



Original Research Article

Quantitative analysis of anti-bacterial properties of Tulsi (*ocimum sanctum*) and Neem (*azadirachta indica*) plant extracts- in vitro study

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ABSTRACT

Oral cavity cohabitates over 750 bacterial species of which only 50% relate to the pathogenesis of oral diseases such as dental caries.¹ Due to the insufficient treatment protocols, new avenues in field of pharmacology are required. As an alternative method of treatment, use of medicinal plants in dentistry has recently drawn attention of many dental researchers. The objective of this study was to quantitatively analyze the anti-bacterial properties of Tulsi (*Ocimum sanctum*) and Neem (*Azadirachta indica*) plants extracts on *Streptococcus mutans* under in vitro conditions. A total of thirty culture plates per plant including 10 plates per extract served as sample size for the study. The leaves, stems and roots of both the plants were shade dried and powdered and extracted by process of successive extraction. Overnight growth of the test organism in Brain Heart infusion was taken and sub-cultured in Blood Agar. The colonies were transferred to Blood Mueller Hilton (BMH) agar and growth pattern was observed the following morning. Three wells of 6mm diameter each were filled with ten micro-litre of the working suspension of different crude extracts of both Neem and Tulsi with the help of micropipettes. Plates were incubated at 37°C for 48 hours and zone of inhibitions (ZOI) were measured. The study illustrated the mean values for the zones of inhibition (mm) for Neem to be 25.50mm, 26.60mm and 25.20 mm, respectively and that for Tulsi to be 22.80 mm, 20.40 mm and 21.20 mm, respectively which were significantly greater than that of Cephotaxime (23.00mm).

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1. Introduction

Oral diseases are among the major health issues wherein dental caries and periodontal diseases are the most commonly occurring problems.¹ Oral cavity is cohabitated by over 750 bacterial species of which only 50% related to the pathogenesis of oral diseases such as dental caries.¹ There has been a rise in the oral disease incidence since 1998, particularly in the developing nations like India due to dietary habits, low awareness among the population regarding the oral health, increased antibiotic resistance

exhibited by the pathogenic bacteria, opportunistic oral infections in subjects with compromised immunity and low per capita income.¹

Normal commensals of the oral cavity such as *Streptococcus mutans* (MS) species actively participate in the initiation and progression of dental caries.²

The bacterial population in oral cavity undergoes constant changes because of changes in local environment (pH, for example) of the oral cavity, salivary effects and changes in colony population mainly due to weak or suppressed immunity.³

Oral disease manifests when the pathogenic bacterial colonization overrides the host immune response and starts

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adhering to the oral mucous membrane and penetrates it, eventually.³

Due to the insufficient current treatment protocols, new avenues in field of pharmacology are required.⁴ As an alternative to current methods of treatment, use of medicinal plants in dental treatment has recently drawn attention of many dental researchers.³ The term ‘Medicinal Plant’ is not a taxonomic term, but based on the utility of the plants.¹ Any plant used in any system of medicine can be categorized as a medicinal plant.¹ Phytotherapy uses complexes of compounds included in plants or substances isolated from plants, which are homogenous compounds of a particular chemical structure.⁴

Various medicines (phytoextracts) like antibacterial, anti-inflammatory and analgesics comprising of valuable simple and complex compounds such as polysaccharides, lectins, tannins, flavonoids, polyphenols, etc. are derived from plants or their parts such as leaves, stem, root, fruits, resin, flowers or barks.³ Phytoextracts are derived mainly from leaves that serve as the dominant source of compounds used in oral care (25.44%), followed by root (20.17%), seed/nut/fruit (18.42%), bark (14.03%), stem (12.28%), whole plant (9.65%) and resin (8.77%).¹ A number of plant products are used as antimicrobial agents such as phenolics, polyphenols, simple phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, coumarins, terpenoids and essential oils.⁵

Herbal products have also shown favorable results when administered to treat oral manifestations of certain systemic disorders and irritational inflammatory lesions.³ Due to these anti-microbial properties, herbal products are used as active components in toothpastes, rinsing solutions and gels.³

