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"Effect of Neem Leaf and Neem Twig Extract on Prevotella Intermedia and Fusobacterium Nucleatum: An Ex Vivo Study."

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I. Introduction

Neem tree (*Azadirachtaindica*) is a very common tree and belongs to the family Meliaceae. It is a tall evergreen tree with clear foliage originally native of India. *Azadirachtaindica*is one of the most widespread tree species in Nigeria.[1]Medicinal plants are rich source of novel drugs that form ingredients in traditional system of medicine, modern medicine, pharmaceuticals intermediate, bioactive principles and lead compounds in synthetic drugs.[2]

In recent years, multiple drug resistance in humans and plant pathogens has developed due to indiscriminate use of synthetic drugs. This drive the need to screen medicinal plants for novel bioactive compound as plant based drug are biodegradable, safe and few side effects. As the public awareness for oral hygiene increases, many people are now turning to traditional medicine for a solution.[3]

In India, this plant is referred to as the village pharmacy because of its ability to cure many disorders ranging from bad teeth and bed bugs to ulcers and malaria. The Neem is of particular interest to the field of dentistry for it has a long history treating teeth and gum problems. In rural areas of India, the twigs are used as toothbrush to prevent gingivitis.[5]

Periodontal diseases is caused due to host- microbial interaction with large number of anaerobic periodontal pathogens contributing in tissue destruction. Pathogenic bacteria are known to liberate their toxins evoking an inflammatory response of the host causing initiation and progression of periodontal disease.[7]

Presently a large number of herbal products are researched for oral use in the form of mouth rinses, gels etc. Herbal extract have an advantage of cost effective, minimal side effect and good patient compliance. Neem leaves are used in the treatment of gingivitis and periodontitis and it is also efficacious in the treatment of oral infections and plaque growth inhibition.[8]

Neem also has broad range antibacterial activity. It removes toxins from the body, purifies the blood and neutralizes damaging free radicals. Neem extract contains Azadiractin the active principal, glycosides which has antimicrobial action, sterols and luminols are anti-inflammatory.[8]

Many studies in the literature have stated about efficacy of different neem extracts on various oral microorganisms but till now no literature have reported on the efficacy of different neem extracts on periodontal pathogens. Hence, this study is designed to assess the effectiveness of neem extract on periodontal pathogens namely *PrevotellaIntermedia* and *FusobacteriumNucleatum*.

II. Materials And Methods

Procurement of material

Fresh neem leaves and neem twigs weighing about 100gm each were obtained from VittalMallya Scientific Research foundation, Bangalore.The plaque sample was collected from 30 subgingival sites from 30 subjects who were clinically diagnosed as chronic generalized periodontitis.

Preparation of neem leaves and neem twig extract

The procedure for the preparation of neem extract was carried at VittalMallya Scientific Research foundation, Bangalore.

Materials required

1. Mouth mirror
2. William's graduated periodontal probe
3. Gracey universal curette 2R-2L and 4R-4L (Hu-Friedy)
4. Reduced transport fluid – RTF (Home made, in the laboratory)
5. Anaerobic Jar (Techno source)
6. Blood agar (Home made, in the laboratory)

7. Kanamycin blood agar (Home made, in the laboratory)
8. Dentaid agar (Home made, in the laboratory)
9. Crystal violet erythromycin agar (Home made, in the laboratory)
10. Aqueous extract of Neem twig (VittalMallya Scientific Research Foundation, Bangalore)
11. Aqueous extract of Neem leaves (VittalMallya Scientific Research Foundation, Bangalore).

100 gramof each fresh neem leaves and neem twigs were taken andcrushed them into powder by using liquid nitrogen.Then at room temperature 300ml methanol was added and stirred at room temperature for 12hrs. The mixture were filtered under vacuum. And the Clearfiltrate were evaporated under vacuum at below -40°C.Complete removal of methanol and dark brown material was done and then transferred to sample bottle. The product were preserved at -20°C.

