Original Research Article

Comparison of effects of Azadricha Indica mouth rinse on Streptococcus Mutans and *P. gingivilis*

Abstract:

Background: Leaves of *Azadirachta indica have been studied vastly to observe their pharmacological properties. The leaves of the* Neem tree and the constituents present in the leaves have reported antibacterial, antihyperglycaemic, immunomodulatory, antiulcer, antimalarial, antifungal, antiviral, antimutagenic, and anticarcinogenic properties. Conventional mouthwashes, containing chlorhexidine, used for their antimicrobial effect against bacteria in the oral cavity have certain adverse effects including alteration in taste, tartar formation and staining of teeth, restoration, and sometimes tongue. Hence the study was aimed to analyze the antimicrobial activity of *Azadirachta indica mouth rinse against Streptococcus mutans* and *P. gingivilis colonies.*

Methodology: It was an in-vivo preclinical experimental study conducted at Baqai Medical and Dental College Karachi., from February 2022 to March 2022. The calculated sample size was n =60. The participants were given an envelope for group randomization. The organisms were grown on appropriate media and extract was prepared and diluted. The extract was diluted in distilled water at 1:4 (Extract: Distilled water) concentration. Study participants were instructed to not brush their teeth before sampling. Study participants were divided into three groups (negative control, positive control, Neem tree extract rinse group) each group had 20 participants. Diluted neem tree extract was given to the experimental group for rinses, distilled water was given to the negative control group and positive controls were given a standard commercially available mouth rinse. The next sample of plaque was collected after two hours to observe the effects of neem tree leaves extract on bacterial colonies.

Results: The growth of colonies was calculated on growth media plates in samples collected before rinsing and samples that were taken after the rinsing. The pre and post samples showed a significant (p-value <0.05) decrease in the number of colonies in the positive control group (conventional rinse) and neem leaf extract group. The intragroup comparison of negative and positive control showed a significant difference in the number of colonies and the same was observed with the neem leaf extract rinse. However, the positive control and the Neem leaves extract comparison was insignificant.

Conclusion: the *Azadirachta indica* leaf extract has better or equal efficacy against oral microflora when compared with the standard chlorhexidine containing conventional mouth wash.

Keywords: *Azadirachta indica,* antibacterial activity, oral microflora, *Streptococcus Mutans, P. gingivilis*

Introduction:

Azadirachta indica is a very common plant in the Asian region including Pakistan, India, China, and other dry regions of the continent (1). *Azadirachta indica* belongs to the Meliaceae family and locally, in Pakistan and India, it is known as the Neem tree (2). Due to its numerous medicinal and health-promoting properties, the Neem tree is also labeled as a "village pharmacy" and “divine traee” in Myanmar and the Indian region (3). Various parts such as seeds, leaves, flowers, and the bark of the Neem tree are being in use as a general folk medicine for years worldwide (4). Neem tree is used to treat multiple diseases in the Unani system of medicine and also in Chinese and European homeopathic (5). Such long-term use of this tree signifies the availability and therapeutic effects of the phytoconstituents present in this plant.

Leaves of *Azadirachta indica have been studied vastly to observe their pharmacological properties. The leaves of the* Neem tree and the constituents present in the leaves have reported antibacterial, antihyperglycaemic, immunomodulatory, antiulcer, antimalarial, antifungal, antiviral, antimutagenic, and anticarcinogenic properties (1). Flowers of *Azadirachta indica are used in aromatherapy and have reported medicinal effects on anorexia, nausea, and intestinal worms (1). Oil extracted from Neem seeds and fruits is used in facial cosmetics such as soap, face wash, and also in hair oil and shampoos (6). Azadirachta indica* seeds are used to treat diseases such as leprosy, diabetes by reducing sugar levels, killing intestinal worms, for birth control, and its oil is used as a facial oil to treat acne, pimples, and other skin diseases. The chemical constituents present in the seeds of the Neem tree such as oleic, palmitic and linoleic acid helps to the glowing and healthy skin (7). In ancient times, Neem twigs were commonly chewed to keep the gums and teeth healthy. While in recent studies, Neem twigs have reportedly ameliorated hyperglycemia, endothelial dysfunction, and systemic inflammation (8). Alongside all these pharmaceutical properties, *Azadirachta indica has reported antibacterial effects against multiple bacteria including* Staphylococcus aureus, Staphylococcus epidermidis, Klebsiella pneumoniae, and Salmonella typhi *(9). Limonoids extracted from the seeds of the Neem tree have reported their antibacterial activity against S. epidermidis, P. aeruginosa*, and *E. coli* (10).