It is suggestible that like any other field of medicine, dentistry should also consider restraining the use of antibiotics as inappropriate and inadvertent administration can result in destruction of physiological bacteria, allergic manifestations, and resistant bacterial strains.⁴ The principal advantages of using plant derived products over allopathic drugs are- low risk of side-effects and cost-effectiveness, making them much more safer and convenient than the artificially synthesized substitutes.⁵

As an alternative to antibiotics, a global interest in the available natural sources for medicine has emerged.⁶ Many plants are used for their medicinal potential and numerous studies (Smith, 2004; Dohare SL, 2012) are carried out over the last two-three decades to detect the chemical and antimicrobial action of various plants and herbs and their modes of action in the treatment of various ailments.^{1,7} Amongst these studied plants are: Tulsi (*Ocimum sanctum*), Neem (*Azadirachta indica*), Guava (*Psidium guajava*), Pudina (*Mentha piperita*), etc.^{8,9}

Tulsi (*Ocimum sanctum*): Tulsi is the traditional Indian herb, also known as “Queen of Herbs” or the “Mother

Medicine of Nature” as it has rich antimicrobial substances (eugenol, methyl eugenol, carvacrol, sesquiterpine hydrocarbon, caryophyllene, linolenic acid, ursolic acid, cirsilineol, etc.) that have been used to treat variety of illnesses like dental caries, gingivitis, bleeding gums, oral ulcers and halitosis.⁹ At a concentration of 4% Tulsi extract, 22 mm zone of inhibition has been observed against *Mutans streptococcus* (A Pooja, 2016).⁸

Neem (*Azadirachta Indica*): “Indian neem/ Margosa tree” or “Indian lilac” is a sacred Indian medicinal Plant.⁹ It is an ingenious medicinal plant that has a wide variety of biological activities such as antioxidant, anti-inflammatory and antibacterial.¹ The aim of this study was to determine and compare the anti-bacterial properties of different phytoextracts obtained from the above-mentioned plants and to evaluate the efficacy of these medicinal plant extracts in effective reduction of cariogenic pathogen namely *Mutansstreptococcus* in oral cavity under in vitro conditions.

2. Materials and Methods

The study was carried out to determine and compare the anti-bacterial properties of three extracts each obtained from two previously mentioned plants i.e., Neem and Tulsi on *Mutans streptococcus*, a cariogenic pathogen.

A total of thirty culture plates per plant including 10 plates per extract served as sample size for the study.

2.1. Armamentarium required

For extract preparation

1. Shade dried plant parts (leaves, stems and roots)
2. Mortar and pestle
3. 80% ethanol
4. Conical glass flasks
5. Sonicator bath (ELMASONIC)
6. Whatman’s filter paper (A1, 125mm)
7. Spherical evaporating flasks
8. Rotary vacuum Evaporator (BUCHI INDIA PVT. LTD.)
9. Eppendorf tubes

For determination of Zones of inhibition

1. Culture petriplates
2. Inoculating loops, tips
3. Hot air oven
4. Incubator
5. Culture media (Brain Heart Infusion Broth, Blood Agar and Mueller Hilton Blood Agar) (Hi-MEDIA, India)
6. Hydrogen peroxide (H₂O₂)

2.2. Methodology

The plant materials belonging to study plants (*Ocimum sanctum* and *Azadirachta indica*) were collected. The main reason for selection of the above mentioned plants for the study were:

1. Easy availability of the plants in the Indian sub-continent.
2. Cost effectiveness.
3. As per some recently studies by Singh AR (2013),¹⁰ Aggarwal P (2014),⁸ these plants were reported to possess anti-bacterial potential by virtue of active biochemical compounds present in them.

Mutans streptococcus was selected as the test organism because it plays a major role in the progression of the process of formation of dental caries and many other dental ailments.

2.3. Identification and authentication of plant material

The plants were identified and authenticated by Janaki Ammal Herbarium (RRLH), CSIR-Indian Institute of Integrative Medicine (IIIM), Jammu, Jammu & Kashmir, India (Acc. No.- 22932/ Coll. No.- 53655 & Acc. No.- 22931/ Coll. No.- 53656, respectively). The fresh leaves were washed with distilled water to remove debris and dried at room temperature (35-40°C) for 10 days to prepare the Herbarium Sheets.