Inclusion Criteria:
1. Patients between 30-50 years of age.
2. Patients with chronic generalized periodontitis.
3. Patients who were systemically healthy (not under any kind of medication).

Exclusion Criteria:
1. Patients with systemic diseases predisposing to periodontitis namely diabetes mellitus and Cardiovascular diseases.
2. Patients undergoing orthodontic treatment.
3. Patients using any form of partial dentures.
4. Patients requiring premedication or on medication such as antibiotic, anticoagulant, steroids, hormonal therapy or any other medications.
5. Patients who are current smokers.
6. Patients who are pregnant and lactating mothers.

Precautions which were taken during collection of plaque sample:

The moderate anaerobes are capable of growth in the presence of oxygen levels as high as 2% to 8%. Among these moderate anaerobes,*Prevotellaintermedia* and *Fusobacteriumnucleatum* are also one of them and could be exposed to room atmosphere for 60 to 90 minutes without appreciable loss of viability according to a study conducted by Loesche W in 1969. The tooth from which the subgingival plaque is to be collected were dried and isolated with cotton rolls. This tooth was made free from any kind of contamination like blood and saliva. A sterile Gracey curette kept inside the sterile pouch and was not opened until the above-mentioned precaution has been completed.

Then, the sterile Gracey curette was taken out of the sterile pouch and inserted subgingivally for the plaque collection and the collected plaque was immediately placed inside the transport media namely; reduced transport fluid (RTF). The RTF was kept as close as possible near the patient's mouth. After putting the plaque inside the RTF, the transport media container was closed immediately and tightened with an adhesive tape and then it was sent to the laboratory.

Methods of collection of plaque sample

30 Subgingival sites from 30 subjects who were clinically diagnosed as chronic generalized periodontitis with pocket depth measuring between 4 – 7 mm were selected and plaque from this site were collected using Gracey universal curette and transported in RTF and were sent to the laboratory [Department of Molecular Biology and Immunology, Maratha Mandal's, NGH Institute of Dental Sciences and Research Centre, Belgaum] for microbial assessment and to check antimicrobial activity of neem. Antimicrobial activity of neemwas checked for the minimum inhibitory concentration of the neem extract against *Prevotellaintermedia* and *Fusobacteriumnucleatum.*

Microbial analysis:

Sample received in the Transport medium (RTF) were first vortexed then inoculated in the culture medium according to the requirement in the enriched and selective medium.

For, *Prevotellaintermedia* Blood Agar was used as an enriched medium and incubated at 37 °C for 3-4 days in anaerobic jar. Kanamycin Blood Agar was used as anaerobic selective medium For *Fusobacteriumnuleatum* Selective medium was CVE (crystal violet Erythromycin agar)and then incubated at 37 °C for 3-4 days in anaerobic jar, these are strictly anaerobe. Then after completion of incubation the plates were removed and noted the colony characters of the required organism were noted and also the colony count was done for quantification.

Then the microbiological examination were performed for Grams staining to check the *PrevotellaIntermedia* and *FusobacteriumNucleatum*. These organisms were confirmed by grams staining and key biochemical - Glucose, sucrose, cellobiose and arabinose.

Minimum inhibitory concentration procedural steps

1. Nine dilutions of each neem extract were performed with Thioglycollate broth for MIC. In the initial tube, 20 microliters of neemwere added into the 380microliter of Thioglycollate broth.
2. For dilutions of 200 microliters of Thioglycollate broth, it was added into the next 9 tubes separately.
3. Then from the initial tube, 200 microliterswas transferred to the first tube containing 200 microliters of Thioglycollate broth. This was considered as 10^{-1} dilution.
4. From 10^{-1} diluted tube 200microliter was transferred to the second tube to make 10^{-2} dilution.
5. The serial dilution was repeated up to 10^{-9} dilution for each neem.
6. From the maintained stock cultures of required organisms, 5microliter was taken and added into 2ml of Thioglycollate broth.
7. In each serially diluted tube, 200 microliter of above culture suspension was added.
8. The tubes were incubated for 48-72 hours in an anaerobic jar at 37°C and observed for turbidity.

