Journal of Experimental Biology and Agricultural Sciences, June - 2024; Volume – 12(3-BIONEXT-2023) page 511 – 521

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Investigating the antimicrobial activity of neem and clove extract on biofilm-producing oral microflora

Tanushree Bhattacharya[1], Rudra Prasad Saha[2], Rajib Majumder[2], Sanmitra Ghosh[1*]

[1]Department of Biological Science, School of Life Science & Biotechnology, Adamas University, West Bengal
[2]Department of Biotechnology, School of Life Science & Biotechnology, Adamas University, West Bengal

Received – December 31, 2023; Revision – March 15, 2024; Accepted – June 15, 2024
Available Online – July 15, 2024

DOI: http://dx.doi.org/10.18006/2024.12(3).511.521

KEYWORDS

Antifungal effect

Clove

Neem

Biofilm

Oral microbiota

Periodontal diseases

ABSTRACT

Periodontal disease, a serious gum infection, is reported to be widespread in the Indian population. A heterogeneous microbial population, predominantly consisting of gram-negative anaerobes such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, and *Porphyromonas intermedia*, is associated with periodontal plaque formation. This condition may be worsened by the invasion of soft tissues by different species of *Candida*. Natural products like clove (*Syzygium aromaticum*) and Neem (*Azadirachta indica*) are very popular and easily available in the Indian climate and have great potential in preventing periodontitis. *Azadirachta indica* (Neem) exhibits versatile modes of action, including reported antimicrobial effects against several species associated with *periodontal* disease. Therefore, this study aims to detect the antimicrobial and antifungal effects of Neem and clove on oral biofilm both before and after biofilm formation. Results of the study revealed that both neem and clove crude extracts and their different dilution showed a significant reduction in the growth of fungal strains (*Candida sp.*) isolated from oral samples from people with poor hygiene and the biofilm produced by them.

* Corresponding author

E-mail: sanmitra.ghosh@adamasuniversity.ac.in (Sanmitra Ghosh)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

The oral microbiota, consisting of hundreds to thousands of unique species of bacteria, viruses, and fungi, plays a significant role in the human microbiome (Arweiler and Netuschil 2016; D'Ambrosio et al. 2023). The various habitats in the oral cavity provide a complex environment for microorganisms on the teeth' hard surfaces and the oral mucosa's soft tissues. It is the second most extensive and diverse microbiota after the gastrointestinal tract (Arweiler and Netuschil 2016; Deo and Deshmukh 2019; Kitamoto et al. 2020). The oral microbiome is crucial in preventing the colonization of harmful bacteria, which could lead to oral disorders such as periodontitis, gingivitis, and caries (John et al. 2017). Additionally, many microorganisms can form biofilms resilient to mechanical stress or antibiotic treatment. Some commensal species may exhibit virulence in response to changes in the oral cavity's environment, behavioral aspects, or an individual's hygiene (Rajhans et al. 2011). There is a growing interest in understanding the role of the oral microbiome in health and disease, and recent advances in metagenomic technologies are being employed to characterize its diversity (Rajhans et al., 2011).

Periodontitis is a complex disease caused by an infection that affects the tissues supporting the teeth, known as the periodontium (Gasner and Schure 2024). It is not a single disease but rather a combination of diseases characterized by the loss and destruction of alveolar bone, deterioration of gums, periodontal ligament, jawbone, and the surfaces of the teeth covered by the gums (Könönen et al. 2019; Van Dyke et al. 2020). Periodontal disease is widespread in the Indian population, affecting over 1 billion people. It is more prevalent in urban areas (22.7%) than in rural areas, and it is also more common in males (42.2%) than in females (34.4%) (Janakiram et al. 2020; Antimicrobial Resistance Collaborators 2024).

The clinical diagnosis of the disease and its categorization involves identifying signs and symptoms in periodontal tissues that help medical professionals identify illnesses based on their origin, pathophysiology, and therapy. In addition to causing tooth loss, this disease also leads to systemic inflammation (Van Dyke et al. 2020). Severe periodontitis is rare in young individuals, while chronic periodontitis primarily affects adults (Zhu et al. 2023). Bacteria are commonly found in saliva and gingival plaque in the oral cavity, while they are present in fewer numbers in keratinized gingiva (Aas et al. 2005). Saliva affects the distribution of microorganisms in different parts of the oral cavity and lacks a stable indigenous biota due to its quick turnover and low amounts of nutrients. Bacteria shed from other oral tissues contribute to the high alpha diversity of salivary microbiota (Janakiram et al. 2020). The initiation and progression of periodontal disease are attributed to a polymicrobial synergy and dysbiosis in the microbial community, leading to the formation of a dysbiotic biofilm, plaque, and overgrowth of key pathological entities in the microbiome (Wilson et al. 2017; Lamont et al. 2018; Cugini et al. 2021). This triggers a destructive and dysregulated host immune response, leading to inflammation, destruction of host tissue, and eventually, the formation of periodontal pockets and bone loss (Hajishengallis and Chavakis 2021, Lamont et al. 2018; Caselli et al. 2020).

The pathologic condition persists in active or dormant phases until the affected tooth is extracted or the microbial biofilm is surgically removed. Environmental and host risk factors, including modifiable (e.g., quitting smoking) and non-modifiable factors (e.g., genetics) which, may determine the severity of periodontal diseases (Van Dyke and Sheilesh 2005).

Dental caries and periodontal disease are two major oral illnesses. Periodontal disease can appear in various forms, with gingivitis and periodontitis being prominent ones. Gingivitis is a common inflammatory disorder of the gingiva, the soft tissue surrounding the teeth, caused by oral microbial plaque (Rathee and Jain 2023). Periodontitis follows severe gingivitis in a smaller number of people, based on an individual's immune response, and is characterized by the destruction of the tooth's skeletal, periodontal, and connective tissue supports (Janakiram et al. 2020; Aas et al. 2005).

A diverse microbial population, predominantly of gram-negative anaerobes like *A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia*, is associated with periodontal plaque formation. This may be exacerbated by the deeper invasion of soft tissues by different species of *Candida*. At a subject-specific and tissue-specific level, a microbiome comprising members like *Neisseria* spp., *C. albicans*, *P. aeruginosa*, *Hafnia alvei*, *Serratia marcescens*, *Filifactor alocis*, and *Enterobacteria* is related to periodontal inflammation and tissue destruction (Paul et al. 2021; Li et al. 2022). Conversely, healthy periodontal tissues are associated with *Lactobacillus acidophilus*, *Fusobacterium necrophorum*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*.

