JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2023/32242

SHORT COMMUNICATION

In vitro Evaluation of Antibiofilm Activity of Methanolic Leaf Extract of *Azadirachta indica* on Cariogenic *Streptococcus mutans*

Deepesh Dinesh[1], Khalid Imran[2*], Sivakumar Vijayaraghavalu[3,4], M. Saifulla Khan[5] and V. V. Nikesh[6]

[1]Department of Life Sciences, Nucleobase Life Sciences Research Laboratory, Bengaluru - 560043, Karnataka, India
[2]Department of Microbiology, Krupanidhi Degree College, Bengaluru - 560035, Karnataka, India; qhalidimran@gmail.com
[3]Department of Life Sciences (Zoology), Manipur University, Imphal - 795003, Manipur, India
[4]Department of Engineering Design, Indian Institute of Technology Madras, Chennai - 600036, Tamil Nadu, India
[5]Department of Pharmacognosy and Phytochemistry, Krupanidhi College of Pharmacy, Bengaluru - 560035, Karnataka, India
[6]Krupanidhi Degree College, Bengaluru - 560035, Karnataka, India

Abstract

Dental caries is a biofilm-dependent disease, and *Streptococcus mutans* is the primary etiological agent involved in the initiation of the disease. The extensive use of a limited range of antimicrobial drugs in dentistry has led to the development of drug-resistant bacteria. There is an increasing need to find new alternatives against drug-resistant bacteria. Globally, there is a continuous effort towards identifying natural anti-caries agents for the prevention and better management of caries. The objective of the present study was to evaluate the antibiofilm potential of *Azadirachta indica* leaf methanolic (ALM) extract against *S. mutans* biofilm. The study employed a standard reference strain of *S. mutans* MTCC 497, for *in vitro* standardisation of biofilm by microtiter plate assay. The antibiofilm activity of the ALM extract was evaluated against the *S. mutans* strain, and the same was confirmed by light and scanning electron microscopy (SEM). The *in vitro* biofilm standardisation results demonstrated that 50 µl/ml of *S. mutans* inoculum concentration exhibited a much superior biofilm formation than the other concentrations employed. Light microscopy and SEM images revealed that ALM extract at 100 mg/ml concentration significantly inhibited the *S. mutans* biofilm. To conclude, the study reports that the *A. indica* leaf extract is a potential source to inhibit the *S. mutans* biofilm. Further studies are warranted to identify the phytochemicals responsible for the antibiofilm activity of ALM extract against *S. mutans* biofilm that aid in the design of natural anti-caries products.

Keywords: Dental Caries, Microtiter Plate Assay, Neem, Phytochemicals, Antibacterial Compounds

1. Introduction

Dental illness, especially dental caries, a biofilm-associated disease, is unquestionably a public health issue and one of the most common diseases in the world[1]. According to the World Health Organisation, cavities affect 60 to 90 percent of school children and nearly all adults worldwide. One of the most significant risk factors for dental illnesses is the colonization of teeth by cariogenic bacteria, with *S. mutans* being the

*Author for correspondence

Article Received on: 27.12.2022 Revised on: 12.01.2023 Accepted on: 21.02.2023

main species associated with the early dental caries process[3]. Strong biofilms produced by *S. mutans* on a tooth's surface are essential for developing dental plaque and cavities[4]. Although there are many acidogenic and aciduric species in dental plaque linked to the development of dental caries, *S. mutans* is the primary producer of extracellular polysaccharides, making these biofilms challenging to manage[5]. *S. mutans* exploits com-dependent quorum sensing systems to regulate various biological functions, such as niche adaptability during host colonization, natural competence, controlling genetic transformation, pathogenicity, and biofilm formation. The *S. mutans* biofilm's structure is a barrier to the chemical anti-biofilm agents, increasing resistance to various antimicrobial elements[6].