Statistical analysis:

The following methods of statistical analysis was used in this study. The results for each parameter (numbers and percentages) for discrete data and averaged (mean + standard deviation) for each parameter were presented in tables and figures.

Mann-Whitney Test:

A non-parametric test (distribution-free) used to compare two independent groups of sampled data. Unlike the parametric t-test, this non-parametric makes no assumptions about the distribution of the data (e.g., normality). This test is an alternative to the independent group t-test, when the assumption of normality or equality of variance is not met. This, like many non-parametric tests, uses the ranks of the data rather than their raw values to calculate the statistic. Since this test does not make a distribution assumption, it is not as powerful as the t-test. The test statistic for the Mann-Whitney test is U. This value is compared to a table of critical values for U based on the sample size of each group. If U exceeds the critical value for U at some significance level (usually 0.05) it means that there is evidence to reject the null hypothesis in favor of the alternative hypothesis. Actually, there are two versions of the U statistic calculated, where $U' = n_1n_2 - U$ where n_1 andn_2 are the sample sizes of the two groups. The largest of U or U' is compared to the critical value for the purpose of the test. In all above test P value less than 0.05 was taken to be statistically significant. The data was analyzed using SPSS (ver 10.5) package.

III. Results

The present study was an ex-vivo conducted to assess the antimicrobial efficacy of neem leaf and neem twig extracts on two organisms namely *Prevotellaintermedia* and *Fusobacteriumnucleatum.*The antimicrobial activity was determined by microbroth dilution metrhod. In each series of tubes, the last tube with clear supernatant was considered to be without any growth and taken as MIC value. Turbidity in the MIC tube indicated growth of the bacteria implying, the organism resistant to the aqueous neem extract.

At 5% and 7.5% of neem leaf extract showed MIC at 50ul whereas 10% and 15% of neem leaf extract demonstrated MIC at 25ul against *Prevotellaintermedia*.(table1)

It was observed that 5% and 7.5% of neem leaf extract revealed MIC at 100ul and 10% and 15% of neem leaf extract demonstrated MIC at 50ul against *Prevotellaintermedia.* (table1)

At 5% and 7.5% of neem leaf extract showed MIC at 100ul and 10% and 15% of neem leaf extract demonstrated MIC at 50ul against *FusobacteriumNucleatum.* (table2)

It showed MIC at 100ul for 5%, 7.5% and 10% of neem twig extract whereas 15% of neem twig extract demonstrated MIC at 50ulagainst*FusobacteriumNucleatum.* (table2)

The difference of effectiveness of neem leaf and neem twig extract of each four concentration against the *Prevotellaintermedia* and *Fusobacteriumnucleatum* demonstrated not statistically significant.(table 3)

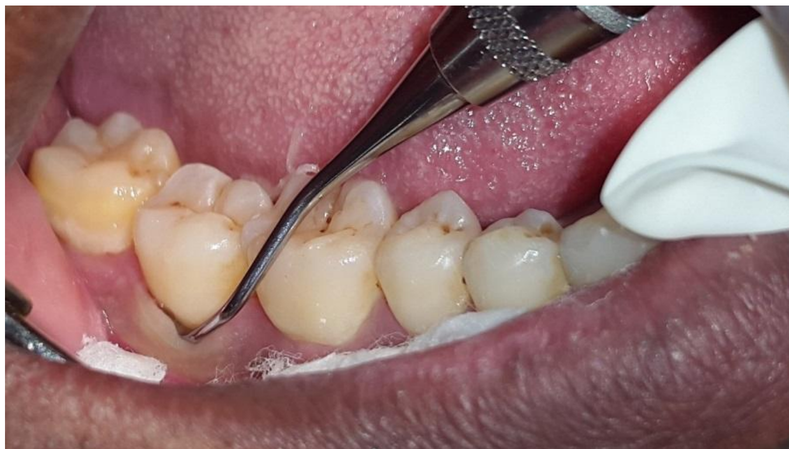


Fig 1: Subgingival plaque collection from molar region.