Conventional mouthwashes, containing chlorhexidine, used for their antimicrobial effect against bacteria in the oral cavity have certain adverse effects including alteration in taste, tartar formation and staining of teeth, restoration, and sometimes tongue also (11). Bad oral hygiene and improper brushing habits may lead to one of the most common and initially asymptomatic diseases, dental caries, which can further lead to gingivitis and periodontitis (12). The bacteria most frequently associated with dental caries, gingivitis and periodontitis are *P. gingivilis*, *Streptococcus mutans*, *Lactobacillus*, and *Actinomycetes (13).* In our study, we are analyzing the antimicrobial activity of *Azadirachta indica mouth rinse against Streptococcus mutans colonies.*

Methodology:

It was an in-vivo preclinical experimental study conducted at Baqai Medical and Dental College Karachi., from February 2022 to March 2022. The calculated sample size was n =60. A consecutive sampling technique was used to recruit the participants. The participants were given an envelope for group randomization. Film of plaque from the labial surface of teeth of study

participants was collected on sterile strips that were transported to the laboratory for culture in sterile containers. For culture, *S. mutant* samples were inoculated in Columbia Agar with 5% sheep blood and incubated for 48 h at 37 °C and increased the level of CO_2. *P. gingivalis* were grown in wilkins-Chalgren anaerobic broth under anaerobic conditions of 5% CO2, 10% H2, and 85% N2 at 37 °C. All bacteria were sub-cultured twice and were grown to the early stationary phase. Neem tree leaves (1000-gram) were purchased from the local market of Karachi and an authentication number i.e. Specimen voucher 1081 was allotted. The leaves were washed and shed dried and lastly ground to powder form. The leaves were soaked in 2500mL of 70% ethanol for 15 days with intermittent shaking. After 15 days the filtrate was filtered with Whatman filter paper (number 1) that was further processed at 60^oC by using a water bath. The mixture was then dried at 50^oC until a well-concentrated extract was produced on the rotary evaporator. The extract was kept in an airtight bottle and stored in a refrigerator till usage. The extract was diluted in distilled water at 1:4 (Extract: Distilled water) concentration. Study participants were instructed to not brush their teeth before sampling. Study participants were divided into three groups (negative control, positive control, Neem tree extract rinse group) each group had 20 participants. Diluted neem tree extract was given to the experimental group for rinses, distilled water was given to the negative control group and positive controls were given a standard commercially available mouth rinse. The next sample of plaque was collected after two hours to observe the effects of neem tree leaves extract on bacterial colonies. ANOVA followed by post hoc Tukey's test was applied to identify the inter and intragroup comparison and Paired t-test was applied as a test of significance for pre and post-experimental comparison, <0.05 p-value was considered as significant at a 95% confidence interval.

Results:

There were sixty participants in study 36 (60%) were males and 24 (40%) were females the mean age of participants was 24 ± 3.5. On asking about brushing habits 42 (70%) participants responded that they brush their teeth daily. The growth of colonies was calculated on growth media plates in samples collected before rinsing and samples that were taken after the rinsing. The pre and post samples showed a significant (p-value <0.05) decrease in the number of colonies in the positive control group (conventional rinse) and neem leaf extract group as shown in table 1. The intragroup comparison of negative and positive control showed a significant difference in the number of colonies and the same was observed with the neem leaf extract rinse. However, the positive control and the Neem leaves extract comparison was insignificant. Table 2 shows the intragroup comparison of the experiment.

