RESEARCH ARTICLE

Antibacterial efficacy of methanolic extract of molave (*Vitex parviflora A. Juss*) leaves against *Streptococcus mutans*

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POSTER PRESENTATION at the 34th Meeting of the International Association for Dental Research – South East Asia Division (IADR-SEA) virtually on 26 – 27 November, 2020.

ABSTRACT

Background: Antibacterial drugs are used for suppressing harmful bacteria. However, some are reported to have side effects which led researchers to investigate plants with antimicrobial properties as potential alternatives. One such indigenous plant is the *Vitex parviflora A. juss*, "molave" or "mulawin" tree.

Objective: This study determined and compared the antibacterial efficacy of 50 mg/ml and 100 mg/ml concentrations of fresh local molave leaves methanolic extract with 0.12% chlorhexidine, distilled water, and 95% methanol on growth inhibition of *S. mutans*.

Methodology: Five hundred grams of fresh molave leaves were collected and subjected to methanolic extraction. *In vitro* antimicrobial susceptibility test by disk diffusion of 50 mg/ml and 100 mg/ml molave extract concentrations, 0.12% chlorhexidine, distilled water, and 95% methanol on 18 Mueller-Hinton agar (MHA) plates inoculated with *S. mutans* was done. For cost-efficiency, the total sample size of 80 plates was reduced by placing 5 test groups in one plate divided into five portions done in 18 replicates. After 48 hours of incubation in anaerobic conditions, resulting zones of inhibition were measured. Data were analyzed through one-way ANOVA and Bonferroni tests.

Results: The mean diameter of inhibition zones produced by 100 mg/ml and 50 mg/ml concentrations of molave methanolic leaves extract and 0.12% chlorhexidine was 15.78 mm, 11.63 mm, and 21.44 mm, respectively. Distilled water and 95% methanol did not inhibit bacterial growth. The 100 mg/ml concentration has stronger antibacterial properties than the 50 mg/ml.

Conclusion: The *Vitex parviflora A. Juss* methanolic leaves extract has the ability to inhibit the growth of *S. mutans in vitro*. Both concentrations were relatively weaker compared to chlorhexidine.

Keywords: *molave, Vitex parviflora A. juss, S. mutans, zone of inhibition*

Introduction

Dental caries is a chronic multifactorial disease that refers to the demineralization of enamel and dentin by acids formed as a result of the metabolism of sugars from one's diet and bacteria in dental plaque [1, 2]. The development of caries involves the interplay of factors such as a susceptible host, a cariogenic oral microflora, and a suitable substrate. This multifactorial concept of caries formation shows that conditions within each of these factors at a given time must be favorable for caries to occur [3]. The consumption of sugars or other fermentable carbohydrates stimulates the growth of oral microbes, specifically *Streptococcus mutans* and *Lactobacilli. S. mutans* are gram-positive, facultative anaerobic bacteria commonly found in the human oral cavity responsible for the initiation of dental caries [4]. The initiation of caries happens when the bacteria outnumber the other oral microflora not associated with the caries process.

Dental caries is considered a global health problem due to its high prevalence and significant social impact [5]. In the Philippines, caries prevalence is reported to be high at 73% [6]. Thus, strategies to address the dental caries problem [7], such as decreasing the growth or activity of *S. mutans*, are important.

The use of oral antimicrobials such as mouth rinses has shown beneficial effects, is a safe component of daily oral health routines, and is a key component in oral health management.