Antibiotics are frequently used for therapeutic and preventive purposes to treat dental caries and other dental-related problems. Antimicrobial resistance has unfortunately emerged rapidly in recent years, along with the usage of antibiotics[7]. Alternative preventative measures and therapeutic approaches that are affordable and safe are greatly needed. According to the World Health Organization, 80% of people in underdeveloped nations rely on ethnomedicine for their primary medical care. Contrarily, 50% of the world's population still uses ethnomedicines derived from plant active components[8]. *A. indica* has been used extensively from prehistoric times to the present[9]. *A. indica* L. is one of many medicinal trees in the Meliaceae family that are commonly found in tropical and semi-tropical areas around the world[10]. Research in food safety, dentistry, virology, bacteriology, parasitology, and mycology is currently being conducted to better understand the wide antibacterial properties of *A. indica*[11]. The tree is well-known for its medicinal properties, which include antifertility, hypolipidemia, antidiabetic, nematicidal, insecticidal, microbicity, antiulcer, anti-inflammatory, antioxidant, antipyretic, hypoglycemic, hepatoprotective, cardioprotective, neuroprotective, and anti leishmaniasis effects[10]. There are hundreds of phytochemicals in the neem tree, and many of them have been proven to be bioactive and have a variety of uses on their own. More than 300 distinct compounds have been found in the neem tree. Some of the more prevalent phytochemicals found in the neem tree, such as azadirachtin, gedunin, and nimbolide, have previously been identified as potential medications with various biological functions[10,12,13]. Flowers, fruits, seeds, leaves, stems, roots, gum, bark, and nearly all other parts of *A. indica* have been utilised as common household treatments for human ailments. Additionally, neem twigs are used by millions of people worldwide as chewing sticks for oral hygiene[14,15]. The potency of plant-derived chemicals against pathogens that cause common and/or fatal illnesses, like *Escherichia coli*[16], *Pseudomonas aeruginosa*[16], *Staphylococcus aureus*[17], and cariogenic bacteria[18], has been thoroughly investigated.

The significant biofilm-forming bacteria, namely *S. aureus*, *Enterococcus faecalis* and *P. aeruginosa,* are commonly associated with human infections, and it is well evidenced that *A. indica* possesses antibiofilm activity against these organisms[19]. Additionally, it is also reported that *A. indica* also possesses antibiofilm properties against methicillin-resistant *S. aureus*, *Helicobacter pylori*, and *Vibrio cholera* organisms[20,21]. Based on the extensive research on various biofilm-forming organisms, *A. indica* may be a potential candidate to combat caries. To the best of our knowledge, there is a lack of studies primarily designed to determine the antibiofilm property of *A. indica* against *S. mutans*. In this context, the present study aimed to evaluate the antibiofilm potential of *A. indica* methanolic leaf extract against *S. mutans* biofilm.

2. Materials and Methods

2.1 Procurement and Revival of Culture

S. mutans freeze-dried culture (MTCC 497) was obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India. The freeze-dried culture was revived in Brain Heart Infusion (BHI) broth and later streaked on BHI agar to obtain single isolated colonies. The pure culture of *S. mutans* was maintained in 80% glycerol stock at –20°C.

2.2 Screening Test for Biofilm

The screening test for biofilm production was performed by tube adherence assay[22]. Briefly, 2 ml of BHI broth with sucrose (5%) was inoculated with an overnight culture of *S. mutans*. A tube with media alone was considered a control. The tubes were incubated at 37°C for 24 hours; after the incubation period, the broth was discarded, washed with phosphate buffer saline, and air-dried. The tubes were stained with crystal violet (0.1%). The excess stain was removed by rinsing it

with deionized water, and the tubes were air-dried by placing them upside down.