3. Preparation of extract

The leaves, stems and roots of both the plants were shade dried and powdered using mortar and pestle and then, extracted by process of successive extraction. Firstly, 60gms each of the powdered plant materials was put into 50ml of 80% ethanol in individual conical flasks. The flasks were rested for 30 minutes and then, subjected to sonication for 30 minutes at 45°C three times to extract the constituents from the plant materials. Prepared extracts were filtered using Whatman's filter paper all three times. The extracts obtained by filtration were transferred into spherical flasks and concentrated to dryness by evaporating the solvent using reduced pressure and heat in a rota-vapour apparatus. Pressure dried extracts were taken out of the flasks by scraping and transferred to Eppendorf tubes (Figure 1).

3.1. Chemicals and culture media

Bacterial cultivation was done on Brain Heart Infusion Broth (Hi-MEDIA, India) and Blood Agar (Hi-MEDIA, India). Thereafter, culturing on Mueller Hilton Blood Agar (Hi-MEDIA, India) was done for analyzing extract's sensitivity.

3.2. Culture preparation methods

3.2.1. For brain heart infusion broth

1. Dissolved 37g media in 1000ml of distilled water.
2. Mixed well and distributed into final containers.
3. Sterilized by autoclaving at 121°C for 15 minutes
4. Introduced test organism into each container with the help of the pure culture sample stick of *Streptococcus mutans* species (ATCC 25175) manufactured by Hi-MEDIA.
5. Kept overnight for growth of the test organism at 37°C.

3.2.2. For Blood Agar (Test Organism Sub-culturing)

1. Prepared the blood agar base as per manufacturer's guidelines.
2. Autoclaved at 121°C for 15 minutes at 15 lbs pressure and transferred to 50°C water bath to cool it down.
3. Added sterile blood and mixed gently to avoid air bubble formation.
4. Dispensed 15ml of prepared agar into sterile petri-plates, aseptically.
5. Labeled the petri-plates and let the agar material to set.
6. The opalescent growth of the test organism obtained after overnight incubation in BHI broth was transferred to Blood agar for sub-culturing for 24 hours at 37°C.

3.2.3. For Mueller Hilton Blood Agar

1. Suspended 38.0 grams of commercial media in 1000 ml of distilled water.
2. Heated to boil and to dissolve the medium completely.
3. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
4. Cooled to 45-50°C.
5. Added 50 ml Human blood obtained from the Department of Microbiology, MMIMSR.
6. Mix well and pour into sterile petri-plates.
7. Replace the lid of each petriplate and store the plates in a refrigerator.
8. The colonies obtained by the overnight growth on blood agar were then transferred to MHB agar and incubated for 1 hour.
9. Three wells of 6mm diameter were bored [Picture 3] in the medium with the help of sterile borer having 6mm diameter and were labeled properly and ten micro-litre of the working suspension i.e., 0.1gm of crude extract/100ml of DMSO (Picture 2) of different crude extracts of both Neem and Tulsi along with 30mcg of Cephalexin (positive control) and ten micro-litre of DMSO (negative control) in different petri-plates, were filled in the wells with the help of micropipette.
10. Plates were left for some time (5-10 minutes) till the extract diffuse in the medium with the lid closed and incubated at 37°C for 48 hours and zone of inhibitions were measured using scale and mean were recorded.

(Figures 3 and 4).

3.3. Organism description

The pure culture samples of *Streptococcus mutans* species (ATCC 25175) manufactured by Hi-MEDIA, were procured for the study from Microbiologics USA. Gram-positive microbial species were analyzed, namely *Streptococcus mutans* using culturing techniques and confirmation was done using Gram staining and catalase positivity test.

4. Observations and Results

In the study the leaf, stem and root crude extracts of Tulsi & Neem were evaluated for antibacterial activity on standard strain of cariogenic bacteria, *Mutans streptococcus*. The antibacterial efficacy of *Ocimum sanctum* and *Azadirachta indica* extracts (0.1g/ml) against *Mutans streptococcus* was evaluated by the agar well diffusion method via determination of surrounding zone of inhibition (Tables 2 and 3). As per the study results, all the three Tulsi extracts (leaf, stem and root) were found to be less active (ZOI= 22.80mm) against tested pathogen, *Mutans streptococcus* when compared to Neem extracts which showed more active zone of inhibition of 26.60mm against *Mutans streptococcus* (Table 4). Also the Neem extracts showed more active zone when compared to Cephalexin (positive control) with a zone of inhibition equal to 23mm. Amongst all the studied crude extracts in concentration of 0.1g/ml, Neem stem extract showed the highest activity with the maximum 26.60 mm zone of inhibition against *Mutans streptococcus* (Table 3).

