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

- **Rephrase the title using the words such as *Effectiveness* or *Efficacy*.**
- **Rather than beginning the title with investigating, it would be better to start as *To evaluate …….***
- **Include which type of study, study setting, study population**

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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

Preferably don't put all the words in Italics as there won't be any difference between the term *Streptococcus mutans* and the others

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

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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.

- **Scientific names should be in Italics**
- **Methodology, not required in the introduction as there is a separate heading for it**
- **Last 2 paragraphs not required in the introduction, If a must to include then remove several terms which shouldn't sound like a discussion or a future recommendation**

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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.

- **Ethical clearance not mentioned**
- **Informed consent not mentioned**
- **Study duration not mentioned**

- **Sample size estimation not mentioned**
- **Statistical analysis not mentioned**
- **Which software used not mentioned**

Study Area

The study was carried out at the Department of Biochemistry and the Microbiology Laboratory of University of Ibadan, Oyo State. Oyo State covers approximately an area of 28,454 square kilometers and is ranked 14th by size. The landscape consists of old hard rocks and dome shaped hills, which rise gently from about 500 meters in the southern part and reaching a height of about 1,200 metres above sea level in the northern part. Some principal rivers such as Ogun, Oyan, Otin, Ofiki, Sasa, Oni, Erinle and Osun River originate in this highland. Oyo State contains a number of natural features including the Old Oyo National Park. In this location there was earlier habitat for the endangered African wild dog, Lycaonpictus; however, this canid is thought to have been locally extirpated at the present. The climate is equitorial, notably with dry and wet seasons with relatively high humidity. The dry season lasts from November to March while the wet season starts from April and ends in October. Average daily temperature ranges between 25 °C (77.0 °F) and 35 °C (95.0 °F), almost throughout the year. Oyowas formed in 1976from Western State, and included Osun State, which was split off in 1991. Oyo State is homogeneous, mainly inhabited by the Yoruba ethnic group who are primarily agrarian but have a predilection for living in high-density urban centres. The indigenes mainly comprise the Oyos, the Oke-Oguns, the Ibadans and the Ibarapas, all belonging to the Yoruba family and indigenous city in Africa. Ibadan had been the centre of administration of the old Western region since the days of British colonial rule. Other notable cities and towns in Ọyọ State include Oyo, Ogbomoso, Ibadan,Isẹyín-Okeogun, Ipapo-Okeogun, Kíṣì-Okeogun, Okeho-Okeogun, Saki-Okeogun, Igbeti-Okeogun, igboho-okeogun, Eruwa-Ibarapa,Iroko, Lanlate, Oje, Owode-Okeogun, Sepeteri-Okeogun, Ilora-Oyo, Jobele-Oyo, Awe-Oyo,Ilẹ́rò-Okeogun, Okaka-Okeogun, Igbo Ora-Ibarapa, Idere. The first degree awarding institution in Nigeria is the University of Ibadan (established as a college of the University of London when it was founded in 1948, and later converted into an autonomous university in 1962).The other universities in the state are: Lead City University, Ibadan, Ajayi Crowther, Oyo, Koladaisi University, Dominican University, Ibadan, Dominion University and Ladoke Akintola University of Technology,

Ogbomoso. The Polytechnic, Ibadan, Oyo State College of Agriculture and Technology Igbo Ora, Adeseun Ogundoyin Polytechnic Eruwa are located in Oyo State.

- **Too much unnecessary matter**

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-heartInfusionBroth

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 121^0C for 15 mins at 15 psi and allowed to cool to 45^0C before pouring 20 ml each into petri dishes. This was left on the bench to solidify and was incubated at 37^0C 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 121^0C for 15 mins at 15 psi and allowed to cool to 45^0C before pouring 20 ml each into petri dishes. This was left on the bench to solidify and was incubated at 37^0C 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 121^0C for 15 mins at 15 psi and allowed to cool to 45^0C before adding 5 % defribinated sheep blood and pouring 20 ml each into petri

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dishes. This was left on the bench to solidify and was incubated at 37^0C 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 121^0C for 15 min at 15 psi and allowed to cool to 45^0C before pouring into petri dishes and allowed to solidify on the bench and was incubated at 37^0C for 24 hrs to ascertain sterility.