Ghannoum et al. (2010) state that a healthy oral mycobiota comprises 74 culturable species and 11 genera of non-culturable fungi. These researchers have reported the presence of specific fungal isolates such as *Candida*, *Aspergillus*, and *Cryptococcus*, which may predispose the host to opportunistic infections and have shown significant inter-individual variance (Ghannoum et al. 2010; Saigal et al. 2011; Padminee et al. 2020). Recent research has revealed that most commensals in saliva are Malassezia species, previously characterized as pathogens of the skin and lungs (Aas et al. 2005; Ghannoum et al. 2010; Nagakubo and Kaibori 2023).

Herbal medicine is an alternative and effective therapy that can be used in conjunction with or instead of conventional chemical drugs

to treat various diseases. Many plant products have antimicrobial, antioxidant, antiviral, antiseptic, and analgesic properties, which greatly interest dentistry. Herbal properties are widely used to alleviate toothaches, gum inflammation, and canker sores (Amanpour et al. 2023). It's important to understand how these phytochemicals work and how they interact with the human body and other medications, as many of these extracts have anti-inflammatory effects and can prevent bleeding, which is vital in dental treatment (Taheri et al. 2011). Herbal products are preferred over-prescribed medicines for treating ailments due to their lower cost and reduced toxicity or side effects (Cruz Martínez et al. 2017; Mosaddad et al. 2023). Recent studies have shown the potential of herbal agents in global dental therapy, especially in the fields of periodontics and endodontics, with plant extracts such as propolis, noni fruit, burdock root, clove, neem leaf, and others (Cruz Martínez et al. 2017; Padminee et al. 2020; Milutinovici et al. 2021; Pasupuleti et al. 2023).

Neem (*A. indica*) from the Meliaceae family is significant in promoting health and is of medicinal importance due to its antibacterial, antihelminthic, anticariogenic, antioxidant, astringent, cytotoxic, and anti-inflammatory properties. Active compounds like Nimbidin, Azadirachtin, Nimbinin, Nimbin, Nimbolide, and Limonoids contribute to these versatile actions (Almas 1999; Subramaniam et al. 2005). The first polyphenolic flavonoids identified in fresh neem leaves were quercetin and ß-sitosterol, known for their antifungal and antibacterial properties (Mathur et al. 2010; Shamsudin et al. 2022). Polyphenols from neem leaf extract adhere to oral surfaces, providing long-lasting antibacterial, anti-inflammatory, and synergistic antioxidant actions, which help combat periodontal disorders (Subramaniam et al. 2005; Buakaew et al. 2021; Wylie et al. 2022). Neem has been demonstrated to have antibacterial properties against various types of *Streptococcus* sp and *C. albicans* (Wolinsky et al. 1996).

Furthermore, Clove (*S. aromaticum* or *Eugenia caryophyllata*) is an aromatic flower bud commonly used as a spice, flavoring, or scent in consumer goods (Afolabi et al. 2008; Danthu et al. 2020; Maggini et al. 2024). Significant constituents of clove oil such as eugenol, β-caryophyllene, caracole, thymol, eugenol, and cinnamaldehyde exhibit antimicrobial activity against Gram-negative (*E. coli*, *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus* sp, *Streptococcus* sp, *Listeria monocytogenes*) bacteria as well as fungal strains like *Aspergillus* sp. (Afolabi et al. 2008; Al-Ahmad et al. 2014; Marchese et al. 2017; Ben Hassine et al. 2021). Clove essential oil also possesses antibacterial, antioxidant, antifungal, and antiviral properties, along with anti-inflammatory, cytotoxic, insect repellent, and anesthetic properties (Dorman and Deans 2000). The phenolic nature of eugenol makes it highly effective against various bacteria. It can easily penetrate the cell walls of gram-positive bacteria, causing the cell wall to degrade (Al-Ahmad et al. 2014), followed by damage to the cytoplasmic membrane, impairment of the bacterial enzyme system, increased permeability, leakage of the cell's contents, and ultimately cell lysis (Prabuseenivasan et al. 2006).

Both neem and clove extracts have been reported to demonstrate antimicrobial activity against common microbes like *C. albicans* and *S. mutans* present in the oral cavity (Barua et al. 2017; Bansal et al. 2019). In countries like India, where there is a rich resource of indigenous flora with medicinal significance, and a significant proportion of the population is affected by periodontal diseases, it is important to investigate the effects of natural, easily available medicinal plants like Neem and clove on the oral biofilm of pathogenic microflora (Zhang et al. 2017). This study aims to explore the impact of plant derivatives on microbes associated with the pathogenicity of periodontitis, with the potential for use as an alternative treatment or in conjunction with existing conventional drugs.

2 Materials and Methods

2.1 Sample collection

We collected oral swab samples from five individuals with poor oral hygiene. We used a sterile swab stick to collect samples by rotating the tip on their teeth and gums before they rinsed their mouths in the morning. The swab sticks were placed in labeled test tubes (OS1-OS5) and transported to the laboratory. We recorded demographic data for each patient. We collected samples from each person thrice and then isolated microorganisms for further analysis (Table 1).

2.2 Revival of samples and culture of microorganisms

The swab samples (OS1-OS5) were placed in 5ml of sterile nutrient broth and left overnight at 37°C to allow any microorganisms in the sample to revive. The next day, 200µl of liquid culture from each sample was spread onto sterile nutrient agar plates and mannitol salt agar plates, and each plate was labeled accordingly. Gram staining was performed on the colonies obtained from OS2-OS4 to establish pure cultures and identify the bacterial morphology and gram characteristics under a light microscope at 40X magnification. To isolate fungi from the oral swab, collected oral samples were plated on potato dextrose agar

Table 1 Details of the sampling Patient's

No. of individuals	Age range (in years)	Sex	Habit
5	11-60	Male-3 Female- 2	Tobacco- 2 (males); Without tobacco- 3; (1 male, 2 females)

(PDA) media and incubated at 30°C overnight. Fungal growth on PDA media was confirmed by staining with lactophenol cotton blue and observing under a light microscope (40X). Pure cultures of colonies obtained from the five samples were prepared and maintained in PDA media and labeled as OS2, OS4A, and OS4B (two types of colonies identified from sample 4). The isolated fungal species from sample 4 were identified as Candida species, which were then cultured on selective Corn meal agar media formulated using 0.2 gm of corn infusion, 0.7ml Tween 80, 1.5 gm agar, and 100 ml distilled water, and autoclaved.

2.3 Preparation of clove and neem extract in the organic solvent

To prepare the extracts, 10 grams of clove were collected and crushed using a pre-sterilized mortar and pestle. Absolute alcohol (99.9%) was added intermittently until the volume reached 20 ml. The sample was soaked for 24 hours, stirred, and left for 24 hours. The upper layer was then separated using a pipette and placed into a sterile falcon tube for further use. Similarly, a 20ml extract of fresh neem leaves was prepared sterilely using absolute alcohol (99.9%), and the upper layer of the supernatant was separated into a sterile falcon tube for future use. The prepared culture stocks were stored at 4°C. For the working solution, 1/2, 1/4, and 1/6 dilutions of each sample were prepared using absolute alcohol as the solvent.