Antimicrobial agents have been one of the most widely and often imprudently used therapeutic drugs worldwide due to increasing demands for suppressing the growth of microorganisms and limiting the transmission of harmful microbes. An example is the amplified usage of oral antimicrobials as chemotherapeutic agents to reduce oral bacteria levels [8]. Chlorhexidine [9] is the most popular among oral antimicrobials capable of destroying or inhibiting the growth of microorganisms in the oral cavity [10]. It is considered the gold standard for oral rinses as it inhibits plaque regrowth and gingivitis. Chlorhexidine is a potent antibacterial that targets a wide array of bacteria including gram-positive and gram-negative. It is also anticariogenic in nature as it acts against *S. mutans* [11]. Although proven effective against the main cause of dental caries which is *S. mutans* [12,13], the prolonged use of chlorhexidine has been reported to have local side effects on the mucous membranes, teeth, and tongue such as alteration of taste sensation and staining [14,10], antimicrobial resistance, and some allergic reactions [10]; thus, there is a need to find alternative indigenous materials with antibacterial properties [15].

Plants are commonly used as remedies, if not as cures, for bacterial diseases [16-18]. Plants contain numerous phytochemicals that exhibit antimicrobial activities. Studies have shown that plant extracts exhibited not only antimicrobial properties but also antifungal and antiviral [19]. Medicinal plants are known to have a wide variety of secondary metabolites such as alkaloids, flavonoids, tannins, and terpenoids, which have *in vitro* antimicrobial properties [20-22]. Alternative antimicrobial agents that are available and safer to use have been investigated to prevent and reduce the number of oral diseases [23]. Of interest in this study are the antibacterial activities of plants [24-28] against *S. mutans*. A number of plants and plant parts have been tested for antimicrobial activity against *S. mutans*, yet, the search for an alternative oral antimicrobial goes on.

Studies on traditional medicine in the Philippines have shown the antibacterial ability of locally available plants [29]. One such indigenous plant is the *Vitex parviflora A. juss*, "molave" or "mulawin" tree [30]. Obico and Ragragio [31] reported that the leaves and stems of *V. parviflora* were being used as an insect repellent by the Aeta indigenous people living in Pampanga. While in the Zamboanga province, the barks and roots of molave have been used as a remedy for "toothache, irregular menstruation, goiter, ulcer, anemia and acidity" as described by Tantengco, Condes, Estadilla, and Ragragio [32]. They further stated that the methanolic crude extracts of molave leaves and stem are effective antibacterial agents for *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, and *Bacillus subtilis*. Crude flavonoid molave extracts have also been tested against human prostate cancer (PC-3) cells *in vitro* [33]. A phytochemical study conducted by Panti *et al*. [34] showed that ethanolic *Vitex parviflora A. Juss* leaves extract contains alkaloids, saponins, diterpenes, phenols, phytosterols, and flavonoids. Flavonoids have been found to be effective against a wide array of microorganisms. This may be possible due to their ability to complex with extracellular and soluble proteins, or with bacterial cell walls. Lipophilic flavonoids have also been found to disrupt microbial membranes [20].

Disk diffusion is the accepted method used for in vitro antimicrobial susceptibility testing in microbiology laboratories. The procedure has been standardized and is summarized as follows: filter paper discs containing the test compound, such as molave leaves extract, are placed on the agar surface of plates previously inoculated with an inoculum of the test microorganism, like streptococci, and then incubated. It is anticipated that as the antimicrobial agent spreads into the agar, it affects the propagation of the test microorganism from which the diameters of zones of inhibition are measured. Results generated from this simple and economical antimicrobial test are easy to interpret and provide a good link between in vitro data and *in vivo* application. However, this method cannot determine if the bacterial growth inhibition kills or just inactivates the microorganism [35].

This study aimed to determine and compare the antibacterial efficacy of 50 mg/ml and 100 mg/ml concentrations of fresh local molave leaves methanolic extract to 0.12% chlorhexidine, distilled water, and 95% methanol on growth inhibition of *S. mutans* through antimicrobial susceptibility testing via the disk diffusion method. The antibacterial efficacy of *Vitex parviflora A. juss* leaf extract on the growth inhibition of *S. mutans* was determined *in vitro* by measuring the mean diameter of zones of inhibition at 50 and 100 mg/ml.

