Comparative Evaluation of Antimicrobial Properties of Three Various Extracts of Achyranthes Aspera and Chlorhexidine against Streptococcus Mutans

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Keywords-
Dental caries, Streptococcus mutans, Anrimicrobial activity, Achyranthes aspera (Aghada)

Abbreviations-
1. ml – Milli liter
2. mm – Milli meter
3. µl – Micro liter
4. yrs – Years
5. °C – Degree Celsius
6. hrs – Hours

Abstract
In the view of pediatric dentistry dental caries is one of the most common of all diseases and partly because of its relatively rapid progress it is the main cause of loss of teeth in children. Keeping in mind the newer etiological concepts of dental caries and the role of Streptococcus mutans various preventive and therapeutic measures are recommended. Medicinal plants are the oldest source of pharmacologically active compounds and have been useful in treating various systemic diseases for centuries. There are numerous study reports in which various plants and their extracts have been previously analyzed and were found to have significant therapeutic properties. This study was designed to palpate the antimicrobial potential of naturally available Achyranthes aspera plant and its derived ethanolic extracts as an alternative to the presently used synthetic agents to combat dental caries. The antimicrobial activity of the leaf, stem and root extracts of Achyranthes aspera (Aghada) was evaluated and compared with chlorohexidine as the standard chemotherapeutic agent against Streptococcus mutans. The observational & statistical findings of the herbal extracts suggest that, the active components of these extracts have definite antimicrobial property against Streptococcus mutans.

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1. Introduction

The most common disease in the view of pediatric dentistry is dental caries and partly because of its relatively rapid progress it is the main cause of loss of teeth in children. Dental caries prevention has long been considered as an important task for the health professionals (Ingle NA., 2014). Various primary preventive chemotherapeutic agents are used to target the causative factors in dental caries among these factors oral microorganisms like S. mutans play a pivotal role in dental caries. Vigorous use of these chemotherapeutics has increased prevalence of side effects of many synthetic antimicrobial agents and reduction in the susceptibility of various microbial strains to the presently being used antibiotics. This has raised the need for newer antimicrobial agents to combat dental caries.

The plant based therapeutics is in great demand in the developed world for primary health care because of their efficacy, safety and minimal documented side effects (Kamboj VP., 2000). Herbal medicine is both promotive and preventive in its approach. This in-vitro study was undertaken to evaluate and compare the antimicrobial properties of leaf, stem and root extracts of Achyranthes aspera (Aghada) and 0.2% Chlorhexidine against Streptococcus mutans serotype c (ATCC 25175). This research aims at finding a natural antimicrobial agent which would prove to be an effective alternative and eventually substitute the available synthetic medications.

2. Review of Literature

Dental caries has been recognized throughout history and exists around the world. During the past decades there was a common consensus from many worldwide studies stating that dental caries had declined significantly. There are however, recent studies that report alarming increases in caries. Over centuries it has shown a significant change in prevalence and pattern. It is a public health problem in India, with a prevalence as high as 60-80% in Indian children (Bali et al., 2004). A retrospective analysis was carried out in 2015 by Kundu H et al, to assess the average dental caries prevalence across different WHO index age groups (5, 12 & 15 years) in India for the past fifteen years. They reported the pooled prevalence of dental caries to be 48.11%, 43.34% and 62.02% in 5, 12 and 15 year olds respectively (Kundu H et al., 2015). The emerging public health issues are related to disparities in prevalence and treatment of dental caries.

The prevention of dental caries has long been considered as an important task for the health professionals. A number of chemotherapeutic agents are also used to target the causative factors in dental caries among these factors oral microorganisms like S. mutans play a pivotal role in dental caries. Chemotherapeutic and antimicrobial agents aiming at these predisposing factors therefore play a significant role in prevention of such oral diseases. The vigorous use of such chemotherapeutic and antimicrobial agents to combat caries led to increased prevalence of side effects like, tooth and restoration staining, increased calculus formation, diarrhea and disarrangements of the intestinal flora (More G et al., 2008). Furthermore, there has been an increase in failure of many popular synthetic antimicrobial agents due to development of multidrug resistant strains of micro-organisms. The recent appearance of strains with reduced susceptibility to antibiotics raises the spectrum of untreatable bacterial infections and adds urgency to the search for new infection fighting strategies. These drawbacks justify the search for new effective anti-cariogenic compounds with minimal or no side that could be employed in caries prevention (Sieradsxi K et al., 1999).