2.4 Paper disc preparation

Paper discs were prepared and sterilized by autoclaving. The sterile paper discs were soaked in the dilutions and crude solutions for 15 minutes, followed by aseptic air drying.

2.5 Antimicrobial activity of neem and clove extract

We used the paper disc method to evaluate the antimicrobial activity of Neem and clove extract. To summarize, we spread 300 µl of an overnight culture of pure culture from OS2, OS4A, and OS4B on a nutrient agar medium and let it absorb on the surface for 30 minutes. We then placed paper discs for the crude extract, respective dilutions (1/2, 1/4, 1/6), and a negative control on each plate. The plates were incubated overnight at 37°C, and observations were made the following day.

2.6 Effect of Neem and clove on preformed biofilm

The study examined the impact of herbal extracts on pre-existing biofilms. Notably, the most significant inhibition zone was observed for OS4B. Consequently, further experiments were conducted exclusively with the pure culture of this sample. To induce biofilm formation, OS4B was inoculated in freshly prepared nutrient broth with 1% glucose and incubated overnight at 37°C. Next, five autoclaved Eppendorf tubes were utilized, with four containing 500 µl of OS4B's overnight culture mixed with 500 µl of nutrient broth, while the fifth served as a negative control with only nutrient broth. The tubes were then placed in an incubator for 24 hours.

The following day, the four Eppendorf tubes containing the culture received 500µl of crude, 1/4, and 1/6 dilutions of clove extraction, respectively, while the fifth tube was left untreated and incubated for an additional 24 hours. The biofilm was subsequently separated from the spent media, washed with 1X PBS, stained with 1% crystal violet solution, and extracted with 33% glacial acetic acid. The absorbance was measured at 600nm using a spectrophotometer. In addition, the effect of clove extract and its dilutions on a 48-hour-old culture of OS4B was assessed through spectrophotometric analysis at 600nm. Similarly, the impact of neem extract on 24 and 48-hour-old biofilms of OS4B was also investigated using the same protocol.

2.7 Effect of neem and clove extract on biofilm formation

Five small sterile Petri plates were taken, and sterile coverslips were placed in each plate aseptically. In plate 1, 2 ml of sterile nutrient broth with 1% glucose was poured, while in the remaining four plates, 1 ml of the same media was added after adding 1 ml of 24-hour-old OS4B liquid culture on the cover slip. This was followed by adding 500 µl of 1/2, 1/4, and 1/6 dilutions of clove extraction in individual plates. The plate with only liquid culture was not treated with any extract. All the plates were then incubated at 37°C overnight. The following day, without disturbing the biofilm, the supernatant was discarded. This was followed by a PBS wash and staining with 1% crystal violet for 15 minutes. After a second PBS wash, the plates were observed under a phase contrast microscope to check for inhibition of biofilm formation. The crystal violet attached to the biofilm was extracted with 33% glacial acetic acid (1 ml) from each plate and transferred to separate Eppendorf tubes. Subsequently, absorbance was measured at 600 nm using a spectrophotometer to estimate the extent of biofilm formation in terms of binding crystal violet. Using the same procedure, the effect of crude neem extract and its dilutions was checked for their impact on the biofilm formation of OS4B.

2.8 Statistical analysis

Experimental data were presented as the mean ± standard error of triplicate measurements and were analyzed using IBM SPSS Statistics version 16. Statistical significance was determined using Student's t-test and one-way ANOVA.

3 Results

3.1 Isolation of microorganisms from oral swab sample

Oral swabs were collected aseptically from five individuals and labeled as OS1-OS4. The swabs were used to streak on sterile nutrient agar

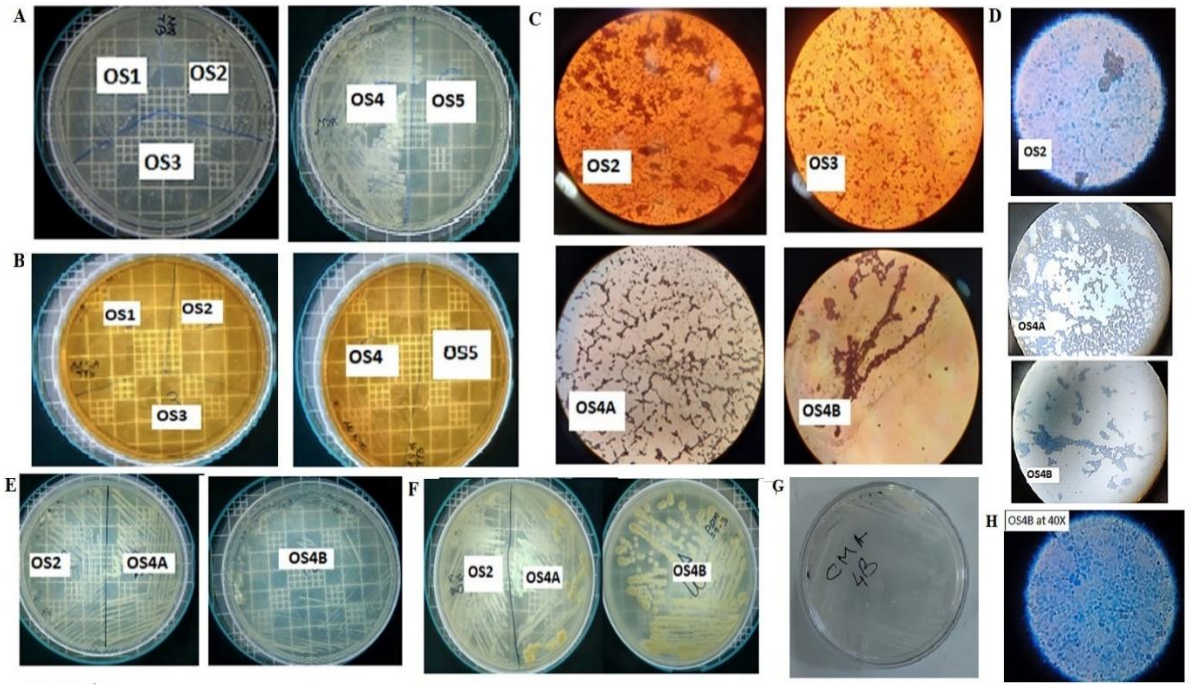

Figure 1 Pictorial representation of: 1A) The growth of samples OS1-OS5 on nutrient agar plates. After 24 hours, samples OS1 and OS5 showed no growth on nutrient agar plates, while samples OS2, OS3, and OS4 exhibited growth; 1B) None of the samples from OS1-OS5 showed growth on MSA plates; 1C) Gram staining of microbial growth from samples OS1-OS5; 1D) Fungal staining with lactophenol cotton blue indicated the presence of fungal culture in OS2 and OS4; 1E) The growth of samples OS2 and OS4 on potato dextrose agar media after 24 hours of incubation; 1F) Pure culture of OS2 and two types of colonies found from sample OS4 (OS4A and OS4B) were grown using PDA media; 1G and 1H) Growth of OS4B samples in corn meal agar media followed by staining with lactophenol cotton blue confirmed the presence of Candida sp. in OS4B sample.