2.3 Standardisation of Biofilm Assay in a 24-Well Microtiter Plate

The biofilm standardisation assay was carried out in a flat-bottom 24-well polystyrene microtiter plate with modifications[23,24]. Briefly, *S. mutans* was inoculated in a test tube containing sterile BHI broth supplemented with sucrose (5%) and incubated at 37°C for 24 hrs. To achieve a 0.5 McFarland standard, the turbidity of the *S. mutans* bacterial suspension was modified. The bacterial inoculum, ranging from 10 to 50 µl/ml (with an interval of 10 µl/ml) was prepared in BHI broth containing 5% sucrose. 1 ml of each bacterial concentration was aseptically added to the individual wells of the microtiter plate, after placing a sterile glass piece (1 X 1 mm) in each of the wells. Inoculated media treated with sterile distilled water was employed as a negative control, while media alone was considered a blank control. The experiment was performed in triplicates. The anaerobic incubation of the microtiter plate was at 37°C for 24hrs. Following the incubation period, the broth was aseptically discarded, and glass pieces were carefully removed with sterile forceps. The glass pieces were rinsed with sterile phosphate-buffered saline and further air-dried. The glass pieces were stained with crystal violet (0.4%) for 5 minutes and were later observed under the microscope to determine the biofilm formation.

2.4 Plant Materials Collection

The leaves of *Azadirachta indica* A. Juss. were collected from the medicinal garden of the University of Agricultural Sciences, GKVK Campus, Bengaluru. The plant materials collected were free from infection. The identification and authentication of the plant material were made by the Head, Centre for Herbal Gardens, The University of Trans-Disciplinary Health Sciences and Technology, Bengaluru.

2.5 Preparation of Powder Extract

The leaves of *A. indica* were washed with sterile distilled water and shade-dried. The leaves were coarsely powdered using a mechanical grinder.

2.6 Reflux Condensation Extraction

50 g of plant material was added to a round-bottom flask containing 300 ml of methanol. Reflux condensation was carried out for two and a half hours. The ALM extract was filtered and dried through rotary evaporation.

2.7 The Antibiofilm Activity of ALM Extract

The antibiofilm property of ALM extract was evaluated in a 24-well polystyrene microtiter plate with modifications[23,24]. The inoculation of the *S. mutans* strain was prepared as described in section 2.3. Briefly, 50 µl /ml bacterial inoculum prepared in BHI broth (5% sucrose) was aseptically added to the microtiter plate after placing a sterile glass piece (1 X 1 mm) in each of the wells of the microtiter plates. The varying concentrations of plant extract with an interval of 20 mg/ml, ranging from 20 to 100 mg/ml, were employed in the study. The experiments were performed in triplicates. Inoculated media treated with chlorohexidine was considered a positive control, the media treated with methanol was a negative control, and the media alone was a blank control. The microtiter plate was anaerobically incubated at 37°C for 24hrs. After the incubation period, the broth was aseptically discarded and glass pieces were carefully removed with forceps. The glass pieces were rinsed with sterile phosphate-buffered saline and air-dried. The glass pieces were stained with 0.4% crystal violet for 5 minutes.

2.8 *In situ* Visualization of *S. mutans*

2.8.1 Light Microscopic Analysis

After staining, the glass pieces were observed under a microscope to determine the biofilm formation. A glass slide was used to mount the glass pieces with the biofilm directed upwards, and they were observed with a light microscope (100 X magnification). The visuals of microscopic images were captured by a digital camera[24].

2.8.2 Scanning Electron Microscope Analysis

Using sterile distilled water, the biofilms on 1×1 mm glass pieces were rinsed, fixed using formaldehyde, and overnight incubated at 20°C. The biofilms on the

glass pieces were dehydrated through graded ethanol treatment, followed by air drying and sputter coated with gold. The biofilms were examined at X-5000 magnification using SEM[25].

3. Results

The freeze-dried culture revived on BHI agar displayed small, pinpoint colonies. The representative image of the pure culture of *S. mutans* is presented in Figure 1.