The results may be used as a reference for baseline information regarding its use as an oral antimicrobial agent. Since *S. mutans* is a vital factor in dental caries formation, this study may be used to infer that controlling its population is one way of affecting the occurrence or disease course of dental caries. If proven effective, this could serve as a potential source of an alternative oral antimicrobial agent that may be developed further to become beneficial for patients with limited ability to perform mechanical cleansing of the teeth.

Methodology

An *in vitro* experimental design was utilized to determine the antibacterial efficacy of *Vitex parviflora A. juss* leaf extract on the inhibition of *S. mutans*. The diameter of zones of inhibition produced after 48 hours by the 50 mg/ml and 100 mg/ml concentrations of molave leaf methanolic extracts, 0.12% chlorhexidine, distilled water, and 95% methanol was measured using the disk diffusion method. A completely randomized sampling design was used in allocating the 5 test solutions to the plates inoculated with *S. mutans*. For cost-efficiency, the initial total sample size of 80 plates needed was reduced by placing the 5 test groups randomly in one plate divided into 5 portions. The test was done in 18 replicates. The study was conducted at the Microbiology Laboratory of the College of Public Health (CPH), University of the Philippines Manila (UPM).

Data Collection Procedure

Preparation of plant extract

Fresh mature leaves from a single tree of *Vitex parviflora A. juss* were collected from the College of Forestry, University of the Philippines Los Baños (UPLB). The leaves were stored in a plastic container and were immediately transported to the Institute of Chemistry, within the UPLB campus, for the methanolic extraction process [36].

The maceration method of extraction was used. The leaves were oven-dried first, then crushed and powdered with a blender. Powdered samples were soaked in 95% methanol, as a solvent, in a covered container. Afterward, a rotary evaporator was used to remove the solvent and obtain the plant extract. The extract was stored in an amber bottle at 4 degrees celsius cold storage, which may be used for a maximum of 30 days after the extraction date.

Preparation of sample microorganism

The test organism used in the study is a strain of *S. mutans* revived from a stock culture provided by the UPM-CPH Department of Medical Microbiology. The majority of the materials utilized in the study, such as anaerobic jars, inoculating loops, and media, were supplied by the same department.

Three anaerobic jars were used throughout the experiment: two Oxoid Anaerobic 3.5L Jar System, one Oxoid Anaerobic 2.5L Jar system, and three BBL GasPak 100 Anaerobic systems. Mueller Hinton Agar (MHA) was used as the agar base for streaking and plating procedures following the recommendations presented in the 2005 National Committee for Clinical Laboratory Standards (NCCLS) Manual of Antimicrobial Susceptibility Testing by Coyle *et al*. [37].

After incubation, the inoculum suspension was prepared through the direct colony suspension method per the procedure described in the 2005 (NCCLS) Manual on Antimicrobial Susceptibility Testing [37]. An inoculating loop was used to pick well-isolated colonies from the plate of re-activated subculture. Selected colonies were suspended in Mueller-Hinton broth in a test tube. For standardization, the prepared inoculum was adjusted to match the turbidity of 0.5 McFarland standard, which corresponds to approximately $1.5x10^8$ CFU/ml. The tubes were placed in front of a Wickerham card to compare the inoculum with the McFarland standard. The 0.5 McFarland standard was prepared by adding 0.5 ml of 1.0% BaCl2 to 99.5ml of 1% H2SO4 solution. The solution was then stirred to maintain a suspension.

Preparation of plant extract dilutions

The extracted solution was checked for the presence or absence of bacteria to confirm its suitability for use in the experiment. The plant extract dilution was prepared manually using 10 ml serological pipets. Two milliliters of pure liquid extract were transferred to a test tube labeled 100 mg/ml. For the 50 mg/ml concentration, 1 ml of the liquid extract was transferred to a test tube and was mixed with 1 ml of distilled water as a dilutant.

