

Research Article

Antibacterial Effects of Chewing Stick Extracts on Streptococcus Mutans

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Abstract

Background: Streptococcus mutans is considered as a major microorganism causing tooth decay, affecting individuals globally. Different types of chewing sticks possess anti cariogenic properties, traditionally been consumed in maintaining dental hygiene. Usage of these sticks can be economical in developing and rural regions where dental caries is a major health concern.

Objective: The current study aims to evaluate antibacterial action of different type of chewing sticks extract on Streptococcus mutans.

Methods: Aqueous extracts of Azadiracta indica (Neem), Melia azedarach (Bakain), Mangifera indica (Mango), Salvadora persica (Peelu), Terminalia chebula (Harhar), Dalbeigia sissoo (Tali) and Juglans regia (Akhrot) plants which have justified folk use were prepared in Punjab University College of Pharmacy, Punjab University. Next, in vitro antimicrobial activity was studied by broth dilution method in Post Graduate Medical Institute, Lahore. Moreover, minimum inhibitory concentration (MIC) as well as the minimum bactericidal concentration (MBC) were quantitatively evaluated on basis of turbidity index.

Results: There is a significant effect of aqueous extracts of plants on the bacterial inhibition. MIC and MBC values were in the range of 6.125 to 100 mg/mL against S. mutans. The notable effect occurred with aqueous extracts of Azadiracta indica showing MIC and MBC values as 6.25mg/mL and 12.5 mg/mL, respectively. Alternatively, Salvadora persica demonstrated 8.33mg/mL and 16.66 mg/mL values of these parameters

Conclusions: Each plant studied exhibited moderate to high antibacterial activity against tested bacterial strain. However, Azadirachta indica and Salvadora persica aqueous extracts showed promising effect against Streptococcus mutans

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Introduction

Dental caries is among the prevalent dental infection caused by acid producing microorganism leading

to the tooth decalcification.^{1,2} Global burden of disease (GBD) report that 60 to 90% of school going children and almost all elderly experience tooth decay.^{1,2,3} Streptococcus mutans, are the main cause of dental caries.⁴ In order to decrease the prevalence of caries, antiplaque or antimicrobial agents has been advocated to dental health care products. One such practice by rural people from old age is use of natural plants to maintain oral



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hygiene.^{2,5} In different regions of the World, different plant sticks serve as natural dentifrice with antibacterial, anti-plaque and anti-fungal properties.^{4,6} World Health Organization (WHO) suggested the practice of chewing stick in 1986 as well as 2000 and concluded that additional work is needed to assess its effectiveness against dental microorganism.^{2,6} In line with the above, different parts of plants have been tested for antimicrobial activity globally.^{6,7} A study conducted in Pakistan in 2014 concluded that Miswak taken from the root of the peelu tree exhibited antimicrobial activity against all the common oral pathogens.² Nevertheless, some knowledge gaps do exist. The current study aims to evaluate antibacterial action of different type of chewing sticks extract on *Streptococcus mutans*.

Variety of in vitro methods have been used to evaluate the antibacterial effect, of chewing sticks including the agar diffusion, Petri dish bioassay, and the use of micro titer plates.^{7,8,9}

Similarly in a study, *Streptococcus mutans* gave an inhibitory zone of 17.33 mm at a concentration of 400µg/ml to alcoholic extract of *Salvadora persica*.⁵

Similarly in a study, 400 mg/mL of aqueous and methanolic extracts of *Salvadora persica* was the most effective on all strains using the agar dilution and minimum inhibitory concentration methods. The methanol extract exhibited a stronger antibacterial activity against Gram-negative (3.3–13.6 mm) than Gram-positive (1.8–8.3 mm) bacteria.⁷ Keeping the above into consideration and lack of evaluation by broth dilution method in previous studies, we aimed to evaluate the antimicrobial potential of natural herbs used by rural people in Pakistan like *Azadiracta indica* (Neem), *Melia azedarach* (Bakain), *Mangifera indica* (Mango), *Salvadora persica* (Peelu), *Terminalia chebula* (Harhar), *Dalbergia sissoo* (Tali) and *Juglans regia* (Akhrot) by broth dilution method so that safe and economical technique can be discovered to limit caries in emerging nations.

