

INVESTIGATING THE ANTIBACTERIAL PROPERTIES OF NEEM PLANT IN THE CONTEXT OF DENTAL CARIES

ABSTRACT

Aim: This study aimed to assess the antimicrobial potential of *Azadirachta indica* (neem) plant extracts on *Streptococcus mutans*, a key bacterium associated with dental caries.

Methods: Neem plant parts (leaves, twigs, and stem bark) were collected from Ibadan, Oyo State, Nigeria, and identified at the Herbarium unit, University of Ibadan. Ethanol and aqueous extracts were prepared using standard procedures. Media including Brain-heart infusion broth, Nutrient agar, Blood agar, Mac Conkey agar, and GSTB medium were used for bacterial culture. Antibiotic susceptibility testing was conducted using Bacitracin and Sodium telluride. The study focused on the isolation and identification of *Streptococcus mutans* from oral specimens, bacterial counts, and the antimicrobial sensitivity profile of neem extracts using the agar well diffusion method.

Results: The study revealed the absence of *Streptococcus mutans* in some specimens. *Staphylococcus* sp. and total bacterial counts varied among the samples. The frequency of occurrence of *Streptococcus mutans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* was documented. The antimicrobial sensitivity profile demonstrated varied zones of inhibition in response to neem extracts against *Streptococcus mutans* strains.

Conclusion: Neem extracts exhibited antimicrobial activity against *Streptococcus mutans*, suggesting their potential role in combating dental caries. The study contributes valuable insights into the therapeutic properties of neem in oral health, paving the way for further research and potential applications in dentistry.

Keywords: Neem Extracts; *Streptococcus mutans*; Dental Caries Antimicrobial Activities; Herbal Remedies

INTRODUCTION:

Dental caries, a prevalent oral health issue globally, is primarily caused by the colonization of bacteria, particularly *Streptococcus mutans*, on tooth surfaces. Despite advancements in dental care, dental caries remains a significant concern, necessitating continuous exploration of alternative approaches for prevention and treatment [1]. Traditional medicinal plants, with their

rich phytochemical composition, have garnered attention for their potential antimicrobial properties against pathogenic bacteria implicated in oral diseases. *Azadirachta indica*, commonly known as neem, is one such plant with a long-standing history in traditional medicine for its diverse therapeutic attributes [2].

The aim of this study is to investigate the antimicrobial activities of neem plant extracts on *Streptococcus mutans*, specifically evaluating the potential of neem in preventing and managing dental caries. The research unfolds in the backdrop of the unique geographical and climatic conditions of Oyo State, Nigeria, which is characterized by an equatorial climate with distinct dry and wet seasons. The choice of Oyo State as the study area is not only influenced by its geographic features but also by its rich biodiversity, including the presence of *Azadirachta indica*, which holds promise as a natural remedy.

The investigation encompasses a multidisciplinary approach, incorporating elements of microbiology, biochemistry, and herbal medicine. Neem, known for its broad-spectrum antimicrobial properties, offers a compelling avenue for exploration in the context of oral health [2]. This research contributes to the existing body of knowledge by delving into the specific impacts of neem extracts derived from different plant parts, namely leaves, twigs, and stem bark, on *Streptococcus mutans*, shedding light on their efficacy in combating the bacteria associated with dental caries [3].

The study area, Oyo State, is characterized not only by its geographic diversity but also by the coexistence of urban and agrarian communities, providing a unique setting to explore the intersection of traditional herbal remedies and contemporary oral healthcare practices. The University of Ibadan, as the focal point of the research, serves as a hub for academic excellence and research initiatives, positioning this study within a context that encourages scientific rigor and exploration.

The methodology employed in this research involves the collection and identification of neem plant materials, preparation of various media for bacterial culture, and the extraction of neem components using both ethanol and aqueous solvents. The antibacterial activities of neem extracts are evaluated through techniques such as the agar well diffusion method, providing a comprehensive understanding of their impact on *Streptococcus mutans* [4,5].