Among all three Tulsi extracts, the leaf extract of Tulsi showed the highest (22.8 mm inhibition zone) as compared to the stem and root extracts of Tulsi (Table 2). This observation is also concordance by many studies including Agarwal et al. in 2010⁸ and Ali H et al. (2012).¹¹ In these studies, it was reported that leaf extract was more potent antimicrobial agent.

Result obtained from the minimum inhibitory concentration (MIC) of different Neem extracts on *Mutans streptococcus* (Table 3) revealed that the all extracts showed the best result with MIC value of 0.1g/ml when compared to Tulsi extracts and positive control (Cephalexin). In the study of Cecily Rosemary Lathe R et al¹² in 2015, they also reported that Neem was effective antibacterial agent and showed the lowest MIC value compared to other extracts which is in approbation of our study (Tables 3 and 4), but are in accordance with the studies carried by Siswomihardjo W et al. (2007)¹³ and F Uwimbabazi et al. (2015).¹⁴

Table 1 illustrates the distribution of total sample. From total 60 samples, 30 (50%) were of Neem (*Azadirachta indica*) whereas rest 50% were of Tulsi (*Ocimum sanctum*). The 30 samples for both Neem and Tulsi further categorised into 10 leaf extract samples, 10 stem extract samples and 10

root extract samples.

Table 2 depicts the zones of inhibition (mm) for leaf, stem and root extracts of Tulsi and Positive control against *Mutans streptococcus*, with mean values equivalent to 22.80 mm, 20.40 mm and 21.20 mm (for Tulsi) and 23 mm, respectively for positive control (Cephalexin).

Table 3 depicts the zones of inhibition (mm) for leaf, stem and root extracts of Neem and positive control against *Mutans streptococcus*, with mean values equivalent to 25.50 mm, 26.60 mm and 25.20 mm (for Neem) and 23 mm, respectively for positive control.

Table 4 demonstrates the multiple comparisons by using Bonferroni's test (Post Hoc Test) and according to this, all stem extracts showed highly significant values ($p=0.000$). The results were also significant when values of Tulsi stem and positive control and root extracts of Neem and Tulsi were compared ($p= 0.004$ & $p= 0.022$, respectively).

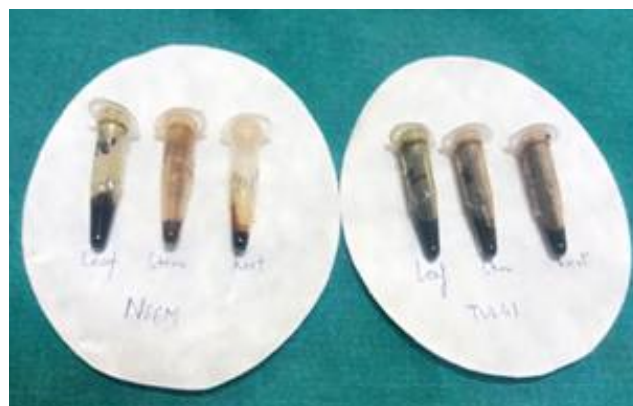


Fig. 1: Three different crude extracts of Tulsi and Neem

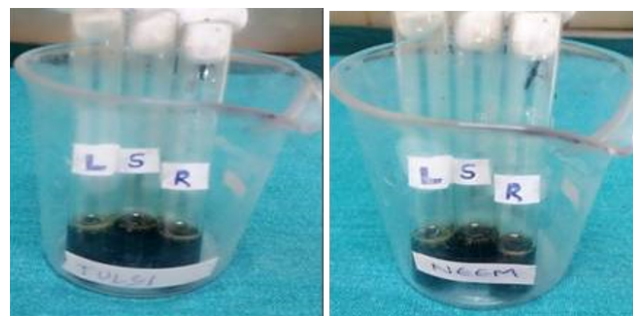


Fig. 2: Different Tulsi and Neem extracts dissolved in DMSO (L- Leaf extract, S- Stem extract & R- Root extract)