Methods

This was cross-sectional study, which was conducted in the Department of Microbiology, Post Graduate Medical Institute and Punjab University College of Pharmacy, Punjab University through non-probability convenient sampling technique.

Standard reference strain of *Streptococcus mutans* ATCC 25175 American Type Culture Collection, USA was used in this study.

The plants used were *Mangifera indica*, *Azadiracta indica*, *Melia azedarach*, *Salvadora persica*, *Terminalia chebula*, *Acacia nilotica*, *Dalbergia sissoo*, *Juglans regia* and were grouped as under:

1. Group 1: Mango (*Mangifera indica*), Group 2: Harhar (*Terminalia chebula*), Group 3: Kikar (*Acacia nilotica*), Group 4: Neem (*Azadiracta indica*), Group 5: Peelu (*Salvadora persica*), Group 6: Bakain (*Melia azedarach*), Group 7: Shisham (*Dalbergia sissoo*)

Chewing sticks were collected from Jinnah Bagh (Lawrence Garden), Lahore and Lalsuhanra Park, Bahawalpur.

The fresh chewing sticks of medicinal plant with appropriate diameter 0.5- 1cm and length 15-20cm were collected from Jinnah bagh (Lawrence garden), Lahore. Peelu (*Salvadora persica*) was collected from Lalsuhanra, Bahawalpur,

Dried chewing sticks of un appropriate diameter and length were excluded from study

Permission was taken from management of Bagh e Jinnah , Lahore and Lalsuhanara Park for sample collection and authenticated by botanist, Government College, Lahore. Sample of the collected plants were deposited as a specimen (voucher No: GC. Herb Bot 1160) in the herbarium, Botany Department, Government College, Lahore, Pakistan. Ethical permission for the study was obtained from Post graduate Medical Institute, Lahore.

Samples were dried and ground into coarse powder. Extracts of the powdered plant material were prepared by maceration method in Punjab University College of Pharmacy, Punjab University.⁵ For this purpose; 100 grams of powdered plant material were added in one liter distilled water, pH 7.0 in a container. Mixture was filtered under UV light lamp through Whatmans filter paper after 24 hours. This process was repeated three times to obtain concentrated extract. The extracts were concentrated in vacuum at 35°C, inoculated a sterile nutrient agar slant with the filtered extract to check the sterility of the extracts and frozen it at -80°C⁹. The frozen extracts were dried at -44°C under

vacuum 0.2 mbar over 3 days using a freeze drier (Christ Lyophilizer). The extracts were kept in screw glass capped bottles and stored in refrigerator till use. Stock solutions of the plant extract were prepared by suspending 600mg of extract in 3ml of distilled water to get 200mg/ml concentration. These stock solutions were stored at -20 °C.⁸

Liofilchem stick containing freeze-dried form of strain (*Streptococcus mutans*) was obtained. Blood agar and Nutrient agar were prepared in Microbiology Laboratory (PGMI). The stick was opened and sub cultured on blood agar and nutrient agar. For 24 hours, at 37 °C plates remained incubated; afterwards bacterial growth obtained was checked by gram staining and catalase reaction.

Using aseptic technique, at least four to five well-isolated colonies were selected from the nutrient agar plate. The top of each colony was touched with a loop, and inoculated into 5 ml of Brain Heart Infusion Broth. After incubation at 35°C, turbidity was checked against the 0.5 McFarland turbidity standard. Turbidity was adjusted to 0.5 McFarland standards by either diluting aseptically with Brain Heart Infusion Broth or further incubation. The inoculums prepared contained approximately $1-2 \times 10^8$ CFU/ml.