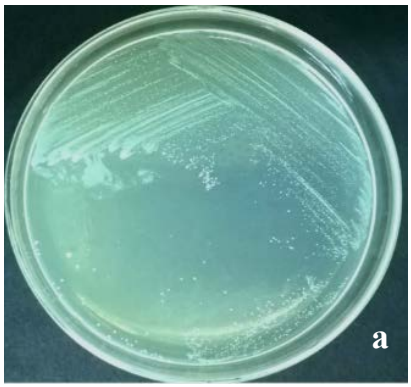

Figure 1. Pure Culture of *S. mutans* MTCC 497 on Brain Heart Infusion Agar.

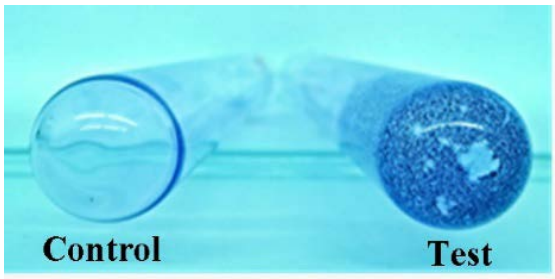

Figure 2. Biofilm screening test of *S. mutans* (MTCC 497).

The reference strain of *S. mutans* demonstrated a positive test for the primary screening test for biofilm formation. Figure 2 represents stained *S. mutans* cells adhered to the bottom of tubes, demonstrating a positive test for biofilm formation.

The results of the standardisation of the biofilm assay displayed that as the concentration of the bacterial inoculum increased, better biofilm formation was observed. Among the five inoculum concentrations, 50 µl/ml of the bacterial inoculum formed a superior biofilm when compared to 10 to 40 µl/ml concentrations. Figure 3 represents the light microscopic images of the *S. mutans* reference strain with respect to biofilm formation at different bacterial inoculum concentrations.

The ALM extract displayed antibiofilm properties against the *S. mutans* reference strain. From the light microscopy images, it is observed that as the concentration of ALM extract increased, the antibiofilm activity also increased, and at 100 µg/ml concentration, a superior antibiofilm activity against *S. mutans* was observed. Figure 4 shows the representative light microscopy images revealing the antibiofilm activity of ALM extract against *S. mutans*.

SEM analysis was employed to elucidate the antibiofilm activity of the ALM extract against *S. mutans* biofilms grown *in vitro* on glass pieces for 24 hrs at 37°C. From the SEM images, it is visualised that the ALM extract possesses a strong antibiofilm activity against *S. mutans* biofilm. ALM extract at a 100 mg/ml concentration significantly disrupted the *S. mutans* biofilm. Figures 5 (a) and (b) represent the SEM images of *S. mutans* biofilms with respect to the control and treated (100 µg/ml ALM extract) samples.