The two concentrations of molave extract served as the treatment group, while the 0.12% chlorhexidine and distilled water served as the positive control and the negative control, respectively. The *S. mutans* were also subjected to 95% methanol treatment to determine if it has no activity on the said organism since it is the solvent used in the extraction process.

Antimicrobial susceptibility testing – disk diffusion method [37]

There were five treatment groups in this study, and the test was done in 18 replicates. Eighteen pre-prepared frozen Mueller- Hinton agar (MHA) plates, each one 4 mm in depth, were allowed to warm up at room temperature to remove excess moisture. The test tube with prepared inoculum suspension was placed in a vortex machine to obtain a well-mixed solution. The tip of a sterile cotton swab was dipped into the suspension, with the swab pressed onto the side of the test tube to remove excess liquid (Fig. 1a). The cotton swab was then used to streak and inoculate the MHA plates, with the tip moved back and forth from edge to edge,

covering the whole surface of the plates. Each plate was rotated 60 degrees, and the same streaking procedure was repeated thrice. Swabbing was also done in a circumferential motion to cover the edges of the agar plates (Fig. 1b). Using single-use micro pipettor tips, each treatment solution was dispensed equally on a 6 mm, sterile paper disc until soaked, one for each treatment solution. Each of the discs was placed at the center of the divided areas of the MHA plates with slight firm pressure to ensure complete and level contact with the media. The plates were then covered, ensuring that the discs were not moved (Fig. 1c). The plates were inverted with the agar side up and incubated for 48 hours at 33-35° and 5% CO2 atmosphere. After 48 hours, the diameter of the inhibited zones was examined under reflected light and measured using a millimeter ruler while viewed a few inches above a black non-reflecting surface. Three researchers measured each plate, and the average diameter of the zones of inhibition per plate was computed and recorded.

Data Processing and Analysis

The data was analyzed using a fixed effect, omnibus, one-way Analysis of Variance (ANOVA) model at p<0.05 (G*Power 3.1 software) [38] followed by a post hoc analysis Bonferroni Test to determine which groups were significantly different, using the R software [39].

Results

The extraction process yielded 19.495 g of plant extract from the 500 g of fresh molave leaves submitted for the extraction. The percent of crude methanolic extract from 500 g of fresh Molave leaves was 3.899%. The resulting product was a mixture of a dark green non-viscous liquid extract with minute plant fragments.

In Table 1, the mean diameter of the zone of inhibition caused by *Vitex parviflora A. juss* extract was 15.78 mm and 11.63 mm at 100 mg/ml and 50 mg/ml concentrations, respectively. Meanwhile, the mean diameter of the positive control (0.12% chlorhexidine) was 21.44 mm. Both the negative control (distilled water) and 95% methanol did not inhibit the growth of the test organism. The results show that the plant extract's higher concentration (100 mg/ml) exhibited a greater zone of inhibition than the 50 mg/ml concentration.

One-way ANOVA test on the zones of inhibition produced by the five test groups obtained a p-value ≤ 0.001 (4.30539935798863E-83), which is less than $\alpha = 0.05$, indicating differences in the means of diameters of the zones of inhibition of *S. mutans* among the five test groups.

The Bonferroni test, a multiple comparisons test, was employed to determine which concentrations differed

Table 1. *Mean of Streptococcus mutans Zones of Inhibition per Treatment Group.*

TREATMENT GROUP	ZONE OF INHIBITION (mm)
50 mg/ml molave extract	11.63[a]
100 mg/ml molave extract	15.78[b]
(+) Control 0.12% chlorhexidine	21.44[c]
(-) Control Distilled water	0[d]
95% methanol	0[d]

*Values with different superscript letters indicate significant differences at p<0.05.

