ANTI-BACTERIAL ACTIVITY OF ACORUS CALAMUS AGAINST STREPTOCOCCUS MUTANS AND LACTOBACILLUS CASEI

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ABSTRACT- Certain plant extracts are being used for their medicinal values for centuries. Microorganisms play a major role in the etiopathogenesis of dental caries through their ability to colonize the tooth surface and form biofilm. In the present study acetone and methanol extracts of Acorus calamus were used to evaluate the anti-bacterial property against Streptococcus mutans and Lactobacillus casei. The findings of the study showed, the acetone and methanol extracts of Acorus calamus to possess anti-microbial activity against Lactobacillus casei, with a mean zone of inhibition of 6mm with methanol extract and 4mm with acetone extract. Also, MIC value was 6.25mg/ml and MBC value was 12.50mg/ml with both the extracts. The acetone and methanol extracts of Acorus calamus showed no anti-bacterial activity against Streptococcus mutans.

Keywords- ACORUS CALAMUS, STREPTOCOCCUS MUTANS, LACTOBACILLUS CASEI

1. INTRODUCTION
World Health Organization (WHO) says that, “plants are the best source for obtaining a variety of drugs” [1, 2]. The secondary metabolites accumulated within the plants serve as the bioactive compounds, most of which have antimicrobial properties [3, 4]. The indiscriminate usage of antimicrobial drugs has resulted in increased antimicrobial resistance in various human pathogens. This has led to the screening of plants for their antimicrobial properties. The target site on which the plant derived compounds act is thought to be different from those that are acted upon by the antimicrobial drugs. Also, these compounds are considered to be safer than the antimicrobials and are also economical [3].

Numerous plant extracts are being used for their various medicinal values for centuries. For example, paste made from Azadirachta indica (Neem) is used in the treatment of various skin infections, juice from the fresh stem of Polyalthia longifolia (Ashoka) is used in the treatment of indigestion [5].

Dental caries affects many people worldwide. Microorganisms play a major role in the etiopathogenesis of dental caries. These organisms can colonize the tooth surface resulting in the formation of biofilm. [6]. Tooth brushing along with mouthwashes prevents biofilm formation. However, mouthwashes can sometimes stain the teeth, alter the taste perception and also cause desquamation of oral mucosa. Therefore, we need alternative sources as adjuvants and various medicinal plant sources are being screened for the same [1].
The leaf extracts of *Piper betle* (Betel), *Saraca asoca* (Ashoka tree), *Ficus benghalensis* (Banyan tree), *Cassia fistula* (Golden shower tree), *Nerium indicum* (Oleander), *Mangifera indica* (Mango tree), *Allium sativum* (Garlic), *Manilkara hexandra* (Milk tree) and *Aegle marmelos* (Wood apple) have shown anti-bacterial activity against the caries causing bacteria, *Streptococcus mutans* [7]. Oil pulling therapy done with coconut oil has shown to reduce the count of *Streptococcus mutans* in the oral cavity [8].

Leaf extracts of *Aegle marmelos* (Wood apple), *Manilkara hexandra* (Milk tree), *Piper betle* (Betel), *Saraca asoca* (Ashoka tree), *Plumeria rubra* (Frangipani), *Ficus virens* (White fig) and *Mangifera indica* (Mango tree) showed anti-bacterial activity against *Lactobacillus acidophilus* and *Lactobacillus casei*. Also, the leaf extracts of *Ficus benghalensis* (Banyan tree) and *Cassia fistula* (Golden shower tree) showed anti-bacterial activity against *Lactobacillus casei* [7].

Various studies on the plant extracts of *Acorus calamus* (vasambu) have shown anti-bacterial activity against *Streptococcus mutans, Staphylococcus aureus, Escherichia coli* and *Shigella flexneri* [9]. The rhizome of *Acorus calamus* is used for various problems like asthma, fever, cough, bronchitis, bloating, gas, colic and poor digestive function [10]. Due to the wide medicinal properties of *Acorus calamus* and their extensive usage in numerous diseases, in this study, the extracts of the rhizome of *Acorus calamus* are evaluated for their anti-bacterial properties against the caries causing pathogens *Streptococcus mutans* and *Lactobacillus casei*.

An important factor affecting the extraction efficiency of bioactive compounds from plant materials is the extraction solvent. Numerous studies are published using petroleum ether, ethanol, ethyl acetate, butanol, chloroform and water, except acetone and methanol as solvents for extraction of *Acorus calamus* rhizome to evaluate its anti-microbial activity [9, 10]. In the present study acetone and methanol are used as solvents to study the anti-bacterial activity of *Acorus calamus*.