Furthermore, the research extends its scope to the isolation and identification of *Streptococcus mutans* from oral specimens, including swabs from patients with dental caries. Bacterial counts, frequency of occurrence, and antimicrobial sensitivity profiles are meticulously documented, creating a nuanced picture of the potential therapeutic applications of neem in oral healthcare.

Conclusively, this project holds the promise of unraveling the antimicrobial potential of neem plant extracts against *Streptococcus mutans*, offering insights that could inform the development of novel strategies for preventing and managing dental caries. By integrating traditional herbal remedies with modern scientific methodologies, this research contributes to the ongoing discourse on the role of natural products in oral health and underscores the significance of exploring indigenous knowledge in the pursuit of effective healthcare solutions.

Methods

Media

The following media was used: Brain-heart infusion broth, Nutrient agar, Blood agar, Mac Conkey agar, GSTB medium.

Antibiotics; The following antibiotic was used: Bacitracin and Sodium telluride.

Study Area

The study was carried out at the Department of Biochemistry and the Microbiology Laboratory of University of Ibadan, Oyo State.

Collection and Identification of Plant Materials

Fresh plant of *Azadirachta indica* (neem) parts such as leaves, twigs and bark were obtained from Airport field in Ibadan, Oyo State. The selected plant were identified and authenticated in the Herbarium unit, Department of Botany, University of Ibadan.

Preparation of Media

All media including, Brain-heart infusion broth, Nutrient agar, Blood agar, Mac Conkey agar, GSTB medium was prepared following manufacturer's instructions.

Brain-heart Infusion Broth

The agar was prepared by weighing and dissolving 9.7 grams of Brain-heart infusion agar powder in 500 ml of distilled water in 1000 ml conical flask. This was properly mixed and heated under flame to dissolve. Then it was sterilized by autoclaving at 121⁰C for 15 mins at 15 psi and allowed to cool to 45⁰C before pouring 20 ml each into petri dishes. This was left on the bench to solidify and was incubated at 37⁰C for 24 hrs to ascertain sterility [6].

Nutrient Agar

Nutrient agar was prepared by weighing and dissolving 14 grams of nutrient agar powder in 500 ml of distilled water in 1000 ml conical flask. This was properly mixed and heated under flame to dissolve. Then it was sterilized by autoclaving at 121⁰C for 15 mins at 15 psi and allowed to cool to 45⁰C before pouring 20 ml each into petri dishes. This was left on the bench to solidify and was incubated at 37⁰C for 24 hrs to ascertain sterility [6].

Blood Agar

Nutrient agar was prepared by weighing and dissolving 14 grams of nutrient agar powder in 500 ml of distilled water in 1000 ml conical flask. This was properly mixed and heated under flame to dissolve. Then it was sterilized by autoclaving at 121⁰C for 15 mins at 15 psi and allowed to cool to 45⁰C before adding 5 % defibrinated sheep blood and pouring 20 ml each into petri dishes. This was left on the bench to solidify and was incubated at 37⁰C for 24 hrs to ascertain sterility [6].

Mac Conkey Agar

This was prepared according to manufacturer's instruction. Exactly 24.3 g of Mac Conkey agar powder was measured and dissolved in 500 ml of distilled water. Then it was sterilized by autoclaving at 121⁰C for 15 min at 15 psi and allowed to cool to 45⁰C before pouring into petri dishes and allowed to solidify on the bench and was incubated at 37⁰C for 24 hrs to ascertain sterility.

TABLE 1: Formulation of GSTB agar (Tanzer *etal.*, 1984).

Component	Amount
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Solution A

Trypticase peptone (BBL)	5g
Yeast extracts (Difco)	5g
K ₂ HPO ₄	5g
Na ₂ CO ₃	0.05g
Agar (BBL)	20g
Salt solution (consisting of 1.15g of MgSO ₄ .7H ₂ SO, 0.19g of MnSO ₄ .H ₂ O, 0.068g of FeSO ₄ .H ₂ O per 10ml of water)	0.5ml
Deionised water	800ml
Boil, cool and adjust pH to 7.2 with HCl	

Solution B

Glucose	50g
Deionised water	q.s.b 100ml

Solution C

Sucrose	50g
Deionised water	q.s. 100ml

KEY: q.s. Quantum satis (sufficient quantity).