plates and mannitol salt agar plates. The plates were then incubated for 24 hours at 37°C. After incubation, samples OS1 and OS5 showed no growth on the nutrient agar plates and were not included in further study. However, samples OS2, OS3, and OS4 showed growth (Figure 1A). None of the samples showed growth on mannitol salt agar plates (Figure 1B). Gram staining of microbial growth did not provide information regarding gram character and morphology (Figure 1C). Fungal staining with lactophenol cotton blue indicated the presence of fungal culture (Figure 1D). Therefore, samples OS2-OS4 were then plated on potato dextrose agar (PDA) media. After 24 hours of incubation, visible growth was observed for OS2 and OS4 on their respective PDA plates. The fungal samples presence in each was further confirmed by staining with lactophenol cotton blue and observing under a light microscope at 40X magnification (Figure 1E). Pure cultures of OS2 and two colonies found from sample OS4 (OS4A and OS4B) were prepared using PDA media (Figure 1F). Further experiments were continued with these three fungal samples only, i.e., OS2, OS4A, and OS4B. To confirm the identity of the fungal strains, they were plated on corn meal agar media, which is used as a selective media for *Candida* sp. The growth of OS4B in this media confirmed the presence of Candida sp., which was further validated by staining with lactophenol cotton blue (Figure 1G and 1H).

3.2 Examination of antimicrobial activity of Neem and clove

To test the antimicrobial effect of Neem on isolated fungal strains, we selected pure cultures of samples OS2, OS4A, and OS4B. We prepared neem crude extract at 1/4, 1/2, and 1/6 dilutions using absolute alcohol as the solvent. We then dipped sterile paper discs in the respective plant extracts to create antimicrobial discs. Each fungal sample was plated on individual plates, and the antimicrobial discs were placed to assess their antifungal activity. For sample OS2, there was no zone of inhibition for the crude extract or any of the dilutions of Neem. However, for sample OS4A, zones of inhibition were observed 0.833±0.115 cm (1/2 dilution), 1.3±0.1 cm (1/4 dilution), and 1.36±0.058 cm (1/6 dilution), with no reported zone for the crude extract or control. As for sample OS4B, the obtained zones of inhibition were 0.9±0.1 cm (1/4 dilution) and 1.1±0.17 cm (1/6 dilution), but no zone of inhibition was reported for the crude extract or the half dilution (Figure 2A and 2B).

A similar experiment was conducted with different clove extracts, and no zone of inhibition for sample OS2 was observed again. However, for the other two samples, OS4A and OS4B, zones of inhibition for the 1/2 dilution (OS4A: 1.3±0.1 cm, OS4B: 1.1±0.1

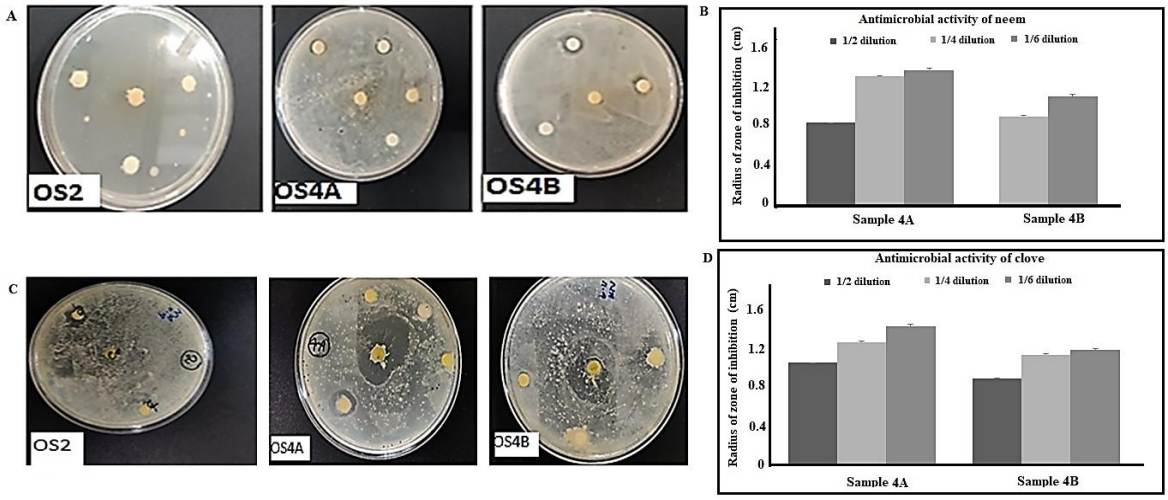

Figure 2 illustrates the zone of growth inhibition of samples OS2, OS4A, and OS4B: 1A) in the presence of neem extract and its dilutions; 1C) in the presence of clove extract and its dilutions; 1B) displaying the zone of inhibition for samples OS4A and OS4B in crude neem extract and its various dilutions (1/4, 1/2, and 1/6 dilutions); 1D) showing the zone of inhibition for samples OS4A and OS4B in crude clove extract and its various dilutions (1/4, 1/2, and 1/6 dilutions).

cm), 1/4 dilution (OS4A: 1.567±0.15 cm, OS4B: 1.4±0.1 cm), and 1/6 dilution (OS4A: 1.766±0.15 cm, OS4B: 1.4667±0.058 cm) were observed (Figure 2C and 2D).

3.3 Effect of neem and clove extract on preformed biofilm

In this experiment, biofilm formation was induced by adding 1% glucose to freshly prepared nutrient broth in five Eppendorf tubes, which were then kept at 37°C for 48 hours (Figure 3A – E). The effects of both clove and neem extracts and various dilutions of these extracts were observed on 24-hour and 48-hour-old biofilms using spectrophotometric analysis at 600nm. The study results indicated that the crude extract of clove didn't cause any change in the biofilm after 24 hours, but after 48 hours, there was a reported 19% reduction in the biofilm ($P<0.001$). Significant reduction in the formed biofilm was observed for different dilutions of clove extract, with a 20% reduction reported after 24 hours and a 41% reduction reported after 48 hours for the 1/4th dilution ($P<0.001$). In addition, the 1/6th dilution resulted in a 50% reduction after both 24 and 48 hours of exposure ($P<0.001$).