### 2. MATERIALS AND METHODS

The present in-vitro study was conducted to evaluate the anti-bacterial activity of crude solvent extracts of the rhizome of *Acorus calamus* against *Streptococcus mutans* and *Lactobacillus casei*. The solvent extraction and anti-microbial bioassay of *Acorus calamus* were done in the Department of Central Research Laboratory, Meenakshi Ammal Dental College & Hospital, Maduravoyal, Chennai.

#### 2.1 MATERIALS USED

**Armamentarium-i**

1. Hot air oven (sterilizer)
2. Incubator
3. Orbital shaking incubator
4. Electronic weighing machine
5. Electric grinder
6. Rhizome of *Acorus calamus* (A14061801C)
7. Solvents
   - Methanol
   - Acetone
8. Glass measuring jar
9. Test tubes
10. Test tube stand
11. Glass funnel
12. Flat bottomed beaker
13. Whatman filter paper (No.1)

**Armamentarium-ii**
1. Inoculating wire loops
2. Sterile cotton swabs
3. Micropipette (V3 variable volume pipette 2 µl - 1000µl)
4. Sterile micropipette tips
5. Standard antibiotic zone measuring scale
6. Sterile glass vials
7. Sterile petri plates
8. Sterile gel puncture needle
9. Test organisms used for the study
   - *Streptococcus mutans* (MTCC 497)
   - *Lactobacillus casei* (NCIM 5304)
   - Culture media
   - de Man rogusa and Sharpe (MRS) broth
   - Mutans Sanguis broth (MS broth)
   - Mueller Hinton Agar (MH Agar)

### 2.2 CRUDE SOLVENT EXTRACTION

**Collection of plant source:** About 500 gms of the rhizome of *Acorus calamus* was purchased from herbal stores and was dried in the shade to become free of moisture. The rhizomes were submitted to the Department of Pharmacognosy, Siddha Central Research Institute, Annanagar, Chennai, for taxonomical identification, (AI4061801C). After species identification the rhizomes were powdered in an electric mixer and stored in an airtight container.

**Preparation of crude solvent extract:** The organic solvents selected for the study were acetone and methanol. The powdered rhizome of *Acorus calamus* was weighed using an electronic weighing machine. 200gms of the powder was dissolved in 600ml of the above-mentioned solvents, in two separate glass bottles with stoppers and were labelled appropriately [Figure-1]. The constituents were mixed well with sterile glass rods and kept in orbital shaking incubator for 48 hours. The solvent extracts were filtered with Whatman filter Paper (No.1) into a flat-bottomed beaker [Figure-2], transferred into pre-weighed petriplates and were left for evaporation for 2 days.

**Preparation of crude solvent extract**
The crude extracts were then reconstituted in dimethyl sulfoxide (DMSO), which is an inert organic solvent, to prepare a concentration of 2000mg/ml. These reconstituted extracts were transferred to sterile labelled vials and were stored in refrigerator at 4°C Celsius, until use.

**Test organisms:** The standard bacterial strains, *Lactobacillus casei* (NCIM 5304) was procured from National Collection of Industrial Microorganisms (NCIM), Pune, Maharashtra and *Streptococcus mutans* (MTCC 497) was procured from Microbial Type Culture Collection and gene bank (MTCC), Chandigarh.

**Antimicrobial bioassay:** The lyophilized cultures were added to Mutans Sanguis broth for *Streptococcus mutans* and de Man, Rogosa and Sharpe broth for *Lactobacillus casei*. The cultures were incubated at 37°C for 2 hours. After incubation, the colonies were sub-cultured in the appropriate media and incubated for 24 hours. The colonies from the primary culture were added to the respective broths. The broths with turbidity were adjusted to McFarland Standard (0.5) and were used for the bioassay.

**Agar well diffusion method:** The anti-bacterial activities of the solvent extracts were tested using the agar diffusion method. Lawn cultures were made onto sterile Mueller Hinton Agar for *Streptococcus mutans* and *Lactobacillus casei*. 
Four wells were punched onto each lawn culture using gel puncture needle. One positive control, one negative control (Dimethyl sulphoxide DMSO) and the solvent extracts in the concentration of 2000mg/ml were added to the wells. The positive controls were azithromycin for *Streptococcus mutans* and *Lactobacillus casei*. The plates were then incubated at 37°C for 24 hours.

The zones of inhibition for each test organism was observed and measured using a standard antibiotic zone measuring scale and recorded in millimetres [Figure-3]. The assay was repeated five times and the mean zones of inhibition were calculated.

**Determination of minimum inhibitory concentration (mic) and minimum bactericidal concentration (mbc):** In the present study, the microbroth dilution technique was used to determine the minimum inhibitory concentration of *Acorus calamus* solvent extracts.

**Procedure:** The Minimum Inhibitory Concentration (MIC) is the lowest concentration of the test solvent extract that is static (prevents the visible growth of bacteria). The Minimum Bactericidal Concentration (MBC) is the lowest concentration at which the solvent extract is cidal (causes microbial death). The MBC is complementary to the MIC.