Tube dilution technique was performed to evaluate In vitro antimicrobial susceptibility testing.⁹ We added 1.0 ml of the culture suspension to all broth dilution tubes. In the first tube, 2ml of plant extract solution was added. From tube number 2-9, 1ml of sterile brain heart infusion was added 1.0 ml of the extract in tube 1 was shifted to the 2nd tube from 1st tube. Process was continued till tube number 8. From 8th tube 1.0 ml was thrown away. Last tube having 1ml of sterile broth served as a control. 1 ml of the culture suspension was added to all of tubes. Subsequent concentration of plant extract was one-half of original concentration in each tube. In this way dilutions of each plant extract were made i.e. 200 mg/ml, 100 mg/ml, 50 mg/ml, 25, 12.5, 6.25, 3.125, and 1.56 mg/ml. Tubes were incubated at 35 °C for 16-20 hours. To prevent drying out, the tubes were sealed with tight fitting screw caps.⁹

Three tubes were included in each batch as control: one tube having distilled water and *Streptococcus mutans*; second tube having brain heart infusion (add in material)

broth and third tube with brain heart infusion broth and *Streptococcus mutans*. The MIC was read as the lowest concentration of the extract that completely inhibited the growth (no turbidity). MIC broth tube without visible growth was used to check the MBC. From the broth dilution tubes without visible growth, standardized loop containing approximately 10^8 CFU/ml of organism were sub-cultured onto appropriately labeled blood agar plates. The plates were then incubated at 37°C for 24 hour. Following overnight incubation, MBC plates were evaluated for the presence or absence of colony growth.⁹ MIC and MBC were repeated thrice to confirm result reliability.

All the tests were performed in triplicate and data were presented as mean deviation. The collected information was analysed by SPSS using one way analysis of variance and compared using Turkey's honestly significant difference (HSD) at 5% level of significance. To meet the normality assumption of the ANOVA, the data were transformed by square root transformation.

Results

The physical properties of aqueous extracts of seven plants, being used as chewing sticks, are given in Table 1. All extracts were blackish and brown colour. *Magnifera indica* shows highest yield extract. All the extracts exhibited neutral to acidic pH. *Azadiracta indica* and *Juglans regia* extracts were almost neutral (Table 1).

The antibacterial activity of different plant extract in terms of MIC and MBC are given in Figure 1 and 2, respectively. The results revealed a significant difference among the different extracts tested ($F=3.81$; $df=8,18$; $p<0.01$). *Azadirachta indica* and *Salvadora persica* proved to be the most effective extracts in terms of minimum inhibitory concentrations, (Figure 2).

The MBC was determined by subculturing the test dilution (used in MIC) on to a fresh solid medium and incubated further for 24 hours. The results revealed a significant difference among the different extracts tested ($F=3.45$; $df=8,18$; $p<0.05$). *Azadirachta indica* and *Salvadora persica* proved to be the most effective extracts in terms of minimum bactericidal concentrations, Moreover, MBC value of different plant extract is almost two fold higher than corresponding MICs.

There is a significant effect of aqueous extracts of plants on the bacterial inhibition. MIC and MBC values are in the range of 6.125 to 100 mg/mL against *S. mutans*. The notable effect occurred with aqueous extracts of *Azadiracta indica* showing MIC and MBC values as 6.25mg/mL and 12.5 mg/mL, respectively. Alternatively, *Salvadora persica* demonstrated 8.33mg/mL and 16.66 mg/mL values (Figure 1 and 2).