The use of plants for healing purposes predates human history and forms the origin of much modern medicine. There are several studies performed to evaluate medicinal properties of several well known medicinal
plants and anti-microbial, anti-fungal, anti-helminthes, anti-inflammatory, wound healing properties are found in these plant extracts. The demand on plant based therapeutics is increasing as they are natural, non narcotic, easily biodegradable, producing minimum environmental hazards and easily available. The major strength of these natural herbs is that their use has not been reported with any side-effects till date (Nagappan N et al., 2012).

*Achyranthes aspera* sp. is one such important medicinal plant used by traditional healers in the treatment of fever, dysentery, asthma, hypertension and diabetes (Shendkar CD et al., 2012). There are studies which scientifically validate the traditional use of *A. aspera* as a natural brush for teeth cleaning and state that phytochemicals of this traditionally used dental caries preventive natural chewing stick plant could be harnessed for dental caries and other biofilm mediated disease management (Murugan K et al., 2013).

3. **Objective of Research**

This study was designed to palpate the antimicrobial potential of naturally available *Achyranthes aspera* plant and its derived extracts as an alternative to the presently used synthetic agents to combat dental caries. Chlorhexidine is the most widely accepted commercially available therapeutic oral rinse which serves as the gold standard when used in comparison with other products. In light of the above discussion, we consider it worthwhile to undertake a study of this well known Indian herbal medicine (*Achyranthes aspera*) for evaluation of its antimicrobial properties. Thus the antimicrobial activity of the leaf, stem and root extract of *Achyranthes aspera* (Aghada) was evaluated and compared with chlorohexidine as the standard chemotherapeutic agent against *S. mutans*.

4. **Materials and Methods**

4.1 **Plant Material**

Plant extracts used in this study were procured from the Department of Rasashastra and Bhaishajya Kalpana, Dr. D. Y. Patil Ayurvedic College and Hospital, Pimpri, Pune. Leaf, root, stem extracts of *A. aspera* were prepared using ethanol as a dilution media. (Figure 1)

![Figure 1: Plant extracts of Achyranthis aspera](image-url)
The values of zones of inhibition of the three different extracts of Achyranthes aspera and 0.2% Chlorhexidine against S. mutans are shown in Table 1. Graphical representation of comparative values zones of inhibition by of the three different extract of Achyranthes aspera and 0.2% chlorohexidine against S. mutans serotype c (ATCC 25175) is shown in Graph 1. Mean values of zones of inhibition exhibited by leaf, stem and root extracts of A. aspera and 0.2% chlorohexidine against S. mutans serotype c (ATCC 25175) are depicted in Table 2 and Graph 2 shows graphical representation of mean values of zones of inhibition exhibited by leaf, stem and root extracts of A. aspera and 0.2% chlorohexidine against S. mutans serotype c (ATCC 25175). Statistical analysis (Post Hoc tests) of the antimicrobial activity and mean zones of inhibitions of the three extracts of A. aspera (Group A=1, B=2, C=3) and 0.2% Chlorohexidine (Group D=4) against S. mutans serotype c (ATCC 25175) is shown in Table 3.

Tables and Graphs

Table 1 – Comparison of zones of inhibition of the three different extracts of Achyranthes aspera and 0.2% Chlorhexidine against S. mutans

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Zones Of Inhibition (in mm) against S. mutans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
</tr>
<tr>
<td>1</td>
<td>2 mm</td>
</tr>
<tr>
<td>2</td>
<td>2.4 mm</td>
</tr>
<tr>
<td>3</td>
<td>2.1 mm</td>
</tr>
<tr>
<td>4</td>
<td>2 mm</td>
</tr>
<tr>
<td>5</td>
<td>2.1 mm</td>
</tr>
<tr>
<td>6</td>
<td>1.9 mm</td>
</tr>
<tr>
<td>7</td>
<td>2 mm</td>
</tr>
<tr>
<td>8</td>
<td>2 mm</td>
</tr>
<tr>
<td>9</td>
<td>2.3 mm</td>
</tr>
<tr>
<td>10</td>
<td>2.1 mm</td>
</tr>
<tr>
<td>11</td>
<td>2 mm</td>
</tr>
</tbody>
</table>
Graph No. 1 – Graphical representation of comparative values zones of inhibition by of the three different extract of Achyranthes aspera and 0.2% chlorohexidine against S. mutans serotype c (ATCC 25175)

Table No. 2 – Mean values of zones of inhibition exhibited by leaf, stem and root extracts of Achyranthes aspera and 0.2% chlorohexidine against S. mutans serotype c (ATCC 25175).

