

# Assessment of Antimicrobial Effectiveness of Neem and Clove Extract against *Streptococcus mutans* and *Candida albicans*: An *In vitro* Study

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## Abstract

**Background:** There is increasing interest to develop antimicrobial aids from alternative sources such as medicinal plants for the treatment of infectious diseases. Neem and clove are known to have antimicrobial properties. **Aim:** The study aimed at detecting the antibacterial and antifungal activity of neem and clove extract against *Streptococcus mutans* and *Candida albicans*. **Materials and Methods:** Strains of *S. mutans* and *C. albicans* and selective media for growing micro-organisms were procured. Antimicrobial activity was assessed using two methods, by determining the minimum inhibitory concentration (MIC) using the broth dilution method and determining the zone of inhibition using well diffusion method on mitis salivarius bacitracin selective for *S. mutans* and Saboraud's dextrose agar plates for *C. albicans*. One way ANOVA with *post hoc* analysis was done to compare the antimicrobial activity of extracts and 0.2% chlorhexidine. **Results:** MIC of neem extract was found to be 4.2 mg/ml and 5.0 mg/ml against *S. mutans* and *C. albicans*, respectively. While for cloves, it was 5.5 mg/ml for both. Neem had the highest antibacterial activity with a mean zone of inhibition of 11.4 mm followed by chlorhexidine and cloves whereas antifungal activity was highest for chlorhexidine (14.4 mm) followed by neem and clove. **Conclusion:** The result of the study established that both plant extracts possess antimicrobial activity against common microbes present in the oral cavity.

**Keywords:** Clove, minimum inhibitory concentration, neem, zone of inhibition

## INTRODUCTION

At present, there is a renewed interest in traditional or the "green medicine" that is safe and more dependable than the costly synthetic drugs, many of which have adverse side-effects.<sup>1</sup>

Increasing disease incidence and economic considerations for alternative treatment and prevention options for safe, effective, and economical way of control of diseases in developing countries.<sup>2</sup> In addition, because prolonged use of antibiotics creates microbial resistance and exposes those immunocompromised to a variety of opportunistic infections. New drug molecules are therefore urgently needed.

Neem tree is also known as *Azadirachta indica* belongs to botanic family Meliaceae and is commonly referred to as "Village Dispensary."<sup>3</sup> Different parts of neem have

been used for their various pharmacological activities such as antioxidant, antimutagenic, anti-inflammatory, anticarcinogenic, antidiabetic properties.<sup>4</sup> Neem is known to inhibit the bacterial adhesion to saliva-conditioned hydroxyapatite, a composite of bone and enamel. Neem extract also inhibited insoluble glucan synthesis, thereby reducing the adherence of streptococci to tooth surfaces. Azadirachtin, Terpenoid chief constituent of neem, is mainly responsible for the antibacterial properties of neem.<sup>5</sup> Antibacterial activity of

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neem extract of varying concentration has been assessed using disc diffusion method and measuring zone of inhibition which displayed variable results in the previously reported literature.

Cloves (*Syzygium aromaticum*) are dried aromatic unopened floral buds, belonging to the family *Myrtaceae*. Clove is a natural antibiotic with broad antibacterial, antifungal, antiviral and antimycosal activity.<sup>6</sup> It has been used by dentists as a dressing in dentistry for minor wounds, and an analgesic in painful and infective diseases of the oral cavity. It has also been used as an analgesic, antispasmodic, and as a general antiseptic in medical and dental practices.<sup>7</sup>

Screening of these medicinal plants for bioactive compounds may lead to the development of less expensive new antimicrobial agents with improved safety and efficacy. These two herbs are widely available in rural and urban areas of India and are accepted conventionally by the majority of people.

As dental caries in the adult are associated with *Streptococcus mutans* and *Candida albicans* have a role in early childhood caries, the present research was conducted with an intention to investigate antibacterial and antifungal properties of neem and clove. The aim of this study was to determine the antibacterial and antifungal activity of neem and cloves against the *S. mutans* and *C. albicans* and to compare it with 0.2% chlorhexidine.

## MATERIALS AND METHODS

The present study was conducted for a period of 3 months (March 2018 to May 2018) in the Department of Public Health Dentistry after obtaining ethical clearance from the institutional ethical committee of the institution.