Procedure: Solutions A, B and C are autoclaved separately at 121⁰C for 20 min, combined and allowed to cool to 50 to 55⁰C before adding 1.0 ml of 1% potassium tellurite (Difco) and 300 U of bacitracin (Sigma), both filter and sterilized. After through stirring at 55⁰C, plates are poured. GS medium is made identically, except that tellurite and bacitracin deleted [8].

Preparation of Extracts**Crude Ethanol Extraction**

The different dried pulverized neem plant parts were soaked in absolute ethanol. 300g, 300g and 650g of the dried pulverized leaf, twig and stem bark respectively were extracted for 72 hours

using 2000ml, 2000ml and 5000ml of absolute ethanol respectively while stirred at every 2hrs. The solvent (now containing the extract) was collected using muslin bag and the filtrate was further filtered using Whatman filter paper 1mm. This process was repeated on the shaft with same quantity of solvent for maximum extraction. The combined filtrate was then concentrated with the aid of rotary evaporator (Heidolphlaborota 400 efficient, made in Germany, model 517-01002-002) set at 40⁰C, after which the concentrate was further concentrated using a vacuum oven set at 40⁰C with a pressure of 700mmHg.

Aqueous Extraction

Different dried pulverized samples of neem plant parts were soaked in distilled water. 290g, 220g and 600g of the dried pulverized leaf, twig and stem bark respectively were extracted for 24 hours using 4000ml, 4000ml and 8000ml of distilled water respectively while stirred at every 2hrs. The extract was collected using muslin bag and the filtrate was further filtered using whatman filter paper 1mm. The combined filtrate was concentrated using rotary evaporator (Heidolphlaborota 400 efficient, made in Germany, model 517-01002-002) set at 50⁰C, after which the concentrate was further concentrated using a vacuum oven set at 40⁰C with a pressure of 700mmHg [9].

Processing of Clinical Specimens

The collected swab specimen inside a peptone transport medium was suspended in a sterile Nutrient agar, Blood agar, Mac Conkey agar and into GSTB medium specifically for the growth of *Streptococcus mutans* and incubated at 37⁰C for 18- 24 hours. Pure cultures of the bacteria isolate was carefully examined macroscopically for their cultural morphology and cellular characteristics respectively. Isolates was characterized based its colonial morphology (such as colour, shape). And petridishes was observed for *Streptococcus mutans*.

Antibacterial Activities of Neem Extracts against *Streptococcus mutans*

Agar well diffusion method was used for antibacterial assay. The extracts (ethanol and aqueous extracts of twigs, leaves and bark) were dissolved in DMSO (Dimethyl sulfoxide) to form a concentration of 20 mg/mL. DMSO was used as control. The turbidity standard of each of the *Streptococcus mutans* strains were prepared and compare to McFarland standard. With sterile

swab sticks, lawns of the standard were made on Brain-heart infusion broth and sterile cork borer of 7 mm was used to make wells on the plates. A micropipette machine was used to dispense 200 μ L of the crude extracts and their SNPs into their respective labeled wells. The plates were incubated at 37⁰C for 24 hours and the zones of inhibition (mm) were measured and recorded [10].

RESULTS and Discussion:

Isolation and Identification of *Streptococcus mutans* from the Oral Cavity

Table 2: Total Streptococcus sp. count on Glucose-Sucrose-Potassium tellurite-Bactracin Medium (GSTB)

Patient	X103 CFU/ml
1.	2.2
2.	1.4
3.	1.1
4.	2.5
5.	1.3
6.	1.5
7.	0
8.	1.7
9.	0

Table 2 showed the total Streptococcus sp. count on Glucose-Sucrose-Potassium tellurite-Bactracin Medium (GSTB). The specimen/ patient 7 and 9 did not contain any growth. This shows that *Streptococcus mutans* was absent.