<table>
<thead>
<tr>
<th></th>
<th>Sample size</th>
<th>Mean</th>
<th>Standard Deviation</th>
<th>F</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>20</td>
<td>2.090</td>
<td>.1410</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>20</td>
<td>3.110</td>
<td>.1553</td>
<td>5906.62</td>
<td>&lt;0.001</td>
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<tr>
<td>Group C</td>
<td>20</td>
<td>3.105</td>
<td>.1638</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group D</td>
<td>20</td>
<td>8.070</td>
<td>.1658</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Graph No. 2- Graphical representation of mean values of zones of inhibition exhibited by leaf, stem and root extracts of *Achyranthes aspera* and 0.2% chlorohexidine against *S. mutans* serotype c (ATCC 25175).

![Graph of zones of inhibition](image)

Table No. 3 - Statistical analysis (Post Hoc tests) of the antimicrobial activity and meanzone of inhibitions of the three extracts of *Achyranthes aspera* (Group A=1, B=2, C=3) and 0.2% chlorohexidine (Group D=4) against *S. mutans* serotype c (ATCC 25175)

<table>
<thead>
<tr>
<th>(I) group</th>
<th>(J) group</th>
<th>Mean Difference (I-J)</th>
<th>Sig.</th>
<th>95% Confidence Interval Lower Bound</th>
<th>Upper Bound</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>2</td>
<td>-1.0200*</td>
<td>.000</td>
<td>-1.154</td>
<td>-.886</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>-1.0150*</td>
<td>.000</td>
<td>-1.149</td>
<td>-.881</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>-5.9800*</td>
<td>.000</td>
<td>-6.114</td>
<td>-5.846</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.0200*</td>
<td>.000</td>
<td>.886</td>
<td>1.154</td>
</tr>
<tr>
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<td>3</td>
<td>.0050</td>
<td>1.000</td>
<td>-.129</td>
<td>.139</td>
</tr>
<tr>
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<td>4</td>
<td>-4.9600*</td>
<td>.000</td>
<td>-5.094</td>
<td>-4.826</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1.0150*</td>
<td>.000</td>
<td>.881</td>
<td>1.149</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>-.0050</td>
<td>1.000</td>
<td>-.139</td>
<td>.129</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>-4.9650*</td>
<td>.000</td>
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<tr>
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<td>5.846</td>
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<tr>
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<td>2</td>
<td>4.9600*</td>
<td>.000</td>
<td>4.826</td>
<td>5.094</td>
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<tr>
<td>4</td>
<td>3</td>
<td>4.9650*</td>
<td>.000</td>
<td>4.831</td>
<td>5.099</td>
</tr>
</tbody>
</table>

4.2 *Standard antimicrobial agent*

The zones of the inhibition of each plant extract are compared with 0.2% Chlorhexidine (Chlorhexidine mouthwash-Hexidine) using it as a gold standard [Jones et al., 1997].

4.3 *Microbial strains*

4.4 *Sampling*

The total sample size for the study was N=80, which was divided into four study groups as follows-
Group A – Leaf extracts of Achyranthes aspera (N_A = 20)
Group B - Stem extracts of Achyranthes aspera (N_B = 20)
Group C - Root extracts of Achyranthes aspera (N_C = 20)
Group D - 0.2% Chlorhexidine (N_D = 20)