Table. 1 Paired t test analysis showing the number of colonies before and after intervention

	Negative Control	**Positive control**	**Neem Leaf extract**

Streptococcus Mutans			
Before	$9 \pm 1 \times 10^4$	$10 \pm 2 \times 10^4$	$10 \pm 2 \times 10^4$
After	$8 \pm 2 \times 10^4$	$6 \pm 1 \times 10^4$	$5 \pm 1 \times 10^4$
P value	0.831	0.002*	0.001*
P. gingivalis			
Before	$17 \pm 2 \times 10^3$	$16 \pm 2 \times 10^3$	$17 \pm 3 \times 10^3$
After	$16 \pm 2 \times 10^3$	$10 \pm 2 \times 10^3$	$9 \pm 2 \times 10^3$
P value	0.538	0.041*	0.001*
*significant p-value			

Table 2. ANOVA followed by post hoc tukys`s Analysis on post interventional results

Groups wise comparison *Streptococcus Mutans*				**p- value**
Negative control	9×10^4	**Positive control**	6×10^4	0.003*
Negative control	9×10^4	**Neem leaf extract**	5×10^4	0.001*
Positive control	6×10^4	**Neem leaf extract**	6×10^4	1.000
Groups wise comparison *P. gingivalis*				
Negative control	$16 \pm 2 \times 10^3$	**Positive control**	$10 \pm 2 \times 10^3$	0.021*
Negative control	$16 \pm 2 \times 10^3$	**Neem leaf extract**	$9 \pm 2 \times 10^3$	0.001*
Positive control	$10 \pm 2 \times 10^3$	**Neem leaf extract**	$9 \pm 2 \times 10^3$	0.036*
*significant p-value				

Discussion:

Azadirachta indica or the Neem tree and other members of *Meliaceae* family have been reported vastly for their therapeutic effects against various ailments especially their antibacterial properties (14). The results of our study showed a remarkable decrease in bacterial colonies that were comparable to conventionally used synthetic mouth wash, containing chlorhexidine as a

vital antibacterial agent. We conducted our study against two of the major bacteria, involved in oral cavity diseases such as dental caries and gingivitis that may lead to periodontitis, which are *Streptococcus mutans* and *Porphyromonas gingivalis* (15). *S. mutans* is a major contributor to tooth decay as being a potent initiator of dental caries (16). The second oral microbe of our study, *P. gingivalis*, has a vital role in the pathogenesis of periodontitis which is an inflammatory disease of supporting tissues of teeth (17). This also eventually leads to tooth loss and further complications.

The participants of our study were told not to brush their teeth before sample collection. Plaque from the labial surface of the teeth was collected and bacterial growth was studied. As expected, our results did not show any significant decrease in the number of *S. mutans* and *P. gingivalis* colonies before and after intervention in the negative control group (rinses with distilled water). In the positive control group (rinses with conventional mouth wash) there was a significant decrease in the number of *S. mutans* colonies and the recorded p-value was 0.002*. Another study has reported remarkably higher antibacterial effects of chlorhexidine containing conventional mouth wash against *S. mutans* when compared with aqueous form and fluoride mouth wash (18). Various other studies have reported such antimicrobial effects of conventional mouth wash against *S. mutans* (19-21). The Neem leaves extract has also shown remarkable antimicrobial activity against *S. mutans* with a p-value as significant as 0.001* which was comparable with the activity of synthetic mouth wash. Another study compared the chlorhexidine-containing mouth wash with the *Azadirachta indica* leaves extract against *S. mutans*. Both showed a significant and comparable zone of inhibition around their respected wells, against *S. mutans* (22).

Against *P. gingivalis*, the negative control group did not show any major decrease in colonies but the positive control group has shown a noteworthy decrease in the number of colonies with a p-value of 0.04*. Another study showed significant inhibition zones by standard mouth wash against *P. gingivalis* (23). In our study, the Neem leaves extract showed significant activity against *P. gingivalis* colonies with a remarkable p-value of 0.001*. The Neem tree leaves extract showed even better antibacterial activity against *P. gingivalis* when compared with the standard mouth wash (positive group). Another study has also reported the bacteriostatic effects of the Neem oil against *actinomycetemcomitans*, *porphyromonas gingivalis* and *fusobacterium nucleatum* and the Neem oil has been suggested as an alternative for the management of alveolitis (24). Multiple other studies have also reported the antimicrobial activity of *Azadirachta indica* against *P. gingivalis* (25, 26).

Post-interventional group-wise comparison was also done between the 3 groups. A significant decrease in the number of bacterial colonies was recorded when negative and positive control groups were compared against both *S. mutans* and *P. gingivalis* showing a p-value of 0.003* and 0.021* respectively. This denotes the antibacterial potential of synthetically produced mouthwashes containing chlorhexidine. The comparison between the Neem leaf extract group and negative control group was also found significant against both *S. mutans* and *P. gingivalis*, showing a significant p-value of 0.001* for both. This represents the marked antibacterial activity of the Neem leaves against the two major oral microbes.