Table 3: Staphylococcus sp. Count on Manitol Salt Agar (MSA)

Patient	X10 ⁴ CFU/ml
1.	7.0
2.	4.3
3.	2.4
4.	1.6
5.	1.1
6.	6.0
7.	2.7
8.	1.2
9.	5.0

Table 3 showed the Staphylococcus sp. Count for 9 samples on Manitol Salt Agar (MSA). The counts were 7.0, 4.3, 2.4, 1.6, 1.1, 6.0, 2.7, 1.2 and 5.0 respectively.

Table 4: Total Bacterial Count from 9 Patients with Dental Caries

Patient	X10 ⁸ CFU/ml
1.	2.3
2.	7.2
3.	6.9
4.	4.6
5.	5.3
6.	6.6
7.	6.5
8.	4.2
9.	1.5

Table 4 above showed the bacteria count from carious lesion of 9 samples collected from Maxillofacial and surgery clinic of University College Hospital. From patient 1 through 9, the bacterial count was 2.3, 7.2, 6.9, 4.6, 5.3, 6.6, 6.5, 4.2 and 1.5 respectively.

Table 5: Frequency of Occurrence of Bacteria in the Oral Specimen

PATIENT	<i>Streptococcus mutans</i>	<i>Staphylococcus aureus</i>	<i>Staphylococcus epidermidis</i>
1.	2	4	2
2.	3	5	4
3.	5	2	3
4.	2	6	2
5.	4	3	1
6.	3	5	4
7.	0	2	0
8.	5	2	2
9.	0	3	1
Total	24	32	19

Table 5 showed the frequency of occurrence of bacteria in the oral specimen. *Streptococcus mutans* count was 24, *Staphylococcus aureus* was 32. While *Staphylococcus epidermidis* was 19.

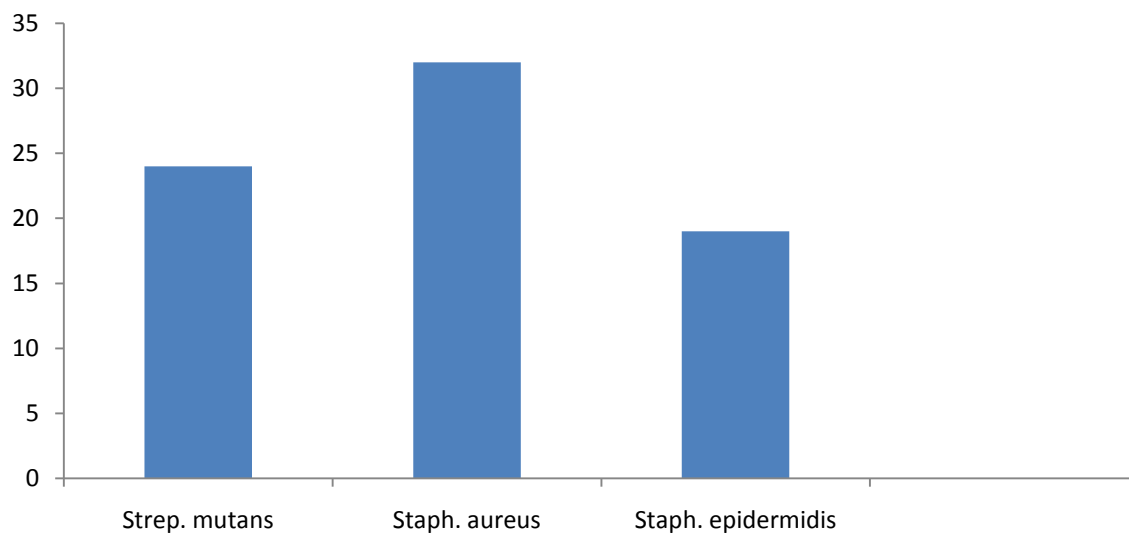


Fig 1: A bar chart representing the frequency of occurrence of bacteria in the oral specimen.