4.5 Microbiological laboratory processing
The microbial inhibition assay was performed using the agar well diffusion method (Deshpande RR., 2012). Muller Hinton Agar was used as the culture medium. Adequate amount of Muller Hinton Agar was dispensed into sterile plates and allowed to solidify under aseptic conditions (Deshpande RR et al., 2010). The agar plates were prepared in sterile petri-dishes and kept overnight for sterility at 37° C. The test samples of strains of S. mutans serotype c (ATCC 25175) were then inoculated with a sterile spreader on the surface of solid Muller Hinton Agar medium in plates (Deshpande RR et al., 2013). After the media was solidified; wells were punched in the plates with the help of a cupborer (5.0mm) (Deshpande RR et al., 2013). The well was filled with 80µl/well concentrations of the extract and 0.2% Chlorhexidine. Ethanol was used as the dilution media (200mg/1ml) and analysis of its antimicrobial activity against S. mutans was performed on a control plate. Plates were incubated at 37±0.1ºC for 24 hours. All the tests were performed under sterile conditions. After incubation, the plates were observed for zones of growth of inhibition and the diameters of these zones were measured in millimeters by using bacterial inhibition zone reading scale and the diameter of the well was excluded while measuring the zones of inhibition (Deshpande R R et al., 2013).

5. Results

The antimicrobial activity was evaluated by measuring the zones of inhibition produced by the three different extracts of A. aspera plant and standard chemotherapeutic agent 0.2% chlorohexidine by using agar diffusion method in the lab (Figure 2). Ethanol was used as the dilution media (200 mg/1ml) and analysis of its antimicrobial activity against S. mutans was performed on a control plate. It does not exhibit formation of zone of inhibitions concluding lack of any antimicrobial activity in the ethanol used for dilution of plant extracts (Figure 3). The values of zones of inhibition were calculated on the agar plates and subjected for statistical analysis using one way ANOVA and Post Hoc analysis. The comparison of the antimicrobial potential leaf, stem, root extracts of the plant and chlorohexidine against S. mutans was done using these tests with their corresponding values of zones of inhibition.

Discussion

There exists voluminous literature on the status of the dental caries in the Indian population. A very extensive and comprehensive National Health Survey conducted in 2004 throughout India has shown dental caries in 51.9% in 5 year-old children, 53.8% in 12 year-old children and 63.1% in 15 year-old teenagers (Deshpande RR et al., 2013). Hence meticulous use of several measures to control caries process is now an important component in the maintenance of dental health of all individuals (Bagramian R et al., 2009).

The concept of dental caries has undergone many changes especially pertaining to its etiological factors. The acidogenic Mutans streptococci group, including Streptococcus mutans and Streptococcus sobrinus are among the cariogenic microorganisms that reside in the oral cavity, and represent the most predominant bacteria associated with dental caries. (Fejerskov O., 2004). Keeping in mind these etiological factors associated with dental caries use of various preventive and therapeutic measures are recommended.

Several antibiotics such as ampicillin, sanguinarine, entnmidazole, phenolic antiseptics and quaternary ammonium-antiseptics, among others, have also been
very effective in preventing dental caries (Porto TS et al., 2009; Leung KW., 2004). There are reports indicating that the there is not an effective reduction in the levels of mutans streptococci or plaque formation with the currently being used antimicrobial agents and the clinically important outcome is reduction in caries incidence (Gold AJ., 2008). Chlorhexidine, an antimicrobial agent that can suppress the growth of mutans streptococci, has been considered to have the potential to prevent dental caries (Emilson CG., 1994). However, there is a lack of consensus on evidence-based treatment protocols and controversy regarding the role of chlorhexidine in caries prevention among dental educators and clinicians (Rijkom HM et al., 1996). Further chlorhexidine comes with its own set of disadvantages. Repeated use of chlorhexidine can lead to tooth & restoration staining and systemic effects like diarrhea (Southern EN., 2006). In such a scenario, the need for a preventive measure, which will reduce the caries activity as well as can be made available to the common mass becomes more glaring.

Plants are the oldest source of pharmacologically active compounds and have provided man with many medically useful substances for centuries. Hence medicinal plants now form the most important source for prospecting of new bioactive molecules (Ekor M., 2013). Medicinal plants like Tulsi, Neem, Turmeric, Triphala, Aloe Vera and many others well known and established plants have long been evaluated worldwide by many conventional researchers. But there are many other plants which are still less palpted for the presence of antimicrobial properties against oral diseases. In India, most of these medicinal plants are commonly found in the evergreen to moist deciduous forests of the Western Ghats region. Achyranthes aspera is one of the many plants which are being evaluated for their medicinal properties. In demonstrating antimicrobial action, active ingredients were obtained from different parts of the plant like leaves, stem, roots, fruits etc. These active ingredient procured from the different parts of a plant were further evaluated individually and compared against each other for their medicinal properties against Streptococcus mutans, the primary etiological micro-organism in dental caries.