The comparison between positive group (mouth wash) and the Neem leaf extract group against *S. mutans* showed almost similar results and the p-value was also recorded as non-significant as 1.000. These results show the equal and comparable antibacterial activity of Neem leaf extract with the standard mouth wash. While the same comparison of the Neem leaf extract and positive control but against *P. gingivalis* showed a quite significant p-value of 0.036*. This denotes that the Neem leaf extract has comparable but better antibacterial activity against *P. gingivalis* than the standard mouth wash. Another study reported that the *Azadirachta indica* oil has better antibacterial activity against *P. gingivalis* (27). Another study showed better antibacterial activity of herbal mouthwash containing *Azadirachta indica* when compared with the standard chlorhexidine-containing mouth wash (28)(29).

Conclusion: Based on these results we can conclude that the *Azadirachta indica* leaf extract has better or equal efficacy against oral microflora when compared with the standard chlorhexidine containing conventional mouth wash. So, the Neem leaf extract can be used as an herbal mouthwash as an alternative to chlorhexidine which has different adverse effects including staining of the tongue, teeth, dentures or restorations, taste alteration, and an increase in tartar or calculus accumulation

Reference

1. Shareef M, Akhtar MSJMSM. Neem (Azadirachta indica) and its potential for safeguarding health, prevention and treatment of diseases. 2018;2(1):04-8.
2. Ahmad S, Maqbool A, Srivastava A, Gogol SJEBMH. Biological detail and therapeutic effect of azadirachta indica (neem tree) products-a review. 2019;6(22):1607-12.
3. Islas JF, Acosta E, Zuca G, Delgado-Gallegos JL, Moreno-Treviño MG, Escalante B, et al. An overview of Neem (Azadirachta indica) and its potential impact on health. 2020;74:104171.
4. Rahmani A, Almatroudi A, Alrumaihi F, Khan AJPR. Pharmacological and therapeutic potential of neem (Azadirachta indica). 2018;12(24).
5. Quraishi HA, Islam N, Iqbal A, Bhat SA, Ahmed J, Ashraf SS, et al. Therapeutical and medicinal properties of Neem (Azadirachta indica) in context of Unani system of medicine: a review study. 2018;8(6-s):394-9.
6. Moin MS, Siddiqui JI, Alam MA, Khatoon F, Khan S, Minhajuddin A. Ethnomedicinal potential of widely used plant Azadirachta indica A. Juss: A comprehensive review. 2021.
7. Kharwar RN, Sharma VK, Mishra A, Kumar J, Singh DK, Verma SK, et al. Harnessing the phytotherapeutic treasure troves of the ancient medicinal plant Azadirachta indica (Neem) and associated endophytic microorganisms. 2020;86(13/14):906-40.
8. Pingali U, Ali MA, Gundagani S, Nutalapati CJD, Metabolic Syndrome, Targets O, Therapy. Evaluation of the Effect of an Aqueous Extract of Azadirachta indica (Neem) Leaves and Twigs on Glycemic Control, Endothelial Dysfunction and Systemic Inflammation in Subjects with Type 2 Diabetes Mellitus–A Randomized, Double-Blind, Placebo-Controlled Clinical Study. 2020;13:4401.
9. Thakur B, Kumar A, Kumar DJSAJoB. Green synthesis of titanium dioxide nanoparticles using Azadirachta indica leaf extract and evaluation of their antibacterial activity. 2019;124:223-7.