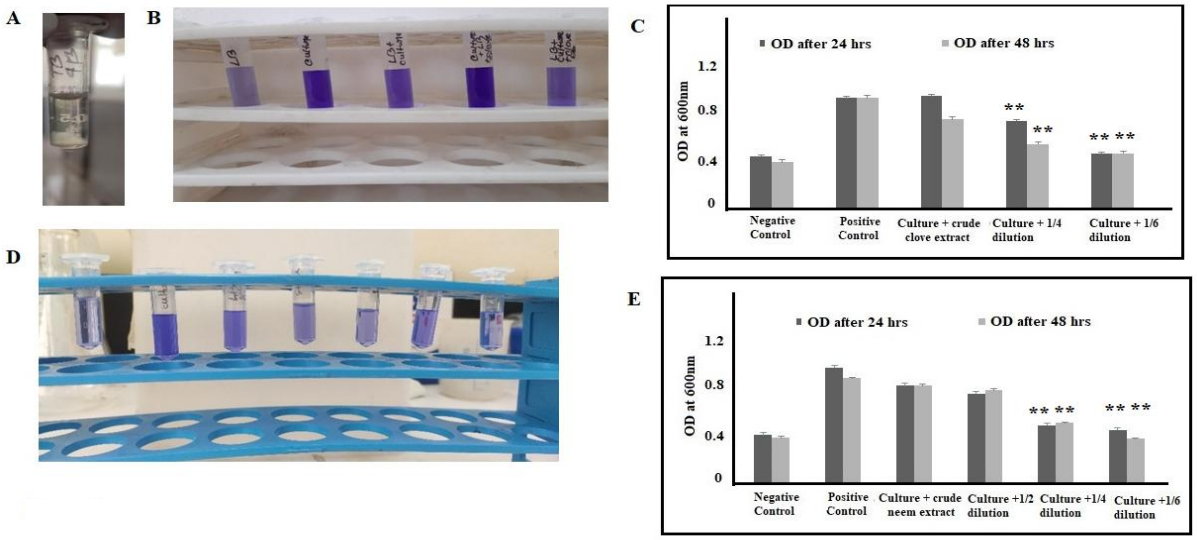

Figure 3A) Biofilm formation in Eppendorf for sample OS4B; 3B) color variation obtained after extraction of crystal violet stain from preformed biofilms of OS4B after treatment with crude extract and increasing dilutions of clove in a series of Eppendorfs; 3C) reduction in OD reading at 600nm with increasing dilution of clove for OS4B; 3D) color variation obtained after extraction of crystal violet stain from preformed biofilms of OS4B after treatment with crude neem extract and dilutions; 3E) reduction in OD reading at 600nm with increasing dilution of Neem for OS4B; ** indicates P value < 0.001

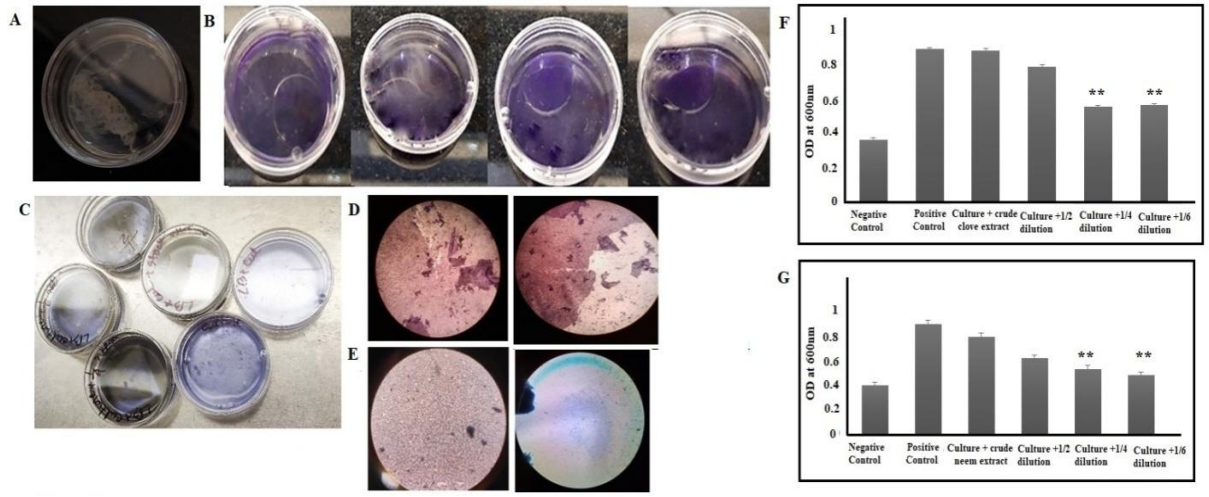

Figure 4A: Biofilm formation in a petri plate for sample OS4B; 4B & C: Variation in color obtained after extracting crystal violet stain from preformed biofilms of OS4B after treatment with crude extract and various dilutions of clove and Neem, respectively; 4D: Microscopic view of biofilm without (left panel) and with a 1/6 dilution of clove and Neem (right panel); 4E & F: A significant reduction in OD reading at 600 nm with increasing dilution of clove and neem extracts respectively; ** indicates P value<0.001

As for the effect of neem extract on the biofilm, the crude extract showed a 15% reduction after 24 and 48 hours of exposure (P<0.01). Different dilutions of neem extract also demonstrated a significant reduction in the formed biofilm, with a reported 23% and 20% reduction after 24 and 48 hours, respectively, for the 1/2th dilution (P<0.001) and a 49% and 47% reduction after 24 and 48 hours, respectively for the 1/4th dilution (P<0.001). The 1/6th dilution of neem extract showed a 54% and 56% reduction after 24 and 48 hours of exposure (P<0.001). In conclusion, both clove and neem extract at 1/6th dilutions showed the greatest capacity to disrupt biofilm formation compared to the crude extracts or the 1/2th and 1/4th dilutions of the extracts.

