



## Effect of neem and betel leaf against oral bacteria

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### ABSTRACT

The antimicrobial effect of neem (*Azadirachta indica*) and betel (*Piper betel*) leaf on oral bacteria were examined. The saliva samples were collected from 50 patients suffering from dental caries and gingivitis. The bacteria present in the samples were isolated and identified by morphological and biochemical characteristics. The isolated bacteria were subjected to antimicrobial assay by disc diffusion method against different concentrations of aqueous and methanol extract. Both aqueous and methanol extract showed inhibitory effect against the tested bacteria even at lower concentration. Methanol extract exhibited larger zones of inhibition against all the isolated organisms compared to aqueous extract. Betel leaves extracts showed more activity compared to neem leaves extracts. In conclusion it can be said that both neem and betel leaves have potent antimicrobial effect on oral bacteria that lead to infection in mouth or normally present in mouth. Thus these plant materials can be used for treatment of dental caries and gingivitis after detailed pharmacological investigation which will be safe and economically viable.

Key words: neem, betel leaves, oral bacteria

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### INTRODUCTION

Pathogens presence in mouth is responsible for oral and dental infections including periodontal diseases, gingivitis, pericoronitis, endodontitis, peri-implantitis, and postextraction infections. Because of the increased bacterial resistance to antibiotics, toxic and harmful effects of few common antibacterial agents, there is a continuous need for alternative therapies which are affordable, not toxic and effective, such as Botanicals (Palombo 2009; Rishton 2008).

*Azadirachta indica* commonly known as neem is an evergreen tree, cultivated in several parts of Bangladesh, India and Pakistan. Every part of the tree is used as traditional medicine for household remedy against various human ailments, from ancient period (Chopra et al., 1958; Kirtikar et al., 1975; Thakur et al., 1981; Koul et al., 1990;

Chatterjee and Pakrashi 1994). The antibacterial activity of neem has been evaluated in past (Chaurasia and Jain, 1978; Chawla et al., 1994). Neem has been considered to have various activities including treatment of pyorrhea and other dental diseases. Leaves of the neem have been used in the treatment of gingivitis and periodontitis (Husain et al., 1992). Neem has also showed better efficacy in the treatment of oral infections and plaque growth inhibition in treating periodontal disorders (Patel and Venkatakrisna, 1988). Combination of neem and mango chewing sticks may provide the maximum benefit to mankind to prevent dental caries (Prashanth et al., 2007). Neem had showed good in-vitro broad range antibacterial activity (Rao et al., 1986) and found effective against *Enterococcus faecalis* and *Candida albicans* etc. Its antioxidant and antimicrobial properties makes it a potential agent

for root canal irrigation as an alternative to sodium hypochlorite (Bohora et al., 2010).

*Piper betle* L. Piperaceae) leaf is widely used as a mouth freshener after meal. Betel leaf has been described from ancient times as an aromatic, stimulo-carminative, astringent and aphrodisiac (Chu, 2001; Sudrik et al., 2012). The leaves are credited with wound healing property (Rahman 2009). Betel leaf is traditionally known to be useful for the treatment of various diseases like bad breath, boils and abscesses, conjunctivitis, constipation, headache, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, swelling of gum, rheumatism, cuts and injuries (Agarwal et al., 2012). The fresh betel leaves possess antimicrobial, ringworm, antifungal, antiseptic and antihelminthic effects (Sarkar et al., 2000). The leaf has a significant antimicrobial activity against broad spectrum of micro-organisms (Jesonbabu et al., 2012) including *Streptococcus pyrogen*, *Staphylococcus aureus*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeruginosa* etc., beside this the leaf extract also poses the bactericidal activity against the urinary tract pathogenic bacteria such as *Enterococcus faecalis*, *Citrobacter koseri*, *Citrobacter freundii*, *Klebsiella pneumoniae* etc (Chakraborty and Shah, 2011; Agarwal and Singh, 2012).

This study was conducted to observe the antibacterial activity of the aqueous and methanol extract of neem and betel leaves against bacteria present in saliva of patients suffering from dental caries and gingivitis.