Antimicrobial Sensitivity Profile of Neem Extracts on *Streptococcus mutans*

Table 6: Antibacterial Activities of Aqueous and Ethanol Extract of Neem against *Streptococcus mutans* strain.

Strep. mutans in 14 petridish	Zone of Inhibition (mm)					
	ENT	ANT	ENL	ANL	ENB	ANB
S.M 1A	14.0	6.0	10.0	2.0	6.0	4.0
S.M 1B	22.0	16.0	18.0	10.0	14.0	6.0
S.M 2A	14.0	8.0	4.0	2.0	6.0	10.0
S.M 2B	12.0	7.0	2.0	6.0	8.0	6.0
S.M 3A	6.0	6.0	2.0	4.0	8.0	6.0
S.M 3B	11.0	8.0	6.0	4.0	10.0	8.0
S.M 4A	18.0	14.0	13.0	10.0	14.0	12.0
S.M 4B	0.0	2.0	0.0	2.0	4.0	4.0
S.M 5A	6.0	4.0	0.0	4.0	8.0	6.0

S.M 5B	5.0	8.0	4.0	4.0	6.0	4.0
S.M 6A	6.0	6.0	0.0	4.0	8.0	7.0
S.M 6B	4.0	2.0	1.0	6.0	7.0	12.0
S.M 8A	6.0	6.0	4.0	2.0	6.0	6.0
S.M 8B	6.0	4.0	4.0	4.0	0.0	0.0

KEY:

ENT: Ethanol extracts of neem twig

ANT: Aqueous extracts of neem twig

ENL: Ethanol extracts of neem leaf

ANL: Aqueous extracts of neem leaf

ENB: Ethanol extracts of neem bark

ANB: Aqueous extracts of neem bark

S.M: *Streptococcus mutans*

Dental caries is steadily increasing in the underdeveloped and developing countries. Treatment is expensive and not a realistic option for the poor. Hence, there is an urgent need to promote traditional preventive measures that are acceptable, easily available, and cost effective. Chewing sticks have been widely used in the Indian subcontinent, the Middle East, and Africa since ancient times. The chewing stick can be a good alternative to the toothbrush as a means of preventing oral and dental disease. It is suitable for cleaning the teeth, costs little, possesses various medicinal properties, and is easily available in the rural areas of the developing countries. It is also an oral hygiene tool that requires no expertise or special resources for its production[11].

The antimicrobial activity was assessed by observing the formation of an inhibition zone around the well containing the extracts, and the measurement of the zone of inhibition was conducted in millimeters using vernier calipers. Duplicate subcultures of each cultured sample in GSTB medium were performed, as outlined in Table 1.

Among the six extracts evaluated, both ethanol and aqueous extracts exhibited varying degrees of antimicrobial activity against *Streptococcus mutans*. Notably, exceptions were observed in *Strep. mutans* plates S.M 4B of ENT, S.M 4B, 5A, 6A of ENL, S.M 8B OF ENB, and ANB, where zero inhibition was recorded. This suggests that certain strains of *Strep. mutans* were not susceptible to the tested extracts, possibly due to the inability of plant metabolites to disrupt the bacterial cell wall.

The neem extract from ENT proved to be the most effective, displaying the highest antibacterial activity against *Streptococcus mutans* with inhibitions measuring 22.00mm and 18mm. Following closely, the neem extract from ENL exhibited an inhibition zone of 18mm, while the ANT extract of neem demonstrated an inhibition zone of 16mm. This enhanced effectiveness may be attributed to the presence of bioactive metabolites in the extracts, obtained through the use of ethanol and water in the extraction process.

A study conducted by Khalid [12] in Saudi Arabia supports these findings, as it explored the antimicrobial efficacy of aqueous neem extracts at various concentrations. Khalid reported that neem exhibited significant effectiveness against *Streptococcus mutans* at a 50% concentration.

The findings of this study reveal promising insights into the antimicrobial potential of neem plant extracts, specifically focusing on their impact on *Streptococcus mutans*, a key bacterial species associated with dental caries. The discussion encompasses a comprehensive analysis of the medical implications of the results and integrates relevant references to contextualize the findings within the existing scientific literature.