3.4 Effect of neem and clove extraction on the formation of biofilm

A fresh culture of OS4B was inoculated in sterile petri plates containing freshly prepared nutrient broth with 1% glucose. The growth was allowed to spread on sterile coverslips. After 48 hours, the impact of natural extracts on biofilm production was evaluated. This was done by crystal violet staining followed by observation under a phase-contrast microscope and measurement of absorbance using a spectrophotometer (Figure 4 A-E). Compared to the positive control plates with no extracts (Figure 4D, E), a significant reduction in biofilm formation was noticed in plates with 1/6 dilutions of clove and neem extracts, respectively (Figure 4D, E). Analyzing the effect of clove extract on biofilm formation, the crude extract showed no effect, while only an 11% reduction was noted for the 1/2 dilution. However, a significant reduction in biofilm formation was observed with 1/4 (37%) and 1/6 dilution (36%) (Figure 4F). Similarly, with neem extract, there was a 12% reduction with the crude extract, whereas a more significant reduction was noted with 1/2 dilution (31.5%), 1/4 dilution (41.6%), and 1/6 dilution (46.7%) (Figure 4G).

4 Discussion

Herbal remedies have been widely used in dentistry to treat tooth pain, periodontal inflammation, and oral mucosal diseases caused by sores and microbial infections. Medications of herbal origin, made up of natural ingredients, are considered safe with minimal toxicity and side effects on humans and the environment. Additionally, being indigenous, they are readily available and inexpensive, making them a useful resource for medicinal purposes. In this study, the effectiveness of two popular herbal products, neem, and clove, was evaluated against biofilm-producing pathogens isolated from the oral cavity of people with dental problems and an unhygienic lifestyle. Natural ingredients extracted from these herbal products in organic solvents were found to have antimicrobial activity against fungal strains isolated from oral swabs collected. The fungal strain identified as a species of *Candida* showed rapid biofilm production, which is characteristic of symptoms for different periodontal diseases. Both neem and clove extracts effectively disrupted preformed biofilms of *Candida* sp. and prevented biofilm formation. The efficiency was higher with more prolonged incubation for 48 hours than 24 hours. It is also worth mentioning that the effectiveness of anti-biofilm activity was enhanced significantly with higher dilutions (1/4, 1/6 dilution) compared to crude extract or 1/2 dilution. This finding is consistent with reports stating that within heterogeneous mixtures of bioactive compounds derived from plants, antimicrobial combination effects may include synergy and antagonism (Vaou et al. 2022). Similar observations were reported against both bacterial and fungal strains isolated from the oral

cavity and other sources using extracts of both neem and clove (Agarwal et al. 2010; Harjai et al. 2013; Nagarajappa et al. 2018; Barua et al. 2017; Bansal et al. 2019; Batiha et al. 2020). The antagonism between bioactive compounds in essential oils can occur during antimicrobial interactions in herbal extracts when (i) a combination of bacteriostatic and bactericidal antimicrobials exists, (ii) antimicrobials target the same site, or (iii) antimicrobial natural components interact with each other (Roller, 2003). The results of this study indicated that the antagonistic effect that masks the activity of the prime antimicrobial component may be relieved with higher dilutions. An example of such alteration of antagonism has also been reported for the antimalarial agent artemisinin. In this case, the flavone casticin exhibited antagonistic activity at a 1:3 ratio. However, the in-vitro activity of artemisinin significantly increased with higher dilutions (Liu et al. 1992; Vaou et al. 2022). However, further validation at a larger scale of experiments is needed for confirmation.

Conclusion

The use of herbal medication in dentistry has proven to be effective. Studies have shown that neem and clove extracts exhibit antifungal properties against *Candida* sp., commonly found in individuals with dental diseases. These extracts can reduce preformed biofilms and prevent biofilm formation, making them promising candidates for treating oral Candida infections. Neem and clove extracts are non-toxic and have significant antifungal activities, which could complement existing commercial medications for treating periodontitis and other biofilm-related oral disorders. However, further research with larger sample sizes and detailed investigation of the mode of action of these herbal extracts is needed before their actual application as prescribed medication.

Conflict of Interest

Nil

Authors' Contribution

Conception: Tanushree Bhattacharya, Rudra Prasad Saha, Rajib Majumder, and Sanmitra Ghosh; Literature review: Tanushree Bhattacharya and Sanmitra Ghosh; Data interpretation: Tanushree Bhattacharya, Rajib Majumder, and Sanmitra Ghosh; Manuscript drafting: Tanushree Bhattacharya and Sanmitra Ghosh; Supervision: Sanmitra Ghosh.

Funding

Nil

References

Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43*(11), 5721–5732. https://doi.org/10.1128/JCM.43.11.5721-5732.2005

Afolabi, O. C., Ogunsola, F. T., & Coker, A. O. (2008). Susceptibility of cariogenic *Streptococcus mutans* to extracts of Garcinia kola, *Hibiscus sabdariffa*, and *Solanum americanum*. *West African journal of medicine*, *27*(4), 230–233.

Agarwal, V., Lal, P., & Pruthi, V. (2010). Effect of plant oils on *Candida albicans*. *Journal of Microbiology, Immunology and Infection*, *43*(5), 447-451.

Al-Ahmad, A., Ameen, H., Pelz, K., Karygianni, L., Wittmer, A., et al. (2014). Antibiotic resistance and capacity for biofilm formation of different bacteria isolated from endodontic infections associated with root-filled teeth. *Journal of Endodontics*, *40*(2), 223-230.

Almas K. (1999). The antimicrobial effects of extracts of Azadirachta indica (Neem) and Salvadora persica (Arak) chewing sticks. *Indian journal of dental research: official publication of Indian Society for Dental Research*, *10*(1), 23–26.

Amanpour, S., AkbariJavar, M., Sarhadinejad, Z., Doustmohammadi, M., Moghadari, M., & Sarhadynejad, Z. (2023). A systematic review of medicinal plants and herbal products' effectiveness in oral health and dental cure with health promotion approach. *Journal of Education and Health Promotion*, *12*, 306. https://doi.org/10.4103/jehp.jehp_1297_22

Antimicrobial Resistance Collaborators (2024). The burden of bacterial antimicrobial resistance in the WHO African region in 2019: a cross-country systematic analysis. *The Lancet Global Health*, *12*(2), e201–e216. https://doi.org/10.1016/S2214-109X(23)00539-9

Arweiler, N. B., & Netuschil, L. (2016). The Oral Microbiota. *Advances in experimental medicine and biology*, *902*, 45–60. https://doi.org/10.1007/978-3-319-31248-4_4

Bansal, V., Gupta, M., Bhaduri, T., Shaikh, S. A., Sayed, F. R., Bansal, V., & Agrawal, A. (2019). Assessment of antimicrobial effectiveness of neem and clove extract against *Streptococcus mutans* and *Candida albicans*: an in vitro study. *Nigerian Medical Journal: Journal of the Nigeria Medical Association*, *60*(6), 285.

Barua, D. R., Basavanna, J. M., & Varghese, R. K. (2017). Efficacy of neem extract and three antimicrobial agents incorporated into tissue conditioner in inhibiting the growth of *C. albicans* and *S. mutans*. *Journal of Clinical and Diagnostic Research*, *11*(5), ZC97.