10. Lu X-F, Lin P-C, Zi J-C, Fan X-NJZZyzzZzzCjoCmm. Limonoids from seeds of Azadirachta indica and their antibacterial activity. 2019;44(22):4864-73.
11. Singh O, Reddy VK, Pradhan D, Sharma LJJoIAoPHD. Comparative evaluation of green tea and chlorhexidine mouthwashes on gingivitis: A randomized controlled trial. 2019;17(4):269.
12. Hazra M, Shetty V, Suresh LRJJoH, NU AS. Salivary Antioxidant Capacity and Oral Health Status in Children with Visual Impairments—A Cross-Sectional Study. 2022.
13. Théophile O, Christian KTR, Alain KY, Pascal ADC, Reine BGS, Diane BF, et al. Natural chemical compounds from plants extract for prevention and treatment of oral infections: a review.
14. Bodiba DC, Prasad P, Srivastava A, Crampton B, Lall NSJPM. Antibacterial activity of Azadirachta indica, Pongamia pinnata, Psidium guajava, and Mangifera indica and their mechanism of action against Streptococcus mutans. 2018;14(53):76.
15. Achmad H, Handayani H, Amin A, Oktawati SJJoID, Research M. Mouthwash product development based on ethanol extract of white rice bran (Oryza sativa L.) as antibacterial of Streptococcus mutans and Porphyromonas gingivalis. 2019;12(3):985-90.
16. Lin Y, Chen J, Zhou X, Li YJCrim. Inhibition of Streptococcus mutans biofilm formation by strategies targeting the metabolism of exopolysaccharides. 2021;47(5):667-77.
17. Bunte K, Kuhn C, Walther C, Peters U, Aarabi G, Smeets R, et al. Clinical significance of ragA, ragB, and PG0982 genes in Porphyromonas gingivalis isolates from periodontitis patients. 2021;129(3):e12776.
18. Malvania EA, Sharma AS, Sheth SA, Rathod S, Chovatia NR, Kachwala MSJJCDP. In vitro analysis of licorice (glycyrrhiza glabra) root extract activity on streptococcus mutans in comparison to chlorhexidine and fluoride mouthwash. 2019;20(12):1389-94.
19. Jacob B, Nivedhitha MJJoC, Research D. Comparative Assessment of the Antibacterial Efficacy of Natural Products and Chlorhexidine Mouthwash against Streptococcus Mutans: A Systematic Review. 2018;12(12).
20. Padiyar B, Marwah N, Gupta S, Padiyar NJIJoCPD. Comparative evaluation of effects of triphala, garlic extracts, and chlorhexidine mouthwashes on salivary Streptococcus mutans counts and oral hygiene status. 2018;11(4):299.
21. Badri SM, Felemban EH, Alnajjar GK, Alotaibi FM, Aljahdali ST, Maher YA, et al. Effectiveness of probiotic lozenges and Chlorhexidine mouthwash on plaque index, salivary pH, and Streptococcus mutans count among school children in Makkah, Saudi Arabia. 2021;33(7):635-41.
22. Nivedhitha MJAJoP, Technology. Antimicrobial Efficacy of Azadirachta indica against Streptococcus mutans-An In vitro Study. 2019;9(3).
23. Vaidya Jasmine M, Ravishankar P, Visithiriyan G, Preethikaa GJAotRSfCB. An In-Vitro Evaluation of the Efficacy of the Essential Oils from Leptospermum Scoparium and Melaleuca Alternifolia as an Antimicrobial Agent against P. Gingivalis. 2021:10291-6.
24. Marie Elvire N, Hortense G, Emmanuel N, Rose N, Francine MJIJBS. Evaluation of the in vitro antibacterial activity of Azadirachta indica used for the treat-ment of alveolitis. 2020;2(2):119.
25. Gul-e-Nazish MS, Ali L, Johnson NJJoP, Phytochemistry. Effectiveness of Azadirachta indica bark extract in periodontitis induced rabbit model. 2020;9(2):15-21.
26. Fasher Bory W, Hammad M, Idris AJM, Diseases I. Evaluation of in vitro antimicrobial effects of Azadirachta indica (Neem) leaves extracts against selected pathogens. 2021.

27. Mohapatra S, Doraikannan SS, Indiran MA, Rathinavelu PKJIJoPHR, Development. Comparison of Antimicrobial Efficacy of Chlorhexidine Mouthwash, Lemongrass (Cymbopogon) Oil and Neem (Azadirachta indica) Oil Against Oral Microflora: An in Vitro Study. 2019;10(10).
28. Vinod K, Sunil K, Sethi P, Bandla RC, Singh S, Patel DJJoISoP, et al. A novel herbal formulation versus chlorhexidine mouthwash in efficacy against oral microflora. 2018;8(2):184.
29. Pandiyan I, Rathinavelu PK, Arumugham MI, Srisakthi D, Balasubramaniam AJC. Efficacy of Chitosan and Chlorhexidine Mouthwash on Dental Plaque and Gingival Inflammation: A Systematic Review. 2022;14(3).