Fig 2 :- Photograph showing Microbial growth in culture media

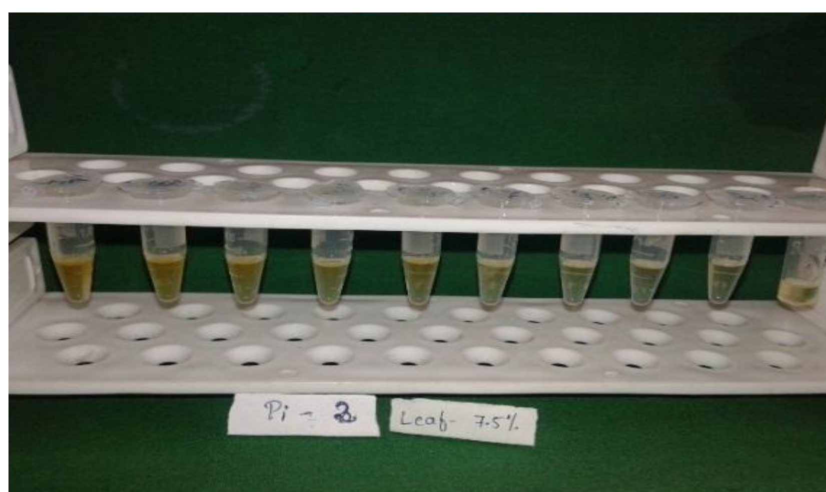


Fig 3: minimum inhibitory concentration

Fig 4: Neem twig powder

Fig 5: Neem leaf powder

Table no.1: Distribution of sensitivity of *Prevotellaintermedia*and *FusobacteriumNucleatum*in concentration at 5%, 7.5%, 10% and 15% of neem leaf extract respectively.

Neem extract	Type	Concentration	MIC								
			100	50	25	12.5	6.25	3.12	1.6	0.8	0.4
Neem leaf	Prevotellaintermedia	5%	S	S	R	R	R	R	R	R	R
		7.5%	S	S	R	R	R	R	R	R	R
		10%	S	S	S	R	R	R	R	R	R
		15%	S	S	S	R	R	R	R	R	R
	Fusobacteriumnucleatum	5%	S	R	R	R	R	R	R	R	R
		7.5%	S	R	R	R	R	R	R	R	R
		10%	S	S	R	R	R	R	R	R	R
		15%	S	S	R	R	R	R	R	R	R