This study evaluated the bacterial growth inhibition of the derived ethanolic extracts of leaves, stem and roots of Achyranthes aspera plant using the disc diffusion method on agar plates. The concentration used for all the three extracts of the plant was 80µl/ well on agar plates inoculated with Streptococcus mutans, serotype C ATCC (25175). The measured values of zone of inhibition exhibited against the tested samples of S. mutans by Group A, Group B, Group C and Group D are tabulated in Table No. 1 and Graph No. 1 shows the pictorial presentation of the same.

Observationally, the comparative evaluation of antimicrobial activity of plant extracts viz. leaf, stem and root of A. aspera (Table No. 1) shows that stem and root extracts exhibit almost equivalent antimicrobial potential which is significantly higher than that of the leaf extracts (Graph No. 1). The antimicrobial activity of the standard 0.2% chlorohexidine gluconate is the highest when compared with all the three extracts of A. aspera (Table No. 2) at tested the concentration (80 µl) which is statistically significant. The results show that the mean values of zone of inhibition for herbal leaf stem and root extracts of A. aspera are 2.090 mm, 3.110 mm and 3.105 mm respectively (Table no. 2 and Graph no. 2). The results indicate that the mouth-rinse we evaluated, (0.2% CHX) demonstrated consistent inhibitory effects on the test bacteria. The test samples showed CHX had an average zone of inhibition of 8.070 mm against S. mutans and it is statistically significant with the p value <0.001 (Table No. 3). However, the observational & statistical findings of the herbal extracts also suggest that, the active components of these extracts have definite antimicrobial property against S. mutans. Stem extract showed
highest activity among the plant extracts which was almost equivalent to that of the root extract of the plant and comparable with chlorhexidine.

In the present study, results show the antimicrobial property of the extracts tested using only one concentration (80 µl/well). The demonstration of antimicrobial activity by ethanol extracts of A. aspera proves the antimicrobial potential of the plant. Further evaluation with higher concentrations of the plant extracts and different dilution media can enhance the activity of this extract. This investigation has increased the possibilities for the implementation of the antimicrobial properties of this plant and its bioactive molecules in drug development and use as a preventive and therapeutic agent in diseases like dental caries.

**Conclusion**

Many advances in the treatment and prevention of dental caries have been introduced over the past century. Medicinal plants are pluripotent in nature and hence offer a great therapeutic measure for the health of the mankind. Combining the strengths of the knowledge based traditional systems such as “Herbal Medicine” with the power of science can provide new functional leads to reduce time, money and toxicity - the three main hurdles in drug development.

The demonstration of significant antimicrobial activity by the derived ethanolic leaf, stem and root extracts of Achyranthes aspera provides the scientific basis for the presence of medicinally active compounds in this plant which can further be useful as preventive and therapeutic measure in the hard and soft tissue diseases of oral cavity. The effect of this plant on more pathogenic organisms, evaluation of further higher concentrations for toxicological investigations and further purification however needs to be carried out.

**Research Highlights**

This research aims to develop newer effective and efficient antimicrobial agents with minimal side effects which would prove to be an alternative to currently being used synthetic antimicrobials. In the dentistry, these effective plant extracts can be formulated into preparations such as dentifrices, mouth washes, gum paints or as an intra-canal medicament and may be used where an antimicrobial agent is required. This research would help support the use of natural herbs, as medicine, which is already in a great demand in developing world.

**Limitations**

This study was carried out on an in-vitro basis, although there are minimal reported side effects of Aghada at systemic level, any toxic effects if present, and local factors like tooth discoloration, taste, smell, which are important specifically when considering pediatric dental usage need to be evaluated in-vivo (Srivastav S et al., 2011; Hasan S., 2014). Adverse effects if any, due to increase in concentration of the extract need to be carefully studied before applying it as a regular antimicrobial agent in dentistry.

**Acknowledgements**

Department of Rasashastra and Bhaishjiya Kalpana, Dr. D. Y. Patil Ayurvedic College and Hospital, Pimpri, NAFARI Lab, Pune,

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