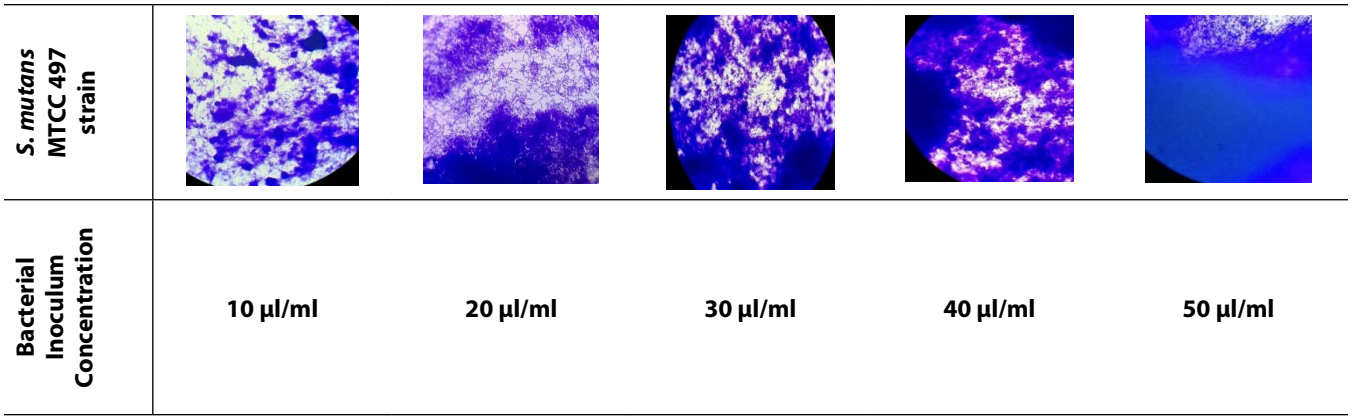

Figure 3. Light microscopy images of *in vitro* biofilm formation of *S. mutans* at different bacterial inoculum concentrations.

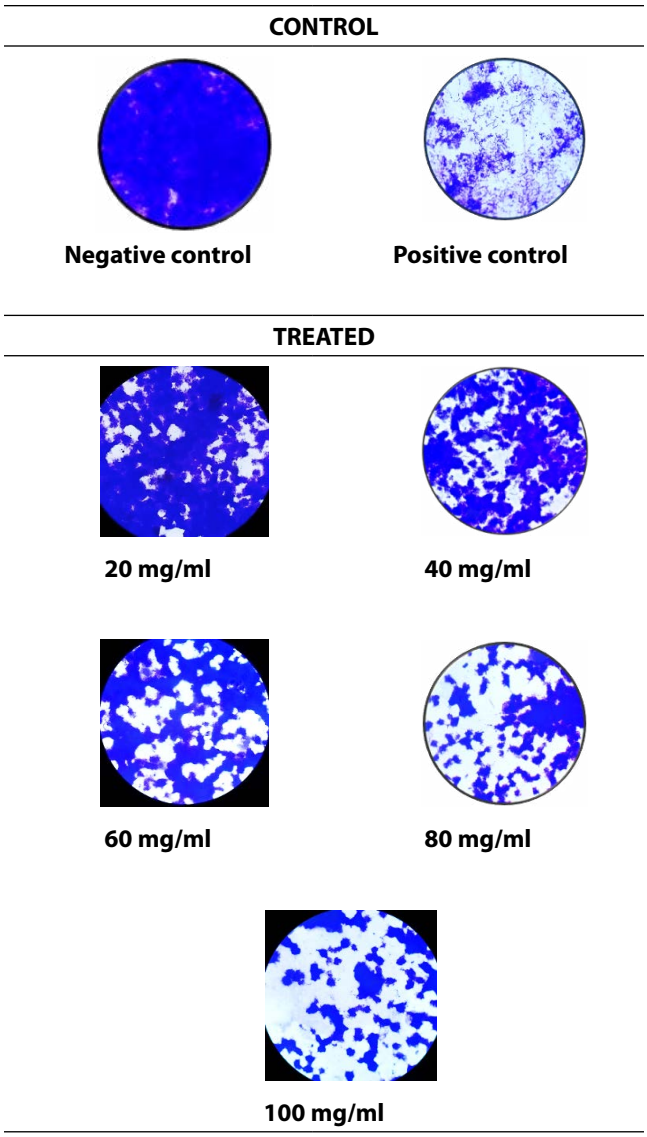

Figure 4. Representative images of light microscopy displaying antibiofilm activity of ALM extract at different concentrations.

4. Discussion and Conclusion

In the last two decades, several natural products have been identified that possess inhibition properties against *S. mutans* to name a few: cranberry, curcumin, green tea extracts, propolis, and many others[4,27-29]. Despite these extensive investigations, it is still elusive to identify a potential candidate for the prevention and management of caries.

A. indica is an omnipotent plant possessing a wide range of antimicrobial activity. Investigators have recommended to include more standardized research methodologies to test the potential of neem derived products and its individual compounds against untested organisms that include both *in vitro* and *in vivo* studies[11]. To the best of our knowledge, there is a lack of studies designed for *in vitro* evaluation of *A. indica* antibiofilm activity against *S. mutans*.