S= Sensitivity and R= Resistance

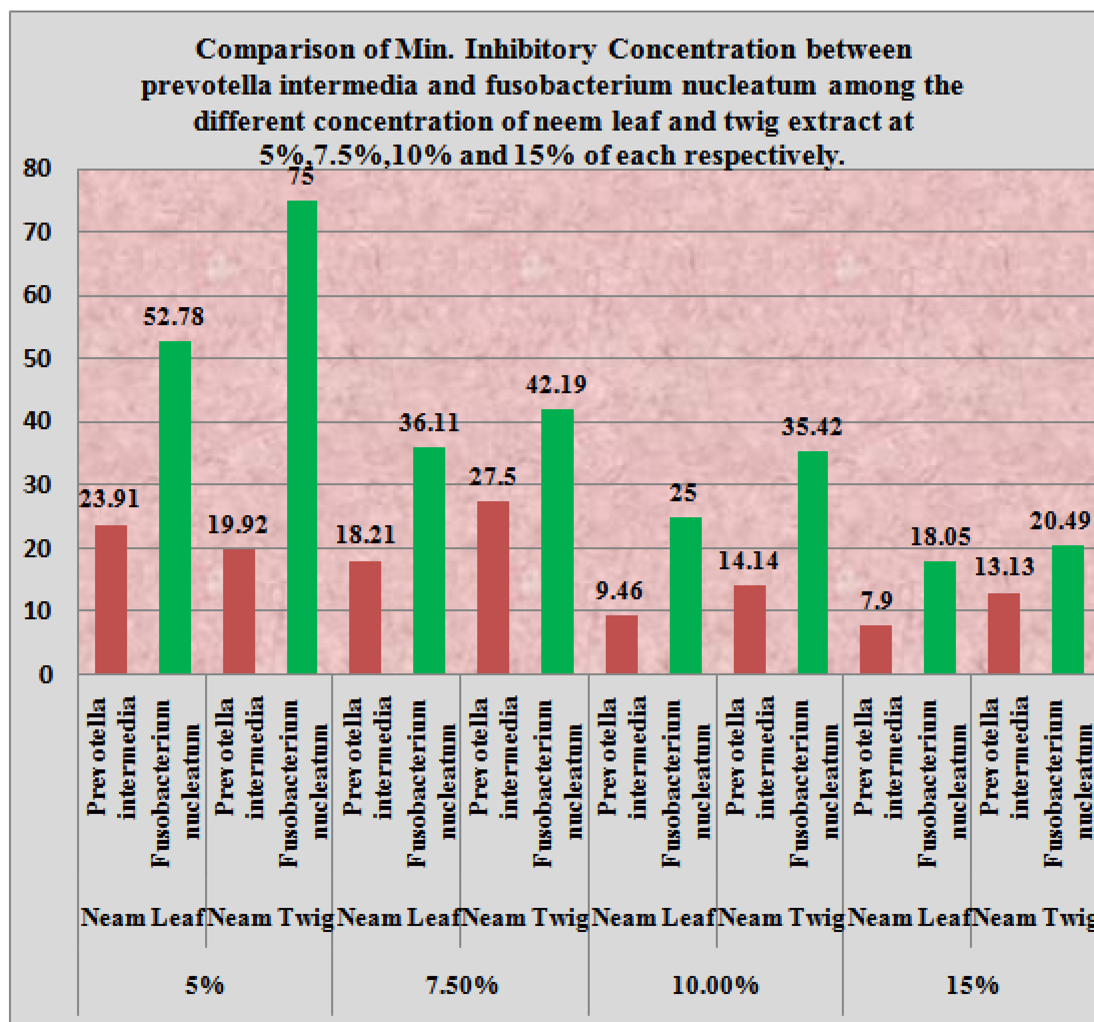
Table no. 2: Distribution of sensitivity of *Prevotellaintermedia* and *Fusobacteriumnucleatum* in concentration at 5%, 7.5%, 10% and 15% of neemtwig extract respectively.

Neem extract	Type	Concentration	MIC								
			100	50	25	12.5	6.25	3.12	1.6	0.8	0.4
Neem twig	Prevotellaintermedia	5%	S	R	R	R	R	R	R	R	R
		7.5%	S	R	R	R	R	R	R	R	R
		10%	S	S	R	R	R	R	R	R	R
		15%	S	S	R	R	R	R	R	R	R
	Fusobacteriumnucleatum	5%	S	R	R	R	R	R	R	R	R
		7.5%	S	R	R	R	R	R	R	R	R
		10%	S	R	R	R	R	R	R	R	R
		15%	S	S	R	R	R	R	R	R	R

S= Sensitivity and R= Resistance

Table no. 3: Comparison of effectiveness between neem leaf and twig extract of each concentration at 5%, 7.5%, 10% and 15% on *Prevotellaintermedia* and *Fusobacteriumnucleatum.*