## MATERIALS AND METHODS

### Collection of plant materials and processing

Neem leaves were collected from local neem tree and betel leaves (*Piper Betle*) were collected from local market. The leaves were washed with clean tap water, shade dried and ground into powder using a blender. The resulting powder of each was then stored at room temperature in a clean, air-tight container.

### Preparation of extracts

Aqueous extracts of both neem and betel leaves were prepared by boiling small pieces of the fresh leaves of the plants in distilled water for several hours until the final volume was one third of the initial volume. For methanol extraction equal volume of leaves powdered and methanol were mixed well in sterile conical flask and kept in shaker for 24 hours. Both the extracted materials were filtered separately by using Whatman no.1 filter paper and then concentrated by evaporation at 60°C. The concentrated extracts were weighed into sterile micro-centrifuge vials and prepared into stocks of 20 mg/ml using distilled water.

### Collection of clinical samples

The saliva samples from the patients suffering from dental caries, gingivitis were collected using sterile cotton tipped swabs placed in the floor of the mouth. It was then placed in a sterile container with saline (2 ml) and was used to inoculate on the agar plates.

### Isolation and identification of bacteria from samples

The saliva sample was plated on the plate count agar for the enumeration of total count of bacteria. The sample was immediately inoculated on to blood agar using streaking method and incubated at 37°C for 24 hours. The colonies that developed were identified using microscopy and biochemical tests (Barrow and Feltham, 1993).

### Morphological characterization of the isolates

From the colonies that developed on blood agar and nutrient agar a smear was made on a clean glass slide using sterile wire loop. It was dried and heat fixed. The smear was flooded with crystal violet solution for 60 seconds. This was then tipped off with Lugol's iodine for 60 seconds and washed. The smear was then decolorized with 70% ethanol and washed. This was counter stained with safranin solution for 1 minute followed by rinsing with distilled water. This was then allowed to air dry before viewing under the microscope using oil immersion objective (x100).

### Biochemical characterization of the isolates

Relevant biochemical tests were carried out to aid in the identification of the bacteria down to species level using standard procedures, described by Barrow and Feltham (1993).

### Anti-microbial assay

The antibacterial activity of the leaf extracts was tested using disc diffusion method (Bauer et al., 1966). Samples were taken from the multiple colonies and examined microscopically for identification of the isolated bacteria. The identified bacteria from respective colonies were grown overnight on nutrient broth at 37 °C and diluted with sterile saline to achieve an approximate density of  $1 \times 10^8$  colony forming units (cfu) per ml. The isolated organisms were then subjected to sensitivity screening against different concentrations of plant extracts.

The paper disc diffusion method was employed. Samples of each aqueous and methanol extracts (20 mg) were dissolved in respective solvents (1 ml). Sterile 5 mm diameter filter paper discs were impregnated with these extracts of different concentrations ranging from 100 µg to 1000 µg per disc. Adequate amount of Muller Hinton Agar were dispensed into sterile plates and allowed to solidify under aseptic conditions. The test organisms isolated from multiple colonies (0.1ml) were inoculated with a sterile spreader on the

surface of solid medium in plates. The agar plates inoculated with these test samples were incubated for one hour before placing the extract impregnated paper discs on the plates. Following this, the sterile discs impregnated with different extracts were placed on agar plates. The bacterial plates were incubated at 37°C for 48 hours. After incubation all the plates were observed for zones of inhibition and the diameters of these zones were measured in millimeters. All tests were performed under sterile conditions in triplicates. Chlorhexidine (1%) was used as positive control and distilled water as negative control.

### RESULTS AND DISCUSSION

The morphological and biochemical examination of saliva samples from 50 patients (age 12-55 years) provided evidence for presence of *Streptococcus mutans*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Lactobacillus* sp. (table 1). The antimicrobial activity of neem and betel leaves against these isolated bacteria is presented in table 2 and 3. Both aqueous and methanol extract showed inhibitory effect against the bacteria in antimicrobial assay. Methanol extract exhibited larger zones of inhibition compared to aqueous extract. Betel leaves extracts showed more activity against all organisms compared to neem leaves extract. The zones of inhibition were comparatively smaller in neem leaves treatment than that of betel leaves.