The leaves of neem and buds of clove were identified and collected from a botanical garden, washed and dried in sunlight and then powdered to prepare a fine powder of 500 g. An aqueous extract was prepared by heating dried powder in 1000 ml of distilled water. Then extract was filtered through Whitman filter paper no 4 and centrifuged at 15,000 rpm for 15 min. Successive concentration, filtration, and extraction of this filtrate powders were done with the help of the Soxhlet apparatus. The concentrated extract was stored at 4°C in airtight bottles for further use.

### Procuring microbes and revival of microorganisms

Strains of *S. mutans* (ATCC 25175) and *C. albicans* were obtained from the laboratory in Mumbai and cultured fresh on Selective media for the purpose of this study. Vial-containing *S. mutans* was broken, and powder-containing lyophilized bacteria was added to the flask containing autoclaved enriched nutrient broth in the Laminar Air Flow chamber. The flask was then kept in the incubator for 48 h at 37°C. After 48 h, it was checked for turbidity on the surface indicative of the revival and growth of bacteria. Similar procedure was carried for the revival of *C. albicans* with simple nutrient broth.

### Identification of bacteria

The bacterial and fungal isolates were suspended in peptone broth and incubated at 37°C for 3–4 h were used as inocula.

One loop full colony of *S. mutans* was picked up using inoculating loop and streaked over the surface of cooled mitis salivarius bacitracin (MSB) plates and then plates were placed in the anaerobic gas jar using anaerobic gas packs, for 24 h. Similarly, colonies of *C. albicans* were picked up using sterile gauze sticks and spread over the slants of Saboraud's Dextrose agar and incubated aerobically for 48 h in an incubator. They were then confirmed on the basis of morphological and colonial characteristics.

### Determination of antimicrobial activity

Antimicrobial and antifungal activity of extracts against *S. mutans* and *C. albicans* was determined using minimum inhibitory concentration (MIC) and zone of inhibition created. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.<sup>6</sup>

MIC was determined using the broth dilution method. Serial dilution of the extract was done in a concentration ranging from 70% to 10%. One loop full colony of *S. mutans* was picked up using inoculating loop from the prepared culture and inoculated in all the test tubes prepared for serial dilution. Suspensions of pure colonies in broth at 10<sup>6</sup> CFU/ml from growth on the plates were made using McFarland's turbidity standards. All the tubes were then vortexed for 10 s in a sterilized chamber. They were then placed in a test tube rack and allowed to stand in incubator at 37°C for 48 h and checked for turbidity at the surface. The test tube showing minimum turbidity was recorded as MIC of neem and cloves for *S. mutans* and *C. albicans*. This particular concentration of the extract was preserved and used to compare the zone of inhibition with chlorhexidine.

### Zone of inhibition

Well diffusion method was employed to determine the zone of inhibition against bacteria and fungus. A total of 5 plates each of MSB and SDA were prepared to assess the mean zone of inhibition of extracts carrying MIC of neem and clove comparing it with 0.2% chlorhexidine. A sterile cotton swab was inserted into the bacterial and fungal suspension, rotated, and then compressed against the wall of the test tube to express any excess fluid. The swab was then streak on the surface of the agar plate twice or thrice to ensure a uniform, confluent growth.

The agar plates were allowed to dry, and three wells (6 mm diameter) were punched out on each plate with a sterile borer in the inoculated agar. A micropipette was used in the wells to pour 100 µl of neem and clove extract. Chlorhexidine 0.2% was used as a positive control and poured in third well. Colonies were then allowed to grow anaerobically for *S. mutans* and aerobically for *C. albicans*. The diameter of the zone of inhibition is measured, and the antimicrobial activity of the extract was reported accordingly.

### Statistical analysis

The job of data entry, validity checks, and formation of desired results (as per the analysis plan) was done using the IBM SPSS

version 22.0 (IBM Corporation, Statistical Package for the Social Sciences. N.Y., USA). Mean and standard deviation were compared by using one-way ANOVA with *post hoc* Tukey's analysis for the antimicrobial activity of extracts and control chlorhexidine.  $P < 0.05$  was considered statistically significant.

## RESULTS

MIC of neem was more for *Candida* (5 mg/ml) as compared to *S. mutans* (4.2 mg/ml). MIC of the clove was equal against both the test organisms (5.5 mg/ml).