Minimum inhibitory concentration (MIC) of *Acorus calamus* solvent extracts against *Streptococcus mutans* and *Lactobacillus casei* were determined by 96 well microtitre broth dilution methods.

Each row of the plate corresponded to a specific solvent extract and one well in each row served as a negative control (extract without test organism) and another as a positive control (broth with the test organism). The 50µl/ml of broth and different concentrations of solvents (100, 50, 25, 12.5, 6.25, 3.12 and 1.5 mg/ml per well) were pipetted to the corresponding well to which the preparations of the test organisms were added. The mixtures were incubated at 37°C for 24 hours and analyzed for turbidity using spectrophotometer. The dilution at which there is complete absence of visible growth is recorded as Minimum Inhibitory Concentration (MIC).

Further, micro-spot assay in agar plate was done from dilution corresponding to minimum inhibitory concentration and higher concentrations. The dilution at which there is complete absence of the bacterial colonies is recorded as the Minimum Bactericidal Concentration (MBC).

**3. RESULTS**

The antibacterial activity of the rhizomes of *Acorus calamus* in different solvent extracts like acetone and methanol were evaluated against *Streptococcus mutans* and *Lactobacillus casei*. The experiment was repeated five times by Agar well diffusion method.

The results were interpreted by measuring and recording the zone of inhibition for each test organism in millimeters. Quantitative variables obtained in the study were assessed using Shapiro- Wilk test. The mean value was calculated, and the Data was statistically analyzed by one way annova. All the statistical analysis was done using SPSS version 16 (IBM Corporation, Chicago, IL, USA).

The zones of inhibition of *Acorus calamus* against *Streptococcus mutans* and *Lactobacillus casei* are depicted in table-1.

The mean values of zones of inhibition of *Acorus calamus* solvents against different microorganisms are depicted in table-2. Azithromycin was used as the
standard antibacterial agent for positive control. Dimethyl sulfoxide (DMSO) was used as the negative control.

Minimum inhibitory concentration (MIC) of *Acorus calamus* extracts against different microorganisms is depicted in table-3. The minimum bactericidal concentration (MIC) is depicted in table-4.

Table 1: Zones of inhibition of *Acorus calamus* extracts (acetone and methanol) against different microorganisms.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Zones of inhibition in acetone extract in mm</th>
<th>Zones of inhibition in methanol extract in mm</th>
<th>Positive Control in mm</th>
<th>Negative Control in mm</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>NA, 3, NA, NA, 2</td>
<td>NA, 4, NA, NA, NA</td>
<td>18, 19, 18, 18, 20</td>
<td>NA</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>3, 5, 4, 3, 5</td>
<td>8, 6, 5, 7, 6</td>
<td>15, 17, 16, 17, 15</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA- No activity.

In Table-1, the zones of inhibition ranged from 0 to 5 mm with acetone and 0 to 8 mm with methanol solvent extract showing no zone of inhibition against *Streptococcus mutans* with both solvents and a maximum zone of inhibition of 5 mm with acetone solvent and 8 mm with methanol solvent against *Lactobacillus casei*.

Table 2: Mean values of zones of inhibition of *Acorus calamus* solvent extracts (acetone and methanol) against different microorganisms.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Extracts</th>
<th>Mean zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>Acetone</td>
<td>1.0000</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>0.8000</td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td>Acetone</td>
<td>4.0000</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>6.4000</td>
</tr>
</tbody>
</table>

Independent sample t test: In Table-2, the mean zones of inhibition ranged from 0.8 to 6.4 mm. The minimum zone of inhibition of 0.8mm was shown by methanol extract against *Streptococcus mutans*. The maximum zone of inhibition of 6.4 mm was shown by methanol extract against *Lactobacillus casei*.

Table 3: Minimum inhibitory concentration of *Acorus calamus* solvent (acetone and methanol) extracts against *Lactobacillus casei*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Microorganism</th>
<th>MIC (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td><em>L. casei</em></td>
<td>6.25</td>
</tr>
<tr>
<td>Methanol</td>
<td><em>L. casei</em></td>
<td>6.25</td>
</tr>
</tbody>
</table>

Table-3 shows that both the acetone and methanol extracts of *Acorus calamus* shows moderate inhibition against *Lactobacillus casei* with static effect at a dilution of 6.25 mg/ml.

Table 4: Minimum bactericidal concentration (MBC) of solvent (acetone and methanol) extracts against *Lactobacillus casei*
Table 4 shows that both the acetone and methanol extracts of *Acorus calamus* shows bactericidal activity against *Lactobacillus casei* at the same concentration of 12.50mg/ml.