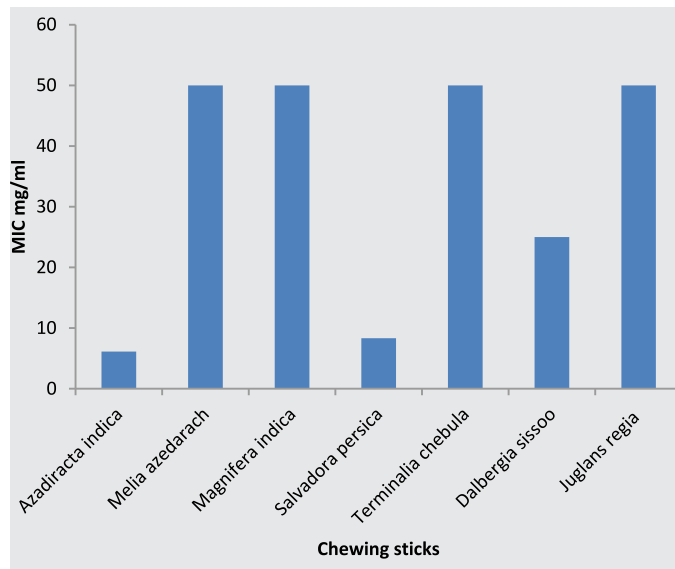


Figure 1: Minimum Inhibitory Concentration of different Chewing Sticks Extracts

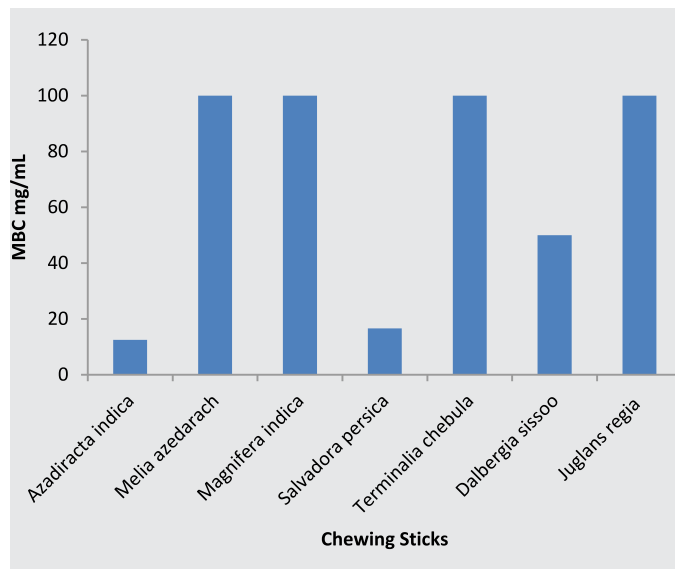


Figure 2: Minimum bactericidal concentration of different chewing sticks extracts

Table 1: Physical property of extracts of plants used as chewing sticks

Name	Color	Yield	pH
Azadiracta indica	Yellowish brown	14.23	6.91
Melia azedarach	Orange brown	11.74	5.56
Magnifera indica	Brownish black	17.47	4.96
Salvadora persica	Reddish brown	13.3	5.1
Terminalia chebula	Brown	10.8	4.81
Dalbergia sissoo	Brown	7.27	5.94
Juglans regia	Brown	15.61	6.35

Discussion

The hypothesis of the present study was proved by the results that commonly used miswak in Pakistan possess antimicrobial activity against *Streptococcus mutans* as their potential anti-plaque effect likely complement mechanical plaque-removing property of chewing sticks. Presence of antibacterial activity in chewing stick extract is consistent with general antibacterial activity reported in the past.^{2,10,11,12,13}

A study was conducted in 2018, and showed very good antibacterial activity of *Mangifera indica* and *Azadiracta indica* against *Streptococcus mutans*. *Mangifera indica* and *Azadiracta indica* had the highest antibacterial activity at concentrations of 0.3 mg/ml and 6.25 mg/ml, respectively. Whereas in the present study *Azadiracta indica* showed good antibacterial activity as compared to *Mangifera Indica*. The difference in the results may be because all plant parts like bark, stem, root, fruit and leave have various active ingredients that don't have reliable, precise and constant constituents.¹⁴

In the present study, the MIC of *Mangifera Indica* aqueous extract was found to be 50mg/ml and MBC was calculated 100mg/ml respectively. Similar results were found in study conducted by Ravi et al¹¹ in 2017 in which they studied antibacterial effects of mango and eucalyptus twigs extracts, Pudina and garlic extracts against *Streptococcus mutans*. They concluded that *Mangifera indica* twig extracts exhibit highest antimicrobial potential against *Streptococcus mutans* than eucalyptus twigs at a lower concentration. Extracts of garlic and Pudina didn't demonstrate substantial antimicrobial effect at same concentrations. In this study, each plant studied exhibited moderate to high antibacterial activity against *Streptococcus mutans* using the macro tube dilution method. MIC and MBC values were in the range of

6.125 to 100 mg/mL quantitatively assessed on the basis of turbidity index and sub culturing test dilution tubes.