Batiha, G. E., Alkazmi, L. M., Wasef, L. G., Beshbishy, A. M., Nadwa, E. H., & Rashwan, E. K. (2020). *Syzygium aromaticum* L.

(Myrtaceae): Traditional Uses, Bioactive Chemical Constituents, Pharmacological and Toxicological Activities. *Biomolecules*, *10*(2), 202. https://doi.org/10.3390/biom10020202

Ben Hassine, D., Kammoun El Euch, S., Rahmani, R., Ghazouani, N., Kane, R., Abderrabba, M., &Bouajila, J. (2021).Clove Buds Essential Oil: The Impact of Grinding on the Chemical Composition and Its Biological Activities Involved in Consumer's Health Security. *BioMed research international*, *2021*, 9940591. https://doi.org/10.1155/2021/9940591

Buakaew, W., Sranujit, R. P., Noysang, C., Sangouam, S., Suphrom, N., Thongsri, Y., Potup, P., & Usuwanthim, K. (2021). Evaluation of Mouthwash Containing *Citrus hystrix* DC., *Moringa oleifera* Lam. and *Azadirachta indica* A. Juss. Leaf Extracts on Dental Plaque and Gingivitis. *Plants (Basel, Switzerland)*, *10*(6), 1153. https://doi.org/10.3390/plants10061153

Caselli, E., Fabbri, C., D'Accolti, M., Soffritti, I., Bassi, C., Mazzacane, S., & Franchi, M. (2020). Defining the oral microbiome by whole-genome sequencing and resistome analysis: the complexity of the healthy picture. *BMC Microbiology*, *20*(1), 120. https://doi.org/10.1186/s12866-020-01801-y

Cruz Martínez, C., Diaz Gómez, M., & Oh, M. S. (2017). Use of traditional herbal medicine as an alternative in dental treatment in Mexican dentistry: a review. *Pharmaceutical Biology*, *55*(1), 1992-1998.

Cugini, C., Ramasubbu, N., Tsiagbe, V. K., & Fine, D. H. (2021).Dysbiosis from a microbial and host perspective relative to oral health and disease. *Frontiers in microbiology*, *12*, 617485.

D'Ambrosio, F., Santella, B., Di Palo, M. P., Giordano, F., & Lo Giudice, R. (2023). Characterization of the Oral Microbiome in Wearers of Fixed and Removable Implant or Non-Implant-Supported Prostheses in Healthy and Pathological Oral Conditions: A Narrative Review. *Microorganisms*, *11*(4), 1041. https://doi.org/10.3390/microorganisms11041041

Danthu, P., Simanjuntak, R., Fawbush, F., Tsy, J. L. P., Razafimamonjison, G., et al. (2020). The clove tree and its products (clove bud, clove oil, eugenol): prosperous today but what of tomorrow's restrictions?. *Fruits*, *75*(5), 224-242.

Deo, P. N., & Deshmukh, R. (2019). Oral microbiome: Unveiling the fundamentals. *Journal of oral and maxillofacial pathology: JOMFP*, *23*(1), 122–128. https://doi.org/10.4103/jomfp.JOMFP_304_18

Dorman, H. J., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology*, *88*(2), 308–316. https://doi.org/10.1046/j.1365-2672.2000.00969.x

Gasner, N. S., & Schure, R. S. (2023).Periodontal Disease. In *Stat Pearls*. StatPearls Publishing.

Ghannoum, M. A., Jurevic, R. J., Mukherjee, P. K., Cui, F., Sikaroodi, M., Naqvi, A., & Gillevet, P. M. (2010). Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS pathogens*, *6*(1), e1000713. https://doi.org/10.1371/journal.ppat.1000713

Hajishengallis, G., & Chavakis, T. (2021).Local and systemic mechanisms linking periodontal disease and inflammatory comorbidities. *Nature Reviews. Immunology*, *21*(7), 426–440. https://doi.org/10.1038/s41577-020-00488-6

Harjai, K., Bala, A., Gupta, R. K., & Sharma, R. (2013). Leaf extract of *Azadirachta indica* (Neem): a potential antibiofilm agent for *Pseudomonas aeruginosa*. *Pathogens and Disease*, *69*(1), 62-65.

Janakiram, C., Mehta, A., & Venkitachalam, R. (2020). Prevalence of periodontal disease among adults in India: A systematic review and meta-analysis. *Journal of Oral Biology and Craniofacial Research*, *10*(4), 800-806.

John, J. R., Daniel, B., Paneerselvam, D., & Rajendran, G. (2017). Prevalence of Dental Caries, Oral Hygiene Knowledge, Status, and Practices among Visually Impaired Individuals in Chennai, Tamil Nadu. *International journal of dentistry*, *2017*, 9419648. https://doi.org/10.1155/2017/9419648

Kitamoto, S., Nagao-Kitamoto, H., Hein, R., Schmidt, T. M., & Kamada, N. (2020).The Bacterial Connection between the Oral Cavity and the Gut Diseases. *Journal of dental research*, *99*(9), 1021–1029. https://doi.org/10.1177/0022034520924633

Könönen, E., Gursoy, M., & Gursoy, U. K. (2019). Periodontitis: A Multifaceted Disease of Tooth-Supporting Tissues. *Journal of Clinical Medicine*, *8*(8), 1135. https://doi.org/10.3390/jcm8081135

Lamont, R. J., Koo, H., & Hajishengallis, G. (2018). The oral microbiota: dynamic communities and host interactions. *Nature Reviews Microbiology*, *16*(12), 745-759.

Li X, Liu Y, Yang X, Li C, Song Z. (2022) The Oral Microbiota: Community Composition, Influencing Factors, Pathogenesis, and Interventions. *Frontiers in Microbiology*, *13*, 895537.doi: 10.3389/fmicb.2022.895537.

Liu, K. C. S. C., Yang, S. L., Roberts, M. F., Elford, B. C., & Phillipson, J. D. (1992). Antimalarial activity of *Artemisia annua*

flavonoids from whole plants and cell cultures. *Plant Cell Reports*, *11*, 637-640.

Maggini, V., Semenzato, G., Gallo, E., Nunziata, A., Fani, R., & Firenzuoli, F. (2024). Antimicrobial Activity of *Syzygium aromaticum* Essential Oil in Human Health Treatment. *Molecules (Basel, Switzerland)*, *29*(5), 999. https://doi.org/10.3390/molecules29050999

Marchese, A., Barbieri, R., Coppo, E., Orhan, I. E., Daglia, M., et al. (2017). Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. *Critical Reviews in Microbiology*, *43*(6), 668–689. https://doi.org/10.1080/1040841X.2017.1295225

Mathur, A., Dua, V.K., & Prasad, GBKS (2010) Antimicrobial Activity of Leaf Extracts of Murraya koenigii against Aerobic Bacteria Associated with Bovine Mastitis. *International Journal of Chemical, Environmental and Pharmaceutical Research*, *1*(1), 12–16.