In the present study, *in vitro* standardisation of *S. mutans* biofilm formation was demonstrated at 50 µl/ml of the bacterial inoculum in a microtiter plate assay. From the results, it is observed that among the concentrations of ALM extract employed for antibiofilm activity, 100 mg/ml demonstrated the highest antibiofilm activity against *S. mutans*. SEM images confirmed the antibiofilm activity of the ALM extract. From the SEM images, it is interpreted that the ALM extract (100 mg/ml) possesses strong antibiofilm activity against *S. mutans.* By comparing the images of treated and control samples, it can be stated that the *S. mutans* biofilm is suppressed and deformed in the former, while the control sample showed a dense, complex structure with substantial matrix formation. In one of the earlier clinical studies, a dental gel incorporated with *A. indica* leaf extract (25mg/g) demonstrated a significant

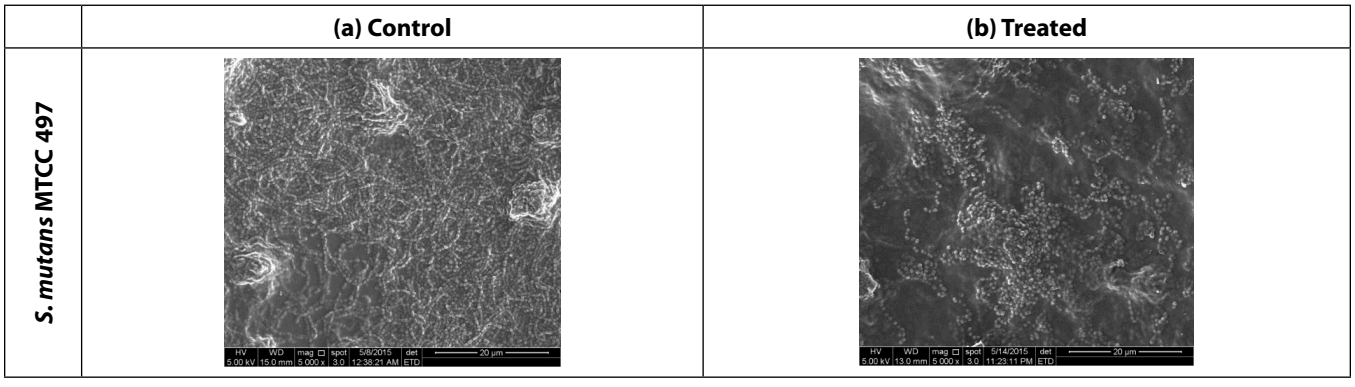

Figure 5. Scanning electron microscopy images of *S. mutans* biofilms (a) Control and (b) Treated sample (ALM extract at 100 µg/ml concentration).

reduction in plaque index and bacterial count compared with the control group. The study proposes the use of *A. indica* leaves for plaque growth inhibition[30]. Based on our findings and previous research, we can conclude that *A. indica* leaf extract is a promising agent for the development of novel anti-caries products.

The quorum sensing (QS) signalling system in *S. mutans* controls the development of biofilms by moderating the expression of virulence proteins in a way that depends on cell density. Therefore, by inhibiting this system, *S. mutans* biofilm formation is inhibited, which in turn can limit the cariogenic process. Destabilizing *S. mutans* QS system has thus been suggested as a modern approach for antibiofilm activity[06]. In this background, the study concludes by stating that further investigations are warranted to identify the bioactive compounds in *A. indica* leaf extract that can be employed as quorum-quenching agents involved in antibiofilm activity, thus preventing caries.