The absence of *Streptococcus mutans* in certain specimens suggests a potential variation in oral microbiota among individuals, reinforcing the multifactorial nature of dental caries. Previous studies have highlighted the heterogeneity in oral microbial communities and their role in oral health disparities [1]. The current research adds to this body of knowledge by emphasizing the importance of considering individual variations in designing preventive and therapeutic interventions.

Staphylococcus sp. and total bacterial counts exhibited variability among samples, underscoring the dynamic nature of the oral microbiome. The diversity in microbial populations in patients with dental caries aligns with the concept of microbial dysbiosis, where an imbalance in

microbial communities contributes to disease states [2]. The interplay between different bacterial species in the oral cavity and their implications for oral health warrant further exploration to develop targeted interventions.

The frequency of occurrence of *Streptococcus mutans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* provides a snapshot of the oral microbiota in the study population. These findings resonate with studies emphasizing the role of *Streptococcus mutans* as a primary cariogenic pathogen [13]. Additionally, the coexistence of other bacterial species such as *Staphylococcus aureus* and *Staphylococcus epidermidis* highlights the polymicrobial nature of oral infections [14]. This observation underscores the importance of addressing the broader microbial context in understanding and managing dental caries.

The antimicrobial sensitivity profile of neem extracts against *Streptococcus mutans* strains demonstrates varying zones of inhibition, indicating differential responses to neem components. This aligns with the notion that microbial strains may exhibit distinct susceptibilities to plant extracts due to variations in their genetic makeup [15]. The diverse pharmacological activities of neem compounds, including antibacterial and anti-inflammatory properties, have been extensively documented [16]. The current study contributes to this body of evidence by specifically assessing the efficacy of neem against *Streptococcus mutans*, highlighting its potential as a natural agent in combating oral pathogens.

The extraction methods employed in this study, utilizing both ethanol and aqueous solvents, offer a nuanced understanding of neem's antimicrobial potential. Ethanol extraction yielded extracts with notable inhibitory effects on *Streptococcus mutans*, emphasizing the importance of solvent choice in optimizing bioactive compound extraction. Similar studies have underscored the impact of extraction methods on the bioactivity of plant extracts, emphasizing the need for standardized protocols [17].

It is essential to acknowledge certain limitations in this study, such as the small sample size and the need for further in-depth characterization of the neem components responsible for the observed antimicrobial effects. Future research should explore the specific bioactive compounds within neem extracts and their mechanisms of action against oral pathogens.

Conclusion: this investigation into the antibacterial properties of neem plant extracts against *Streptococcus mutans*, a key contributor to dental caries, has provided valuable insights into the potential applications of neem in oral health. The study revealed the absence of *Streptococcus mutans* in some specimens, emphasizing the individual variations in oral microbiota. *Staphylococcus sp.* and total bacterial counts exhibited variability among samples, highlighting the dynamic nature of the oral microbiome and the importance of microbial dysbiosis in dental caries.

The frequency of occurrence of *Streptococcus mutans*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* depicted a polymicrobial nature of oral infections, underlining the complexity of microbial interactions in oral health. The antimicrobial sensitivity profile demonstrated varied zones of inhibition in response to neem extracts, indicating their potential efficacy against *Streptococcus mutans* strains. The neem extract from twigs exhibited the highest antibacterial activity, suggesting the presence of bioactive metabolites obtained through ethanol extraction.

These findings contribute to the broader discourse on the role of natural products in oral health, especially in the context of preventing and managing dental caries. The study lays the groundwork for future research endeavors focusing on the specific bioactive compounds within neem extracts and their mechanisms of action against oral pathogens. As dental caries remains a significant global concern, the exploration of alternative, cost-effective preventive measures, such as neem-derived remedies, is crucial for addressing oral health disparities. Integrating traditional herbal remedies with modern scientific methodologies opens avenues for developing novel strategies to combat oral diseases effectively.

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