Milutinovici, R. A., Chioran, D., Buzatu, R., Macasoi, I., Razvan, S., et al. (2021). Vegetal Compounds as Sources of Prophylactic and Therapeutic Agents in Dentistry. *Plants (Basel, Switzerland)*, *10*(10), 2148. https://doi.org/10.3390/plants10102148

Mosaddad, S. A., Hussain, A., & Tebyaniyan, H. (2023). Green Alternatives as Antimicrobial Agents in Mitigating Periodontal Diseases: A Narrative Review. *Microorganisms*, *11*(5), 1269. https://doi.org/10.3390/microorganisms11051269

Nagakubo, D., & Kaibori, Y. (2023). Oral Microbiota: The Influences and Interactions of Saliva, IgA, and Dietary Factors in Health and Disease. *Microorganisms*, *11*(9), 2307. https://doi.org/10.3390/microorganisms11092307

Nagarajappa, S., Bathija, P., Mishra, P., Bansal, V., Gupta, S., & Sontakke, S. (2018). Antibacterial and Antifungal Activity of Neem and Clove Extract Against *S. mutans* and *C. albicans*-An invitroStudy. *World Journal of Pharmaceutical Research*, *7*(5), 1484-93.

Padminee, K., Anusha, R., Lakshmi, K., & Kumar, P. D. M. (2020). Geographic disproportions in dental workforce distribution and its impact on oral disease burden: An Indian perspective. *SRM Journal of Research in Dental Sciences*, *11*(2), 76-81.

Pasupuleti, M. K., Nagate, R. R., Alqahtani, S. M., Penmetsa, G. S., Gottumukkala, S. N. V. S., & Ramesh, K. S. V. (2023). Role of Medicinal Herbs in Periodontal Therapy: A Systematic Review. *Journal of International Society of Preventive & Community Dentistry*, *13*(1), 9–16. https://doi.org/10.4103/jispcd.JISPCD_210_22

Paul, O., Arora, P., Mayer, M., & Chatterjee, S. (2021). Inflammation in periodontal disease: possible link to vascular disease. *Frontiers in physiology*, *11*, 609614.

Prabuseenivasan, S., Jayakumar, M., & Ignacimuthu, S. (2006). In vitro antibacterial activity of some plant essential oils. *BMC complementary and alternative medicine*, *6*, 39. https://doi.org/10.1186/1472-6882-6-39

Rajhans, N. S., Kohad, R. M., Chaudhari, V. G., & Mhaske, N. H. (2011). A clinical study of the relationship between diabetes mellitus and periodontal disease. *Journal of Indian Society of Periodontology*, *15*(4), 388–392. https://doi.org/10.4103/0972-124X.92576

Rathee, M., & Jain, P. (2023).Gingivitis.In *StatPearls*. StatPearls Publishing.

Roller, S. (Ed.). (2003). *Natural antimicrobials for the minimal processing of foods*. Wood head publishing.

Saigal, S., Bhargava, A., Mehra, S. K., & Dakwala, F. (2011). Identification of *Candida albicans* by using different culture medias and its association in potentially malignant and malignant lesions. *Contemporary clinical dentistry*, *2*(3), 188–193. https://doi.org/10.4103/0976-237X.86454

Shamsudin, N. F., Ahmed, Q. U., Mahmood, S., Ali Shah, S. A., Khatib, A., et al. (2022). Antibacterial Effects of Flavonoids and Their Structure-Activity Relationship Study: A Comparative Interpretation. *Molecules (Basel, Switzerland)*, *27*(4), 1149. https://doi.org/10.3390/molecules27041149

Subramaniam, S.K., Siswomihardjo, W., & Sunarintyas, S. (2005). The effect of different concentrations of Neem (*Azadiractha indica*) leaves extract on the inhibition of Streptococcus mutans (In vitro). *Dental Journal: Majalah Kedokteran Gigi*, *38*, 176-179.

Taheri, J. B., Azimi, S., Rafieian, N., & Zanjani, H. A. (2011). Herbs in dentistry. *International Dental Journal*, *61*(6), 287–296. https://doi.org/10.1111/j.1875-595X.2011.00064.x

Van Dyke, T. E., & Sheilesh, D. (2005). Risk factors for periodontitis. *Journal of the International Academy of Periodontology*, *7*(1), 3–7.

Van Dyke, T. E., Bartold, P. M., & Reynolds, E. C. (2020). The Nexus Between Periodontal Inflammation and Dysbiosis. *Frontiers in immunology*, *11*, 511. https://doi.org/10.3389/fimmu.2020.00511

Vaou, N., Stavropoulou, E., Voidarou, C., Tsakris, Z., Rozos, G., Tsigalou, C., & Bezirtzoglou, E. (2022). Interactions between medical plant-derived bioactive compounds: Focus on antimicrobial combination effects. *Antibiotics*, *11*(8), 1014.

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., et al. (2017). Quantitative and Qualitative Assessment Methods for Biofilm Growth: A Mini-review. *Journal of engineering and technology*, *6*(4), 1.25.

Wolinsky, L. E., Mania, S., Nachnani, S., & Ling, S. (1996). The inhibiting effect of aqueous *Azadirachta indica* (Neem) extract upon bacterial properties influencing in vitro plaque formation. *Journal of Dental Research*, *75*(2), 816–822. https://doi.org/10.1177/00220345960750021301.

Wylie, M. R., & Merrell, D. S. (2022). The Antimicrobial Potential of the Neem Tree *Azadirachta indica*. *Frontiers in pharmacology*, *13*, 891535. https://doi.org/10.3389/fphar.2022.891535

Zhang, Y., Wang, Y., Zhu, X., Cao, P., Wei, S., & Lu, Y. (2017). Antibacterial and antibiofilm activities of eugenol from essential oil of *Syzygium aromaticum* (L.) Merr.& L. M. Perry (clove) leaf against periodontal pathogen *Porphyromonas gingivalis*. *Microbial pathogenesis*, *113*, 396–402. https://doi.org/10.1016/j.micpath.2017.10.054

Zhu, L., Tang, Z., Hu, R., Gu, M., & Yang, Y. (2023). Ageing and Inflammation: What Happens in Periodontium?. *Bioengineering (Basel, Switzerland)*, *10*(11), 1274. https://doi.org/10.3390/bioengineering10111274