Type	Concentration	Comparison between Neem extract	N	Mean	SD	Median	Min.	Max.	Mann-Whitney U*	P value
Prevotellaintermedia	5%	Neem Leaf	10	23.91	23.496	15.63	.80	50.00	39.500	0.965
		Neem Twig	8	19.92	19.972	12.50	3.12	50.00		
	7.5%	Neem Leaf	10	18.21	19.926	14.06	.40	50.00	40.500	0.481
		Neem Twig	10	27.50	38.832	12.50	3.12	100.00		
	10%	Neem Leaf	10	9.46	9.653	7.81	.40	25.00	46.500	0.796
		Neem Twig	10	14.14	19.383	4.69	.40	50.00		
	15%	Neem Leaf	10	7.90	9.800	3.93	.40	25.00	48.500	0.912
		Neem Twig	10	13.13	20.003	2.36	.40	50.00		
Fusobacteriumnucleatum	5%	Neem Leaf	9	52.78	36.324	25.00	25.00	100.00	20.000	0.139
		Neem Twig	8	75.00	26.726	75.00	50.00	100.00		
	7.5%	Neem Leaf	9	36.11	39.003	25.00	6.25	100.00	25.500	0.321
		Neem Twig	8	42.19	27.498	37.50	12.50	100.00		
	10%	Neem Leaf	9	25.00	20.252	25.00	6.25	50.00	30.500	0.387
		Neem Twig	9	35.42	30.298	25.00	6.25	100.00		
	15%	Neem Leaf	9	18.05	16.224	25.00	3.12	50.00	34.000	0.605
		Neem Twig	9	20.49	18.562	12.50	3.12	50.00		



Graph 1: Comparison of mean minimum Inhibitory concentration between neem leaf and twig extract of each concentration at 5%, 7.5%, 10% and 15% on *PrevotellaIntermedia* and *FusobacteriumNucleatum.*

IV. Discussion

In the present study,the MIC of the prepared neem leaf and neem twig extract were studied on two periodontal pathogens, i.e. *Prevotellaintermedia* and *Fusobacteriumnucleatum.*

The MIC value in the present study for neem leaf extract against the *Prevotellaintermedia* ranged from 25ul to 50 ul whereas the MIC value for neem twig extract against the same pathogen demonstrated 50ul to 100ul.

The effectiveness of neem leaf and neem twig extract of each four concentration against the *Prevotellaintermedia* and *Fusobacteriumnucleatum* demonstrated not statistically significant.

The antimicrobial activity of neem leaf extract against *Fusobacteriumnucleatum* showed MIC value at 50ul. And the MIC value ranged from 50ul to 100ul for neem twig extract against *Fusobacteriumnucleatum.*Prashant and co-workers revealed that neem stick extract established the maximum zone of inhibition on *streptococcus mutans*at 50% concentration. Even at 5% concentration neem extract showed some inhibition of growth for all the four species i.e. *streptococcus mutans, streptoccussalivarius, streptococcus sanguis*and *streptococcus mutans*. [9]

Owing to the bitter taste associated with this plant, various different formulations have been developed. It was formulated along with the sweetener and flavor to increase the patient compliance and acceptability.[6]

Application of neem chip after scaling and root planing in chronic periodontitis patient demonstrated statistical significant reduction in probing depth at 6weeks and 3 months and a significant reduction in gingival index score after 3 months as well as there was decrease in plaque index scores and gain in clinical attachment levels.[12]

The ethanolic extracts of various leaves namely *MoringaOleifera, Musa Paradisiaca, AzadirachtaIndica, CynodonDactylon, AlternantheraSessils, AnisochilusCarnosu*s were evaluated individually for antimicrobial activity by disc diffusion method against the four selected species i.e. *Escherichia Coli, Bacillus Subtilis, Vibrio Cholera* and*Klebsiella Pneumonia.* The results revealed considerably high effective against Escherichia coli than other extracts.[7]

V. Conclusion

The present study investigated an ex-vivo antimicrobial efficacy of neem leaf and neem twig extract against the two periodontal pathogens namely *PrevotellaIntermedia* and *FusobacteriumNucleatum.* It can be concluded thatfour different concentration of neem leaf extract i.e. 5%, 7.5%, 10% and 15% were effective against the *PrevotellaIntermedia* and *FusobacteriumNucleatum.* Likewise four different concentration of neem twig extract also were effective against the two periodontal pathogens. The effectiveness of the two neem extracts were not statistically significant.

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Conflicts of interest

There are no conflict of interest

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