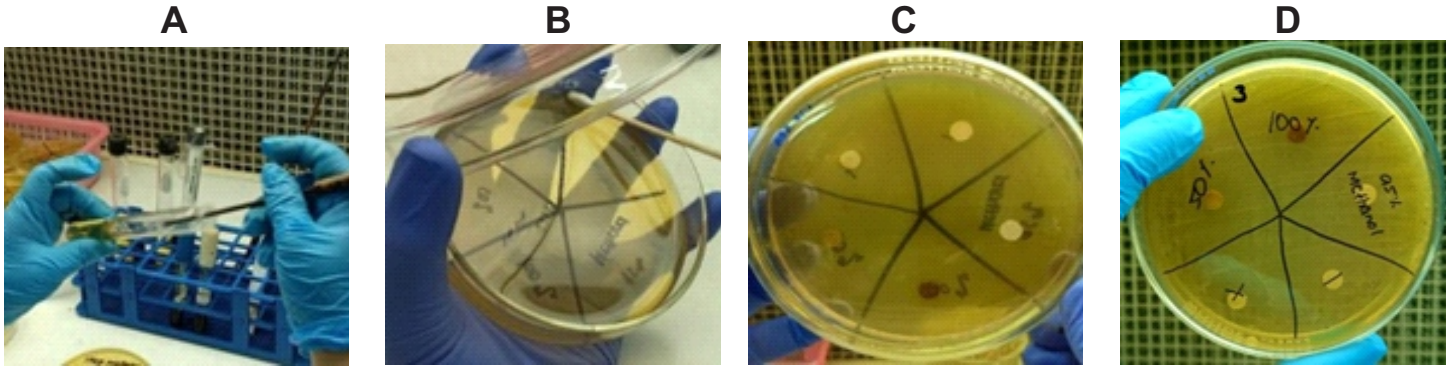

Figure 1. *Actual diagram of the disk diffusion method. (a) Inoculum of S. mutans. (b) MHA plate inoculated with S. mutans. (c) MHA plate with discs soaked in the 5 treatment groups. (d) MHA plate with resultant zone of inhibition after incubation.*

significantly from each other. The paired comparison of chlorhexidine and 100 mg/ml of plant extract and chlorhexidine and 50 mg/ml of plant extract had a p-value less than $\alpha = 0.05$. This means that chlorhexidine has a significantly larger zone of inhibition than the two concentrations of the plant extract (p-value ≤ 0.001). Also, the plant extract's 100 mg/ml concentration has a significantly bigger zone of inhibition than the 50 mg/ml concentration.

Discussion

This study determined the antibacterial activity of two concentrations of *Vitex parviflora A. juss* (molave) leaf extracts and compared it with a commonly used oral antimicrobial, 0.12% chlorhexidine.

The disk diffusion tests showed an antibacterial activity for both concentrations of molave leaves extracts against *S. mutans*. The obtained average zone of inhibition by the molave extract in this study is similar to the results obtained in a previous study by Tantengco *et al.* [32] on another staphylococcus microorganism. In their study, 100 mg/ml and 50 mg/ml of crude methanolic *Vitex parviflora A. juss* leaf extracts were tested against *Staphylococcus aureus*. The average zones of inhibition obtained were 15.70 mm and 13.37 mm, respectively. A disk diffusion test also showed that molave leaf extract has a less antimicrobial effect than 0.12% chlorhexidine.

The antibacterial activity of a plant extract is attributable to the phytochemicals that it contains. The leaves were utilized since it was found that extracts of molave leaves contain phytochemicals [34]. Maceration as the method of extraction was used because this aids in releasing the most active soluble phytochemicals from the plant's cell wall [40]. Methanol was used as the extraction solvent due to its ability to extract hydrophobic and hydrophilic components. In addition to this, Tantengco *et al.* found that the molave extract in the 95% methanol solvent was effective against *S. aureus* and *E. coli* [32].

A phytochemical study on ethanolic *Vitex parviflora A. juss* leaves extract conducted by Panti *et al*. [34] showed that this plant contains alkaloids, saponins, diterpenes, phenols, phytosterols, and flavonoids. Flavonoids, phytochemicals produced by plants in response to microbial infection, have been proven to fight human pathogens in vitro. Flavonoids disrupt microbial membranes and have been found to have antimicrobial properties against a wide array of microorganisms [20]. This may be due to its ability to complex with extracellular and soluble proteins or with bacterial cell walls.