5. References

1. Yadav K, Prakash S. Dental Caries: A Microbiological Approach. J Clin Infect Dis Pract. 2017; 2(1):1-5. https://doi.org/10.4172/2476-213X.1000118
2. Oral Health Database. Available online: https://www.mah.se/CAPP/ (accessed on 14 April 2020).
3. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. Microbiological Reviews. 1986; 50(4):353-80. https://doi.org/10.1128/mr.50.4.353-380.1986
4. Koo H, Duarte S, Murata RM, Scott-Anne K, Gregoire S, Watson GE, Singh AP, Vorsa N. Influence of cranberry proanthocyanidins on formation of biofilms by *Streptococcus mutans* on saliva-coated apatitic surface and on dental caries development *in vivo*. Caries Research. 2010; 44(2):116-26. https://doi.org/10.1159/000296306
5. Forssten SD, Bjorklund M, Ouwehand AC. *Streptococcus mutans*, Caries and Simulation Models. Nutrients. 2010; 2:290–298. https://doi.org/10.3390/nu2030290
6. Pourhajibagher M, Alaeddini M, Etemad-Moghadam S, Rahimi Esboei B, Bahrami R, Mousavi M, Bahador A. Quorum quenching of *Streptococcus mutans* via the nano-quercetin-based antimicrobial photodynamic therapy as a potential target for cariogenic biofilm. BMC Microbiology. 2022; 22(1):1-8. https://doi.org/10.1186/s12866-022-02544-8
7. Haque M, Sartelli M, Haque SZ. Dental infection and resistance - global health consequences. Dentistry Journal. 2019; 7(1):22. https://doi.org/10.3390/dj7010022
8. Oyebode O, Kandala NB, Chilton PJ, Lilford RJ. Use of traditional medicine in middle-income countries: A WHO-SAGE study. Health Policy Plan. 2016; 31:984–991. https://doi.org/10.1093/heapol/czw022
9. Sarkar S, Singh RP, Bhattacharya G. Exploring the role of *Azadirachta indica* (neem) and its active compounds in the regulation of biological pathways: An update on molecular approach. 3 Biotech. 2021; 11(4):1-2. https://doi.org/10.1007/s13205-021-02745-4
10. Saleem S, Muhammad G, Hussain MA, Bukhari SN. A comprehensive review of phytochemical profile, bioactives for pharmaceuticals, and pharmacological attributes of *Azadirachta indica*. Phytotherapy research. 2018; 32(7):1241-72. https://doi.org/10.1002/ptr.6076
11. Wylie MR, Merrell DS. The Antimicrobial Potential of the Neem Tree *Azadirachta indica*. Frontiers in Pharmacology. 2022; 13. https://doi.org/10.3389/fphar.2022.891535
12. Braga TM, Rocha L, Chung TY, Oliveira RF, Pinho C, Oliveira AI, *et al.* Biological Activities of Gedunin-A Limonoid from the Meliaceae Family. Molecules. 2020; 25(3). https://doi.org/10.3390/molecules25030493
13. Nagini S, Nivetha R, Palrasu M, Mishra R. Nimbolide, a Neem Limonoid, is a Promising Candidate for the Anticancer Drug Arsenal. J. Med. Chem. 2021; 64(7):3560–3577. https://doi.org/10.1021/acs.jmedchem.0c02239
14. Brahmachari G. Neem - An Omnipotent Plant: A Retrospection. Chembiochem. 2004; 5(4):408–421. https://doi.org/10.1002/cbic.200300749
15. Gupta SC, Prasad S, Tyagi AK, Kunnumakkara AB, Aggarwal BB. Neem (*Azadirachta indica*): An Indian Traditional Panacea with Modern Molecular Basis. Phytomedicine. 2017; 34:14–20. https://doi.org/10.1016/j.phymed.2017.07.001
16. Reichling J. Anti-biofilm and virulence factor-reducing activities of essential oils and oil components as a possible option for bacterial infection control. Planta Medica. 2020; 86(8):520–537. https://doi.org/10.1055/a-1147-4671
17. Guzzo F, Scognamiglio M, Fiorentino A, Buommino E, D'Abrosca B. Plant derived natural products against *Pseudomonas aeruginosa* and *Staphylococcus aureus*: Antibiofilm activity and molecular mechanisms. Molecules. 2020; 25(21):5024. https://doi.org/10.3390/molecules25215024
18. Freires IA, Denny C, Benso B, de Alencar SM, Rosalen PL. Antibacterial activity of essential oils and their isolated constituents against cariogenic bacteria: A systematic review. Molecules. 2015; 20(4):7329–7358. https://doi.org/10.3390/molecules20047329
19. Vestby LK, Gronseth T, Simm R, Nesse LL. Bacterial biofilm and its role in the pathogenesis of disease. Antibiotics. 2020; 9(2):59. https://doi.org/10.3390/antibiotics9020059