Table 1  
Morphological and biochemical characteristics of bacteria present in saliva

|                 | <i>Streptococcus mutans</i>           | <i>Enterococcus faecalis</i> | <i>Pseudomonas aeruginosa</i> | <i>Lactobacillus</i> sp. |
|-----------------|---------------------------------------|------------------------------|-------------------------------|--------------------------|
| Gram's staining | Gram positive cocci in pairs or chain | Gram positive cocci          | Gram negative rods            | Gram positive rods       |
| Catalase        | -                                     | -                            | -                             | -                        |
| Bile esculin    | -                                     | +                            | -                             | +                        |
| Citrate         | -                                     | -                            | +                             | -                        |
| Motility        | -                                     | -                            | +                             | -                        |
| Sorbitol        | +                                     | -                            | -                             | -                        |
| Mannitol        | +                                     | +                            | +                             | +                        |
| Oxidase         | -                                     | -                            | +                             | -                        |

It is interesting to note that even at the lowest concentration of 100 µg, both the plant extracts

showed antimicrobial activity. Neem showed highest zone of inhibition (25.6 mm) against

*Lactobacillus* sp. followed by *Streptococcus mutans* (25 mm) and *Pseudomonas aeruginosa* (24.3) at 1000 µg concentration of methanol extract and lowest was found against *Enterococcus faecalis* (14.4 mm) (table 2). On the other hand methanol extract of betel leaf was found most effective (27 mm) against *Pseudomonas aeruginosa* followed by *Lactobacillus* sp (26.3 mm) and *Streptococcus mutans* (26 mm) at 1000 µg concentration, whereas low inhibitory effect (14 mm) was found against *Enterococcus faecalis* at this higher concentration. Chlorhexidine as a positive control at 1% concentration found 25 mm diameter inhibition zone against *Streptococcus mutans* whereas 2 mm diameter of zone was found at 00 µg concentration as negative control.

Literature showed that neem extract has antimicrobial activity against *Enterococcus faecalis* and *Streptococcus mutans* (Dhanya et al., 2011; Bohora et al., 2010). Strong antimicrobial

activity of neem has been found against *Streptococcus mutans* with inhibition zone of 18 mm at 500 µg concentrations (Lekshmi et al., 2012). In our study neem leaf extract showed 12.1 mm and 13.8 mm inhibition at 500 µg concentrations of aqueous and methanol extract, respectively. The maximum antimicrobial activity was observed on *Streptococcus mutans* at 1000 µg concentration with inhibition zone of 23.3 mm and 25 mm in aqueous and methanol extract, respectively. However, at lower concentrations, it was less effective on *Enterococcus faecalis*. This result is in agreement with the study by Dhanya Kumar et al. (2011). Neem-based mouth rinse is equally efficacious with fewer side effects as compared to chlorhexidine and may be used as an adjunct therapy in treating plaque induced gingivitis (Chatterjee et al., 2011). Neem leaves have been used in the treatment of gingivitis and periodontitis. The antibacterial effect of neem mouthwash against salivary levels of *Streptococcus mutans* and *Lactobacillus* has been

Table 2  
Effect of neem on microorganisms isolated from mouth

| Organism                      | Average zone inhibition (mm) (n=3) |     |        |      |        |      |        |      |        |      |         |      |
|-------------------------------|------------------------------------|-----|--------|------|--------|------|--------|------|--------|------|---------|------|
|                               | 00 µg                              |     | 100 µg |      | 250 µg |      | 500 µg |      | 750 µg |      | 1000 µg |      |
|                               | A                                  | M   | A      | M    | A      | M    | A      | M    | A      | M    | A       | M    |
| <i>Streptococcus mutans</i>   | 2                                  | 3.2 | 8.2    | 10   | 10.5   | 11.7 | 12.1   | 13.8 | 18     | 21.3 | 23.3    | 25   |
| <i>Enterococcus faecalis</i>  | 2                                  | 2   | 7.5    | 8.6  | 9.2    | 9.8  | 11.3   | 12   | 13     | 13.3 | 13.8    | 14.4 |
| <i>Pseudomonas aeruginosa</i> | 1                                  | 1   | 9      | 9.7  | 11.3   | 13.2 | 15     | 17   | 17.3   | 19.2 | 22      | 24.3 |
| <i>Lactobacillus</i> sp.      | 2                                  | 2   | 10     | 10.6 | 13.3   | 15   | 17.4   | 19.1 | 22.2   | 23.5 | 24      | 25.6 |