Table 1: Distribution of total sample

Total no of sample	Nature of phytoextracts	
	Neem (Azadirachta indica)	Tulsi (Ocimum sanctum)
60	30 (50%) 10 – Leaf sample 10 – Stem sample 10 – Root sample	30 (50%) 10 – Leaf sample 10 – Stem sample 10 – Root sample

Table 2: Antibacterial activity of different Tulsi extracts concentration 0.1gm/ml (10 μ l) on *Mutans streptococcus* in agar well diffusion method.

S. No.	Tulsi Leaf (mm)	Tulsi Stem (mm)	Tulsi Root (mm)	Positive Control (Cephotaxime) (mm)
1	22	22	20	22
2	18	22	22	22
3	26	20	26	20
4	22	22	24	23
5	32	16	18	27
6	20	20	18	23
7	26	20	28	20
8	16	20	18	26
9	24	22	20	24
10	22	20	18	23
Mean	22.80	20.40	21.20	23

Table 3: Antibacterial activity of different Neem extracts concentration 0.1gm/ml (10 μ l) on *Mutans streptococcus* in agar well diffusion method

S. No.	Neem Leaf (mm)	Neem Stem (mm)	Neem Root (mm)	Positive Control (Cephotaxime) (mm)
1	29	26	22	22
2	26	29	29	22
3	28	28	28	20
4	26	28	28	23
5	22	28	29	27
6	20	29	22	23
7	28	26	26	20
8	28	22	22	26
9	22	22	22	24
10	26	28	24	23
Mean	25.50	26.60	25.20	23

5. Discussion

Since ancient times, several plants and their products have been used as sources for curatives in both developed and developing world because of their potential to prevent disease attributed to their anti-microbial as well as other pharmacological properties.¹⁵ Based on these properties, several plants are considered to be the crucial sources of traditional medicines and drug manufacturing today and since the very beginning of civilizations.¹⁶ As per estimation (World Health Organization), at least 25% of all modern drugs are synthesised either directly or indirectly from plants and their products playing an important role in

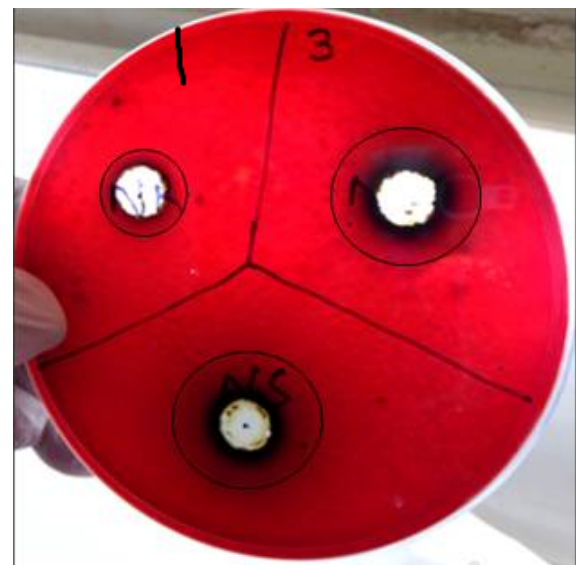
treatment of diseases worldwide and affecting millions of people.¹⁷

A wide range of various plant and herb species with great medicinal potential are found in the tropical and subtropical regions of India. Among the vast variety of secondary metabolites found in these plants are compounds such as flavonoids, terpenoids, tannins, glycosides and alkaloids. These metabolites serve as important agents of antimicrobial crude drugs and source of other anti-infection compounds.¹⁷

Development of antibacterial drug resistant microorganisms is leading to the occurrence of many