Neem had the highest antibacterial activity against *S. mutans* with a mean zone of inhibition of  $(11.4 \pm 4.03 \text{ mm})$  followed by chlorhexidine  $(9.2 \pm 1.095 \text{ mm})$  and Cloves  $(3.8 \pm 3.633 \text{ mm})$  and the difference was found statistically significant ( $P = 0.008$ ) [Table 1]. *Post hoc* test revealed that the antibacterial activity of the chlorhexidine and neem extract was comparable, but a significant difference was found between the antibacterial activity of clove and neem as well as clove and chlorhexidine [Table 2].

The significant difference in antifungal activity against *Candida* was found among the three groups ( $P = 0.004$ ). It was the highest for Chlorhexidine with a mean zone of inhibition of  $(14.4 \pm 5.55 \text{ mm})$  followed by neem  $(5.8 \pm 4.26 \text{ mm})$  and Clove  $(3.88 \pm 2.28 \text{ mm})$  [Table 3]. *Post hoc* test revealed that there was a significant difference between the antifungal activity of clove and chlorhexidine and neem and chlorhexidine and no significant difference found ( $P = 0.78$ ) between clove and neem [Table 4].

## DISCUSSION

The age of herbal therapy is returning, and there is herbal "renaissance" worldwide. They are not only effective in the treatment for infectious diseases but also mitigate many of the side effects that are often associated with synthetic antimicrobials.<sup>8</sup>

The study evaluates the effectiveness of neem and clove extract of plant origin in the inhibition of the growth of *S. mutans* and *C. albicans*, which plays a vital role in tooth decay.

In the present study, neem and clove exhibited antibacterial and antifungal activity against *S. mutans* and *C. albicans*. The MIC of neem and clove was comparable against *Candida*, i.e., 5 mg/ml for both the groups. Aqueous extract of neem exhibited the highest mean zone of inhibition of 11.4 mm against *S. mutans* as compared to chlorhexidine which had a mean zone of inhibition of 9.2 mm and it was only 3.8 mm for clove. Chlorhexidine displayed a better antifungal activity with a mean zone of inhibition of 14.4 mm as compared to neem with 5.8 mm and clove with 3.8 mm.

In the present study, neem and clove exhibited antibacterial and antifungal activity against *S. mutans* and *C. albicans*. The MIC of neem and clove was comparable against *Candida*,

**Table 1: Comparison of mean zone of inhibition (in mm) of neem, clove, and chlorhexidine against *Streptococcus mutans***

Extracts	Number of plates	Mean zone of inhibition	SD	F	P
CHX	5	9.2	1.095	7.47	0.008*
Neem	5	11.4	4.037		
Clove	5	3.8	3.633		

\*Significant. CHX – Chlorhexidine; SD – Standard deviation

**Table 2: *Post hoc* Tukey test comparing anti-bacterial activity against *Streptococcus mutans* between three groups**

Reference group	Comparing group	Mean difference	P
CHX	Neem	-2.20	0.89
	Clove	5.40	0.02*
Neem	CHX	2.20	0.89
	Clove	7.60	0.02*
Clove	CHX	-5.40	0.02*
	Neem	-7.60	0.02*

\*Significant. CHX – Chlorhexidine

**Table 3: Comparison of mean zone of inhibition (in mm) of neem, clove, and chlorhexidine against *Candida albicans***

Extracts	Number of plates	Mean zone of inhibition	SD	F	Significance
CHX	5	14.4	5.550	8.78	0.004*
Neem	5	5.8	4.266		
Clove	5	3.8	2.280		

\*Significant. CHX – Chlorhexidine; SD – Standard deviation

**Table 4: *Post hoc* Tukey test comparing anti-fungal activity against *Candida albicans* between three groups**

Reference group	Comparing group	Mean difference	P
CHX	Neem	8.60	0.02*
	Clove	10.60	0.01*
Neem	CHX	-8.60	0.02*
	Clove	2.00	0.74
Clove	CHX	-10.60	0.01*
	Neem	-2.00	0.74

\*Significant. CHX – Chlorhexidine

i.e., 5 mg/ml for both the groups. Aqueous extract of neem exhibited the highest mean zone of inhibition of 11.4 mm against *S. mutans* as compared to chlorhexidine which had a mean zone of inhibition of 9.2 mm and it was only 3.8 mm for clove. Chlorhexidine displayed a better antifungal activity with a mean zone of inhibition of 14.4 mm as compared to neem with 5.8 mm and clove with 3.8 mm.