4. DISCUSSION

Dental caries is still a public health problem in developed as well as developing countries. Advanced dental caries involves the pulp, which is more painful and warrants extensive and expensive treatment.

The aim of the study was to evaluate the anti-microbial activity of acetone and methanol extracts of *Acorus calamus* rhizome against the *Streptococcus mutans* and *Lactobacillus casei*.

The rhizome of *Acorus calamus* was chosen for the study as it is being used traditionally right from birth, and there are many published studies on its anti-microbial activity, also it is easily available and is economical[11,12,13,14,15]. The antibacterial activity of *Acorus calamus* is due to the specific and non-specific interactions resulting in protein denaturation and enzymes inactivation of the bacteria[16].

The reason for choosing *Streptococcus mutans* is it is the initiator of dental caries. *Lactobacillus casei* was chosen as it is involved in progression of the caries process. [17,18,19]. The other secondary invaders are *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and *Lactobacillus acidophilus*.

Efficiency of the extraction is strongly affected by the solvents used. The extraction solvents affect the extraction yield and the quantity of bioactive compounds in the extract.

Acetone was chosen as one of the solvents for extraction because; it has the property of wide solvency of chemicals. It is one of the least hazardous solvent. Acetone can be easily evaporated. It can be procured easily and is cost-effective.

Methanol was chosen as one of the solvents in the study because; it can extract both hydrophilic and lipophilic compounds. Methanol can effectively extract both polar and non-polar compounds. As methanol has a low boiling point of 65 degree Celsius, it can be easily evaporated during extraction procedure. Moreover, it is cost effective and relatively free of regulation compared to ethanol[16].

The findings of the study showed, the acetone and methanol extracts of *Acorus calamus* to possess anti-microbial activity against *Lactobacillus casei*.

The findings from the agar well diffusion assay showed the acetone and methanol solvent extracts of the rhizome of *Acorus calamus* to possess no antibacterial activity against *Streptococcus mutans* and to possess anti-microbial activity against *Lactobacillus casei*.

The findings also showed the methanol extracts of *Acorus calamus* to possess better anti-microbial effect than the acetone extracts. Methanol as a solvent can extract the maximum concentration of phytochemicals, phenols and flavonoids from the plant materials than acetone can extract[16, 20].

On interpreting the minimum inhibitory concentration (MIC) and the minimum bactericidal (MBC) values, it was observed that the rhizome of *Acorus calamus* to exhibit bacteriostatic activity and bactericidal activity against *Lactobacillus casei*.
In the present study, the acetone and methanol extracts of the rhizome of *Acorus calamus* showed no anti-bacterial activity against *Streptococcus mutans*. Whereas, in the anti-microbial studies using ethanolic extracts with MIC value of \( \leq 3.125 \) \( \mu \text{g/ml} \) \(^{(2)}\), petroleum ether and Dimethyl Formamide (DMF) extracts with mean zone of inhibition of 10mm \(^{(2)}\) and petroleum ether extracts with zone of inhibition of 10 mm \(^{(2)}\) of *Acorus calamus* rhizome showed anti-bacterial activity against *Streptococcus mutans* \(^{(2,22,23)}\).

From the above mentioned studies, the rhizome of *Acorus calamus* is proved to be effective against *Streptococcus mutans*. However, the solvents used for extraction were ethanol, petroleum ether and Dimethyl formamide (DMF). But, in the present study, methanol and acetone were used as solvents and the extracts showed no anti-bacterial effect against *Streptococcus mutans*. The reason can be that ethanol, petroleum ether and Dimethyl Formamide (DMF) extract the biologically active substances from *Acorus calamus* rhizome better than acetone and methanol. Thus, we can find the solvents to be vital in the anti-bacterial activity of *Acorus calamus* rhizome against *Streptococcus mutans* \(^{(22,24)}\).

The present study using acetone and methanol extracts of *Acorus calamus* rhizome against *Lactobacillus casei* is one amongst the very few studies done. Therefore, valid comparisons could not be established.

The study did not use various other extraction solvents. Also, quantification and extraction of the different active compounds of the rhizome of *Acorus calamus* was not done.

Therefore, further research can be done by extracting the active compounds of rhizome of *Acorus calamus*, using different solvents for extraction and also increasing the extract concentration further.

5. CONCLUSION
The purpose of the study was to evaluate the anti-bacterial activity of *Acorus calamus* against *Streptococcus mutans* and *Lactobacillus casei* in acetone and methanol solvents. Results of the study showed no anti-bacterial activity against *Streptococcus mutans* and mild anti-bacterial activity against *Lactobacillus casei*. Between the extracts used, methanol extract had better inhibitory effect than acetone extract. Further studies with other extraction solvents and the quantitative evaluation of the bioactive compounds in the solvents are to be done to analyze the anti-microbial efficacy of rhizome of *Acorus calamus*.

REFERENCES