In 2019, a study was conducted to find antimicrobial activity of herbal medicines on *Streptococcus Mutans*. They concluded that Neem and Aleo vera gel have antimicrobial effect at different concentrations.¹⁵ Pulbutr P et al. also evaluated MIC and MBC of *Derris reticulata* ethanolic stem extract by broth dilution method against *Streptococcus mutans* and found it to be effective at low concentrations.¹⁶ A study is published in 2023, and also concludes that the *A. indica* leaf extract is a potential source to inhibit the *S. mutans* biofilm.¹⁷ Results of studies are consistent with present study.

In 2019 Ali Jahanban-Esfahlan and his co workers¹⁸ found that antimicrobial activity of walnut husk aqueous extracts against gram positive and gram negative bacteria including *Candida albicans* and *Staphylococcus aureus*. Result exhibited that growth of gram positive microorganism was sensitive by wall nut extracts, and *S. Aureus* showed maximum sensitivity. We have also found antibacterial activity of aqueous extract of *Juglans regia*.

Azadirachta indica and *Salvadora persica* aqueous extracts showed promising effect against *Streptococcus mutans*. The maximum antibacterial activity occurred with aqueous extracts of *Azadirachta indica* as compared to other chewing sticks selected. *Salvadora persica* demonstrated MIC and MBC 8.33mg/mL and 16.66 mg/mL values respectively against *Streptococcus Mutans*. In an study conducted in Pakistan, antibacterial effects of Kikar, Neem and Peelu was observed.² It was concluded that anti cariogenic effect of *Salvadora persica* is as beneficial as any antibacterial medicine using agar diffusion method. In 2017 Hanan Balto also examined *S. persica* ethanol and hexane extract against *S. mutans*, *S. Sanguis*, and *S. Salivario*. It was concluded that the methanolic extract of *S Persica* at concentration 200 mg/ml showed broad spectrum activity by Agar well diffusion method.¹⁹ The difference in result could be due to the developing environment like harvesting time, weather inconsistency, desiccation and extraction methods, there could be other conditions like, strain type, storage conditions like light, moisture and temperature.

The present study showed that MIC of aqueous extract of pelu was 8.33 mg/ml. In 2019, Khalil et al. proved

Ethanolic extract of *S Persica* is more effective against all bacteria in comparison to hexane extract. It is more effective on growth inhibition in 25, 50 and 100gm/ml concentration. The minimum inhibitory concentration required for killing of *Streptococcus Sanguis* and *S. Mutans* was 8 mg/ml of ethanol which is in accordance to the current study. Recently Khalil et al. in 2019²⁰

The antibacterial properties of chewing sticks of different plants belonging from different geographical regions have been described (Saleh et al., 2006; Vahabi et al., 2011). Though, they have never been established for samples originating from Pakistan, where extreme climate and weather conditions might lead to dissimilar properties. This study may be the first report regarding in vitro antimicrobial evaluation and contrast of distilled water extracts of different miswaks against *Streptococcus mutans* indigenous to (Lahore, Punjab) Pakistan using broth dilution method.

Overall, this report gives a basis for further in vivo studies and tends to reinforce the use of these extracts as antimicrobial agent in folk medicine. The two plants should be evaluated further in depth to isolate the active components and to clarify their mode of action, hence, their actual effects on denture plaque. However, additional tests, including experimental models and pharmacological applicability, are required before considering these plant extracts as alternative methods in the treatment of oral diseases or for their use in daily oral-care products, gel, rinses, or effervescent tablet preparations, especially for elderly denture wearers.

In vitro susceptibility of chewing sticks extracts was evaluated in present study. In vivo interaction period of the extract with the bacteria in oral cavity is not clear.

Additional work should be conducted including additional microbes: examining the superiority and effectiveness of chewing sticks by in vitro or in vivo tests and associating the incidence of caries amongst chewing stick users and nonusers to elucidate the picture.

Conclusion

Outcomes advocate that chewing sticks can be an effective method for prevention of caries. The aqueous extracts of *Azadirachta indica* and *Salvadora persica* proved to be the most effective in terms of minimum inhibitory and minimum bactericidal concentrations

against *S. mutans*. The aqueous extract of *Azadirachta indica* showed strongest bactericidal activities with MIC and MBC values of 6.125 mg/mL and 12.5 mg/mL, respectively.

Ethical Approval: Given

Conflict of Interest: The authors declare no conflict of interest.

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