Moreover, increasing the concentration of the extract also increased its antibacterial activity.

The difference between the antibacterial activity of the two different concentrations of *Vitex parviflora A. juss* leaves extracts against *S. mutans* may be explained by the increasing abundance of phytochemicals present in the extract. The higher the concentration of the molave leaves extracts, the greater the diameter of the zone of inhibition recorded [32].

On the other hand, the mechanism of action of chlorhexidine on *S. mutans* is to alter the bacterial cell membrane and further penetrate the inner cell membrane. Its ability to bind to the phospholipids in the inner membrane results in the leakage of potassium ions and other low molecular weight compounds. The flow of ions, then, results in the coagulation and precipitation of cell cytoplasm wherein phosphate complexes like adenosine triphosphate and nucleic acids are formed. At this stage, the bactericidal effect is irreversible [11]. While chlorhexidine is the most popular oral antimicrobial and has been proven to be effective against *S. mutans* [11], side effects have been reported with prolonged use [10, 14].

The difference in the mechanism of action of chlorhexidine and molave leaves extract might explain chlorhexidine's significantly higher antimicrobial effect. While the results showed that both concentrations of molave leaves extract were relatively weaker than chlorhexidine, it was still able to inhibit the growth of *S. mutans*, thus providing baseline data on its antibacterial activity. One notable finding is that the antimicrobial effect increased when the concentration of molave leaves extract was doubled from 50 mg/ml to 100 mg/ml. Further studies using higher concentrations of molave leaves extract against *S. mutans* may approximate the results for chlorhexidine.

It is interesting to find that the molave leaves extract exhibits antimicrobial activity against *S. mutans* complementing phytochemical studies. As for the distilled water and 95% methanol, no zone of inhibition was observed. Since distilled water is the negative control, it is expected to not show any antibacterial activity against *S. mutans*. On the other hand, 95% methanol was the solvent used in the extraction process of *Vitex parviflora A. juss* leaves; hence, it was tested to show that this component of the extraction process is not responsible for the antibacterial activity of the extract.

This study shows that the methanolic extract from molave, a plant readily available in the Philippines, may be a viable alternative to chlorhexidine. The medicinal plant could serve as a potential source of an alternative oral

antimicrobial agent that may be developed further to become useful for patients with limited ability to perform mechanical cleansing of their oral cavities.

Conclusion

The disk diffusion method shows that *Vitex parviflora A. Juss* (molave) leaf extracts have the ability to inhibit the growth of *S. mutans* and that higher concentrations produce significantly bigger zones of inhibition. Both concentrations of molave leaf extract were relatively weaker compared to chlorhexidine.

Since molave is a natural product, there are fewer expected adverse effects [17,23]. Studies on its antimicrobial properties may be pursued such as isolating the different components of the plant extract through a phytochemical study and then testing the antibacterial properties of each phytochemical. This will help understand and describe the mechanism of action of the plant extract in inhibiting bacterial growth or determining the distinct active components that play in the mechanism of action against *S. mutans*. The use of ethanolic, aqueous, and other extracts is also recommended. The mutagenicity, toxicity, and other assays for molave leaf extract as an antimicrobial for *S. mutans* may also be explored. Other studies on the applicability of molave extracts as an antimicrobial may also be done by subjecting it to a Time-kill test (time-dependent or concentration-dependent) and flow cytofluorometric methods to determine its bactericidal effect [35]. Findings may be used for future *in vivo* studies or the development of commercial molave extracts as an oral antimicrobial. Plants and plant parts, like molave leaves, play an essential role in drug discovery now that multi-drug resistance is becoming more common. The search for new antimicrobials is an ongoing quest.

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