Results

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

- **Full form required in the heading**

Component	Amount
Solution A	
Trypticase peptone (BBL	5g
Yeast extracts (Difco)	5g
K2HPO4	5g
Na2CO3	0.05g
Agar (BBL)	20g
Salt solution (consisting of 1.15g of MgSO4.7H2SO, 0.19g of MnSO4.H2O, 0.068g of FeSO4.H2O per 10ml of water)	0.5ml
Deionised water	800ml
Boil, cool and adjust pH to 7.2 with HCl	
Solution B	

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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 121^{0}C for 20 min, combined and allowed to cool to 50 to 55^{0}C before adding 1.0 ml of 1% potassium tellurite (Difco) and 300 U of bacitracin (Sigma), both filter and sterilized. After through stirring at 55^{0}C, 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 effiecient, made in germany, model 517-01002-002) set at 40^{0}C, after which the concentrate was further concentrated using a vacuum oven set at 40^{0}C with a pressure of 700mmHg.**(reference?)**

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

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whatman filter paper 1mm. The combined filtrate was concentrated using rotary evaporator (Heidolphlaborota 400 effiecient, made in Germany, model 517-01002-002) set at 50^0C, after which the concentrate was further concentrated using a vacuum oven set at 40^0C 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 37^0C 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*.

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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 µL of the crude extracts and their SNPs into their respective labeled wells. The plates were incubated at 37^0C for 24 hours and the zones of inhibition (mm) were measured and recorded [10].

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 3showed 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

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

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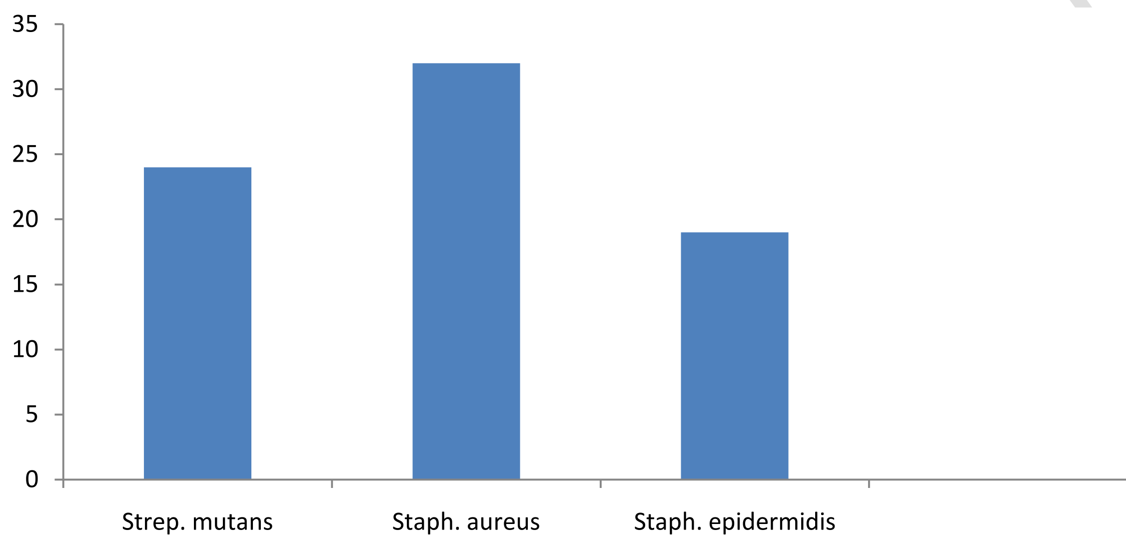

Fig 1: A bar chart representing the frequency of occurrence of bacteria in the oral specimen.

Antimicrobial Sensitivity Profile of Neem Extracts on Streptococcus *mutans*

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

	Zone of Inhibition (mm)					
Strep. mutans in 14 petridish	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

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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 etracts of neem leaf

ENB: Ethanol extracts of neem bark

ANB: Aqueous extracts of neem bark

S.M: Streptococcus *mutans*

Discussion:

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

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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 [11]. Additionally, the coexistence of other bacterial species such as Staphylococcus aureus and Staphylococcus epidermidis highlights the polymicrobial nature of oral infections [12]. 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 [13]. The diverse pharmacological activities of neem compounds, including antibacterial and anti-inflammatory properties, have been extensively documented [14]. 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

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the impact of extraction methods on the bioactivity of plant extracts, emphasizing the need for standardized protocols [15].

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: the results of this study shed light on the potential of neem plant extracts in addressing microbial imbalances associated with dental caries. The variation in oral microbial composition, the prevalence of Streptococcus mutans, and the differential response to neem extracts underscore the complexity of oral health dynamics. Integrating traditional herbal remedies like neem into contemporary oral healthcare practices holds promise for developing effective and culturally relevant strategies for preventing and managing dental caries.

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- **All the headings should be in capital letters and bold**

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