This study measured both zone of inhibition and MIC similar to the studies of Sedighinia *et al.* and Shafiee *et al.*<sup>9,10</sup> In a study done by Rai and Joshi<sup>11</sup> the antimicrobial activity of clove and clove bud oil were investigated by agar well diffusion

method against five dental caries causing microorganisms which reported MIC of clove and clove oil to be 12.5 and 3.5 mg/ml respectively which is lesser than present study. The results of the present study found the zone of inhibition for Clove against *S. mutans* lesser than the result of Sweta and Geetha who found clove extract was more effective against *S. mutans* with a higher zone of inhibition. Kaempferol and myricetin present in clove are supposed to have significant growth inhibitory effects against periodontal pathogens.<sup>12</sup> These inter-study variation might be methods used to prepare the extracts or higher inherent property of killing *S. mutans* ideally. The same method to prepare the herbal extracts of clove or clove bud oil, as well as neem, should be used for *in vitro* tests. Furthermore, same concentrations should be used for testing the comparison of effectiveness.

In the studies done by Dalirsani *et al.*,<sup>13</sup> Sedighinia *et al.*<sup>9</sup> and Shafiee *et al.*,<sup>10</sup> chlorhexidine mouth rinse had a comparatively greater zone of inhibition than herbal mouth rinse which is different from our study which shows neem had a higher zone of inhibition. Agarwal *et al.*,<sup>14</sup> Hegde and Vasavi<sup>15</sup> also found the lesser zone of inhibition for the herbal group than our study which was compared against the Chlorhexidine group. A higher mean zone of inhibition was found in the study of Sajankumar *et al.*,<sup>16</sup> for neem extract (15.6 mm) as compared to 0.2% chlorhexidine, whereas our study found 11.4 mm mean zone of inhibition against *S. mutans*. The antimicrobial activity of neem may be attributed to bioactive compounds such as nimbidin, nimbolide, mahmoodin, margolone, margolonone, and isomargolonone.

Nimbidin, a principle component of neem, is responsible for its antibacterial and anti-inflammatory action. Other bioactive compounds such as nimbolide, mahmoodin, margolone, margolonone, and isomargolonone also play a certain role in Neem's antimicrobial and antifungal properties.<sup>17</sup> Polyphenolic tannins present in the extract, effectively bind to the surface-associated bacterial proteins, resulting in bacterial aggregation and loss of glucosyltransferase activity. This bacterial aggregate effectively reduces the count of *S. mutans*.<sup>18</sup>

Hegde and Kesaria also found that Neem extract had significant effectiveness against *C. albicans* similar to this study.<sup>19</sup> Chlorhexidine is a cationic agent that exhibits broad-spectrum antimicrobial activity. It kills bacteria by disrupting the cell membrane.<sup>20</sup>

In the present investigation, chlorhexidine proved to be a better antifungal than antibacterial. Although it has been used to prevent dental caries for several decades, it is associated with some side effects such as staining of teeth and addiction. Thus, there is no perfect antimicrobial agent to prevent dental caries until now.

In dentistry, *A. indica* has been investigated, due to its antimicrobial potential against oral microorganisms. Furthermore, it also has an anti-adherence activity by altering bacterial adhesion and the ability of the organism to

colonize. The use of neem as an endodontic irrigant might be advantageous because it is a biocompatible anti-oxidant and thus not likely to cause severe injuries to patients that might occur via NaOCl accidents.

When used in appropriate concentrations, herbal drugs do not interrupt or alter the natural flora. Therefore, care must be taken in selecting herbal antimicrobials, with consideration of the effect of herbs in oral tissues, the mechanism of action, and side effects. Herbal medicine forms a comprehensive system, which is both promotive and preventive in its approach. Apart from healing and reducing the microbial count in the oral cavity, they also help in strengthening the overall immunity.

## CONCLUSION

Neem and clove have shown strong antifungal and antibacterial activity. The antibacterial and antifungal properties of neem were comparable to chlorhexidine, and the antimicrobial properties of the clove were lower. The microbial inhibitory potential of neem and clove extract observed in this study opens the door for antimicrobial mouth washing.

Preclinical and clinical trials are needed to evaluate biocompatibility and safety before neem can conclusively be recommended as an antimicrobial mouthwash.

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## Conflicts of interest

There are no conflicts of interest.

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