20. Guchhait KC, Manna T, Barai M, Karmakar M, Nandi SK, Jana D, Dey A, Panda S, Raul P, Patra A, Bhattacharya R. Antibiofilm and anticancer activities of unripe and ripe *Azadirachta indica* (neem) seed extracts. BMC complementary medicine and therapies. 2022; 22(1):1-8. https://doi.org/10.1186/s12906-022-03513-4
21. Wylie MR, Windham IH, Blum FC, Wu H, Merrell DS. *In vitro* antibacterial activity of nimbolide against *Helicobacter pylori*. Journal of Ethnopharmacology. 2022; 285:114828. https://doi.org/10.1016/j.jep.2021.114828
22. Christensen GD, Simpson WA, Bisno AL, Beachey EH. Adherence of slime-producing strains of *Staphylococcus epidermidis* to smooth surfaces. Infection and immunity. 1982; 37(1):318-26. https://doi.org/10.1128/iai.37.1.318-326.1982
23. Hasan S, Danishuddin M, Adil M, Singh K, Verma PK, Khan AU. Efficacy of E. officinalis on the cariogenic properties of *Streptococcus mutans*: A novel and alternative approach to suppress quorum-sensing mechanism. Plos one. 2012; 7(7):e40319. https://doi.org/10.1371/journal.pone.0040319
24. Gowrishankar S, Duncun Mosioma N, Karutha Pandian S. Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and - susceptible *Staphylococcus aureus* biofilms. Evidence-Based Complementary and Alternative Medicine. 2012; 2012. https://doi.org/10.1155/2012/862374
25. Yoshida A, Kuramitsu HK. Multiple *Streptococcus mutans* genes are involved in biofilm formation. Applied and environmental microbiology. 2002; 68(12):6283-91. https://doi.org/10.1128/AEM.68.12.6283-6291.2002
26. Lee HJ, Kang SM, Jeong SH, Chung KH, Kim BI. Antibacterial photodynamic therapy with curcumin and *Curcuma xanthorrhiza* extract against *Streptococcus mutans*. Photodiagn Photodyn Ther. 2017; 20:116–119. https://doi.org/10.1016/j.pdpdt.2017.09.003
27. Xu X, Zhou XD, Wu CD. The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. Antimicrob Agents Chemother. 2011; 55:1229–1236. https://doi.org/10.1128/AAC.01016-10
28. Santiago KB, Piana GM, Conti BJ, Cardoso EO, Murbach Teles Andrade BF, Zanutto MR, Mores Rall VL, Fernandes A Jr, Sforcin JM. Microbiological control and antibacterial action of a propoliscontaining mouthwash and control of dental plaque in humans. Nat Prod Res. 2017; 32:1441–1445. https://doi.org/10.1080/14786419.2017.1344664
29. Lemos JA, Palmer SR, Zeng L, Wen ZT, Kajfasz JK, Freires IA, Abranches J, Brady LJ. The biology of *Streptococcus mutans*. Microbiology Spectrum. 2019; 7(1):7-1. https://doi.org/10.1128/microbiolspec.GPP3-0051-2018
30. Pai MR, Acharya LD, Udupa N. Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel - a 6-week clinical study. Journal of ethnopharmacology. 2004; 90(1):99-103. https://doi.org/10.1016/j.jep.2003.09.035