-isobutylamine, which gives it its insecticidal characteristics, which also has an acidic taste and, therefore, could also stimulate patient salivation (23). However, Leng et al in 2011 (11) found this metabolite especially in the flowers of the plant, which in the present study did not evaluate the presence of the metabolite of Spilanthol but if it was found several other metabolites such as coumarins, tannins and Flavonoids could give it the property of antimicrobial effect that is sought, which also adds that it was the extract that obtained a MIC lower than the others.

Ardoino in 2014 (24) tested the antibacterial activity of tannins, belonging to the group of terpenes, as well as that of steroidal nuclei and triterpenes, saponins, flavonoids, and alkaloids; which agrees with what was found in the preliminary phytochemical screening of Spilanthes Acmella. Narayana in 2001 (25) conducted a study of the effects of flavonoids, where he found that it had an antimicrobial effect against many bacteria, especially gram-positive ones; as well as it was evidenced in the study that there is abundant presence in stems and flowers, and slight presence in leaves and in addition, bactericidal effect was found especially in Streptococcus mutans at 500ppm and Porphyromonas gingivalis at 250ppm. Additionally, Narayana also found that this secondary metabolite has an anti-inflammatory effect, which would help in the resolution or arrest of the evolution of periodontal disease in the oral cavity, which is precisely what is sought to be solved with the present study.

Domingo et al in 2003 (26) found that some secondary metabolites with antimicrobial effects are phenolic compounds, which due to their number of hydroxyl groups can intervene in the toxicity of bacteria; tannins and coumarins which can act at the level of eukaryotic DNA, which would explain their effect on the bacteria. Just as flavones also have an effect by forming complexes between the soluble and extracellular proteins and the cell wall of the bacteria, among which we find Streptococcus mutans; saponins and sugars also have an effect, which contributes to what was found in the experiment where abundant coumarins, tannins, reducing sugars, flavonoids and phenolic compounds were found; and for this reason it could be inferred that the extract of stems could have a better antimicrobial effect due to the presence of a greater number of secondary metabolites; however, they were the flowers with the best antimicrobial effect, which also have the same secondary metabolites as the stems, except for the reducing sugars that the stems have.

In addition, it is observed that the stems, leaves and flowers of Spilanthes Acmella have bactericidal effect in S. Mutans, which is evidenced in the minimum bactericidal concentration (MBC), and bacteriostatic and bactericidal effect with respect to P. Gingivalis in the different components , which is a great contribution for the scientific community and to initiate future and new investigations for the use of this extract in dentistry. As evidenced by Ahmed et al in 2013 (27), finding that Spilanthes Acmella has a strong inhibitory activity with respect to bacterial growth, especially in gram-positive
and gram-negative bacteria, which coincides with the present experiment when finding this effect on Streptococcus mutans (gram-positive bacteria) and Porphyromonas Gingivalis (gram-negative bacteria). Prachayasittikul et al in 2013 (13) found that when performing Spilanthes Acmella tests on agar solution methods they have an inactive antimicrobial effect, even when MIC shows antibacterial activity in Streptococcus pyogenes. Like Holetz in 2002 (9), who by means of studies his results also showed inactive antimicrobial activity for Spilanthes Acmella in comparison with other 13 medicinal plants of Brazil; It also found that it possesses inactive antifungal activity with respect to all the fungi evaluated. However, this is not related to what was found in the present study since microdilutions in agar showed that if they have a bactericidal effect with respect to Streptococcus mutans at 500ppm and in Porphyromonas gingivalis at 250ppm. Although there is not much scientific evidence that speaks specifically about the antimicrobial effect in Streptococcus Mutans and Porphyromonas Gingivalis, it was evidenced that this plant does have both bactericidal and bacteriostatic effects for these bacteria, being the most effective flowers in P. Gingivalis when obtaining a minimum inhibitory concentration at 250ppm. Therefore, S. Amella is a 100% natural extract, which can be used and made, generating low economic costs and contributing to oral health by preventing the colonization and virulence of bacteria such as Streptococcus Mutans and Porphyromonas Gingivalis that intervene in the process of dental caries and periodontal disease. However, it is recommended to carry out further studies regarding the effect it has on bacteria in the oral cavity and, even, to specify what type of active metabolite is causing this property.

CONCLUSION
From the above it can be concluded that the extracts of Spilanthes Acmella, even more flowers, have good inhibitory and bactericidal activity with respect to the bacteria that cause the most prevalent diseases of the oral cavity, Streptococcus Mutans and Porphyromonas Gingivalis, considering them important clinically to use them as antimicrobial agents in dentistry. It was also shown that the three extracts, stems, leaves and flowers, have abundant secondary metabolites cardiotonics, coumarins and tannins; as well as the stems are those that present a greater number of secondary metabolites both in diversity and in abundance.

CONSENT
It is not applicable.

ETHICAL APPROVAL
It is not applicable.

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How To Cite This Article:

Source of Support: Nil

Conflict of Interest: None declared

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