Table 4: Post Hoc Test for multiple comparisons by using Bonferroni's Test

Dependent Variable	Extract I	Extract II	Mean Difference (I-II)	Std. Error	Sig.	Inference
LEAF	Neem	Tulsi	2.700	1.535	0.270	Non sig.
		Control Positive	2.500	1.535	0.345	Non sig.
	Tulsi	Neem	-2.700	1.535	0.270	Non sig.
		Control Positive	-0.200	1.535	1.000	Non sig.
	Positive Control	Neem	-2.500	1.535	0.345	Non sig.
		Tulsi	0.200	1.535	1.000	Non sig.
STEM	Neem	Tulsi	6.200*	1.014	0.000	Highly Sig.
		Control Positive	3.600*	1.014	0.004	Highly Sig.
	Tulsi	Neem	-6.200*	1.014	0.000	Highly Sig.
		Control Positive	-2.600*	1.014	0.049	Sig.
	Control Positive	Neem	-3.600*	1.014	0.004	Highly Sig.
		Tulsi	2.600*	1.014	0.049	Sig.
ROOT	Neem	Tulsi	4.000*	1.375	0.022	Sig.
		Control Positive	2.200	1.375	0.364	Non sig.
	Tulsi	Neem	-4.000*	1.375	0.022	Sig.
		Control Positive	-1.800	1.375	0.605	Non sig.
	Control Positive	Neem	-2.200	1.375	0.364	Non sig.
		Tulsi	1.800	1.375	0.605	Non sig.

**Fig. 3:** Antibacterial activity of different Tulsi extracts at concentration of 0.1gm/ml (10 μ l) on Mutans streptococcus in agar well diffusion method**Fig. 4:** Antibacterial activity of different neem extracts at concentration of 0.1gm/ml (10 μ l) on Mutans streptococcus in agar well diffusion method

bacterial diseases in south Asia, particularly in India. To handle this problem it has become a global challenge to use newer sources to develop other therapeutic reforms.¹⁸

Our present study was done to investigate the antibacterial properties of Tulsi and Neem extracts (folk medicinal plants, being used for centuries in treating local population) against Mutans streptococcus.

The study was aimed at extracting three different types of extracts (leaf, stem and root) from Tulsi and Neem, using sonication and vacuum evaporator. The extracts were used at a dose of 0.1gm/ml by dissolving the crude extract with

the solvent (DMSO).

In an earlier study, Agarwal et al. in 2010⁸ demonstrated that among all the extracts of *Ocimum sanctum*, extract at 4% concentration showed highest antibacterial activity against MS with an inhibition zone of 22mm, almost equivalent to the results of our study where ZOI was found to be 22.80mm for Tulsi (Tables 2 and 3).

In another study by Ali H et al. (2012),¹¹ it was concluded that Tulsi at a concentration of 400mg/ml showed inhibitory zones ranging from 19.55 mm to 20.95 mm

against E.coli, Proteus and other strains of Staphylococcus, showing almost similar results when compared to the current study.

Goyal P et al, 2011¹⁹ demonstrated the antibacterial activity of the steam distilled extract of Tulsi and concluded that the extracts showed zones of inhibition ranging from 30mm – 39mm against different bacteria. However, this study showed significantly greater results when compared to the results of our study (ZOI= 22.80mm). The reason for greater zones of inhibition can be attributed to the technique of steam distillation and the bacterial species studied. Steam distillation leads to the extraction of only the active volatile aromatic compounds like essential oil and eugenol that are responsible for greater antibacterial potential.

In 2015, Cecily Rosemary Lathe R et al¹² in their study demonstrated the antibacterial efficacy of Neem and concluded that Methanolic extracts of Neem showed antibacterial potential with a zone of inhibition of 26mm. The results of their study were similar to the results obtained in our study.

Further in 2014, Soniya A et al²⁰ carried out a study on the antibacterial potential of Neem extracts and observed a maximum inhibitory zones of 20mm diameter, which is significantly smaller than that obtained in our study (ZOI= 26.60mm). The reason for such difference is because of the solvent (methanol) that reduces the antibacterial efficacy of extracts.²¹

As per our current study, when the antibacterial efficacy of Tulsi extracts, Neem extracts and positive control were compared with one another, the antibacterial potential of Neem tree was significantly greater than that of Tulsi and Cephalexin (Table VI).

6. Source of Funding

None.

7. Conflict of Interest

The authors declare that there is no conflict of interest.

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