A-aqueous extract, M- methanol extract

Table 3  
Effect of betel leaf on microorganisms isolated from mouth

| Organism                      | Average zone inhibition (mm) (n=3) |   |        |      |        |      |        |      |        |      |         |      |
|-------------------------------|------------------------------------|---|--------|------|--------|------|--------|------|--------|------|---------|------|
|                               | 00 µg                              |   | 100 µg |      | 250 µg |      | 500 µg |      | 750 µg |      | 1000 µg |      |
|                               | A                                  | M | A      | M    | A      | M    | A      | M    | A      | M    | A       | M    |
| <i>Streptococcus mutans</i>   | 2                                  | 2 | 9.3    | 10.5 | 10.9   | 11.3 | 14.7   | 17   | 18     | 20   | 22      | 26   |
| <i>Enterococcus faecalis</i>  | 2                                  | 2 | 7      | 8    | 9.1    | 9.6  | 11     | 12   | 13     | 13.7 | 13.5    | 14   |
| <i>Pseudomonas aeruginosa</i> | 1                                  | 2 | 10.2   | 12   | 13     | 13.5 | 15.8   | 17.3 | 18.5   | 20.2 | 24      | 27   |
| <i>Lactobacillus</i> sp.      | 2                                  | 2 | 11.3   | 12.5 | 14.1   | 16.3 | 18.6   | 19.9 | 21     | 21.5 | 25.2    | 26.3 |

A-aqueous extract, M- methanol extract

experienced over a period of 2 months (Vanka et al., 2001). Bacterial count was found to be reduced significantly in the groups treated with the neem extract gel compared to the chlorhexidine gluconate mouth wash (Pai et al., 2004). However, the promising antibacterial effect of neem leaf extract in this study suggests that after proper pharmacological investigation of neem ingredients it can be used for treatment of dental infection and gingivitis.

Both aqueous and methanol extract of betel leaves showed inhibition against all isolates even at lower concentration of 100 µg. Potent inhibitions were observed by methanol extract at highest concentration of 1000 µg against all isolated except *Enterococcus faecalis* that were more to the inhibition shown by the Chlorhexidine (1%).

In a study by Khan and Kumar (2011) methanol extract of betel leaf showed maximum zone of inhibition of 17.5 mm against *Pseudomonas aeruginosa* (500 mg/ml) whereas 17.3 mm zone of inhibition was found at 500 µg concentration in this study. Betel leaf has strong effect on gram negative bacteria compared to gram positive as found by Sivasankaridevi et al. (2013). Methanol extract of *Piper betel* var. Bangladeshi showed the maximum zone of inhibition 26 mm against *Pseudomonas aeruginosa* (Agarwal et al., 2012) which is comparable with our study where the maximum inhibition was 27 mm in methanol extract. The betel leaf has a significant antimicrobial activity against broad spectrum of micro-organisms (Jesonbabu et al., 2012) including *Pseudomonas aeruginosa*, *Enterococcus faecalis*, etc. (Agarwal and Singh, 2012; Chakraborty and Shah, 2011) that support this study.

Betel leaf is a second most popular daily consummation item in Asia, which contribute the best oral hygiene to oral cavity (Bissa et al., 2007). The promising antibacterial effects of betel leaves in this study suggest that use of this popular item as a mouth freshener can be a very good substitute for the available drugs after proper pharmacological investigation. Thus present study has an important impact in order to create an

effective and inexpensive oral health intervention for low socio-economic communities.

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