

The antimicrobial activity of *Azadirachta indica*, *Mimusops elengi*, *Tinospora cardifolia*, *Ocimum sanctum* and 2% chlorhexidine gluconate on common endodontic pathogens: An *in vitro* study

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ABSTRACT

Objective: To check the antimicrobial activity of *Azadirachta indica* (Neem), *Ocimum sanctum* (Tulsi), *Mimusops elengi* (Bakul), *Tinospora cardifolia* (Giloy) and Chlorhexidine Gluconate (CHX) on common endodontic pathogens like *Streptococcus mutans*, *Enterococcus faecalis* and *staphylococcus aureus*. **Materials and Methods:** The agar diffusion test was used to check the antimicrobial activity of the Methanolic extracts of the medicinal plants along with CHX. Six different concentrations of the tested agents were used for the study. The values of Zone of Inhibition were tabulated according to the concentration of the tested agent and data was statistically analyzed using ANOVA and Bonferroni *post-hoc* tests. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentrations (MBC) values were also recorded. **Results:** All the plants extracts showed considerable antimicrobial activity against selected endodontic pathogens. At 3mg. concentration, *O.sanctum* was the most effective against *S. mutans*, *M. elengi* showed highest zone of inhibition against *E.faecalis*, whereas CHX was the most effective agent against *S.aureus*. CHX was also the most consistent of all the medicaments testes, showing inhibitory effect against all the tree pathogens at all the selected concentrations. **Conclusions:** The Methanolic extract of *A.Indica*, *O.sanctum*, *M. Elengi*, *T.cardifolia* and Chlorhexidine Gluconate has considerable antimicrobial activity against *S. mutans*, *E. faecalis* and *S. aureus*.

Key words: Antimicrobial activity, chlorhexidine gluconate, endodontic pathogens, medicinal plant extracts

INTRODUCTION

Microorganisms are primary etiological factor in the development of pulpal and periapical diseases.^[1] Endodontic treatment is aimed at complete elimination of microbes form the pulp space. This goal is achieved by thorough chemo mechanical preparation followed by three dimensional obturation of the root canal system. Although mechanical instrumentation

can remove a significant number of bacteria from the root canal system,^[2] the bacteria remaining in the intricacies of the canal can cause or sustain periradicular tissue inflammation.^[3] Therefore, mechanical instrumentation of the pulp space is accompanied by use of different types of irrigation solutions. According to current literature, sodium hypochlorite and 2% chlorhexidine remains to be the most preferred irrigating solutions.^[4]

How to cite this article: Mistry KS, Sanghvi Z, Parmar G, Shah S. The antimicrobial activity of *Azadirachta indica*, *Mimusops elengi*, *Tinospora cardifolia*, *Ocimum sanctum* and 2% chlorhexidine gluconate on common endodontic pathogens: An *in vitro* study. Eur J Dent 2014;8:172-7

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DOI: 10.4103/1305-7456.130591

Sodium hypochlorite has an excellent antimicrobial and tissue dissolution properties. However, it can cause soft-tissue inflammation if expressed out of the confines of the root canal. This event is associated with extreme pain and/or widespread swelling.^[5] In addition, it has an unpleasant odor and taste. Chlorhexidine gluconate (CHX) has a wide antimicrobial spectrum. However, at 2% concentration, which is most commonly used in endodontics, may have toxic effects on host tissues if expressed beyond the confines of the root canal and may impair healing.^[6] It also lacks tissue dissolving capacity.^[7]

Natural products are known to play an important role in human life. Various parts of the plants like root, bark, seed and leaves have been an important source of medicine since thousands of years. In recent years a predominant interest has been observed in evaluating different plant extracts for their antimicrobial properties against bacteria causing dental caries and periradicular pathology. A study by Murray *et al.* evaluated the possible use of *Morinda citrifolia* juice as an alternative to Sodium hypochlorite as an irrigant.^[8] Berberine, a plant alkaloid isolated from many medicinal plants, when combined with CHX was found to be comparable to Sodium hypochlorite in its bactericidal efficacy.^[9] India has a rich flora of medicinal plant species that are widely distributed throughout the country.

Mimusops elengi, locally known as Bakul is a small to large tree found all over India. The plant finds an important place in the indigenous system of medicine and its various parts are used in the treatment of various systemic diseases including dental problems. It has shown significant anti-inflammatory, analgesic and antipyretic activity.^[10] The bark of *M. elengi* is acrid, astringent and is used as a gargle for odontopathy, inflammation and bleeding gums.^[11] The tender stems are used as tooth brushes.^[12]

Azadirachta indica (Neem) is perhaps the most useful traditional medicinal plant. Every part of the tree has been used as traditional medicine for household remedy against various human ailments. The tree is still regarded as "Village dispensary" in India. Most of the parts of the plant such as fruits, seeds, leaves, bark and roots contain compounds with proven antiseptic, antiviral, antipyretic, anti-inflammatory, antiulcer and antifungal properties.

Ocimum sanctum, popularly known as Tulsi is a time-tested premier medicinal herb that is used in ayurvedic medicine since ancient times. It has made

an important contribution to the modern research due to its large number of medicinal properties. Different parts of the plant have shown antimicrobial, anti-inflammatory, analgesic, antipyretic, antiulcer, antidiabetic, antioxidant and anticancer activity.

Tinospora cardifolia is a large deciduous climbing shrub found throughout India. The ayurvedic name of the plant is Guduchi, Giloy or Amrita. In India, the extract of the plant is used as a remedy for many diseases including diabetes, hepatitis etc., The plant finds a special mention for its use in tribal or folk medicine in different parts of the country. The drug has been subjected to extensive phytochemical, pharmacological and clinical investigations and many interesting findings have been reported.

This study was aimed to compare the antimicrobial efficacy of various concentrations of selected medicinal plants like *A. Indica* (Neem), *O. sanctum* (Tulsi), *M. elengi* (Bakul), *T. cardifolia* (Giloy) and CHX against common endodontic pathogens such as *Enterococcus faecalis*, *Streptococcus mutans* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Procurement of material

The leaves of Neem and Tulsi, stem of Giloy and Bark of Bakul were collected from the courtyard. All the plant materials were identified by the senior professor at L. M. College of Pharmacy, Ahmedabad. Plant materials were washed with distilled water and dried under the shade for 10-12 days. All the material was ground in an electric grinder to produce a powder.

Preparation of extracts

The powdered material was again dried in an oven at 40°C for 4 h and used for extraction. Accurately weighed 50 g of powdered leaf sample was extracted with 500 ml methanol. This process was repeated until the residual marc got exhaustively extracted and finally extracts were pooled and evaporated in rota-evaporator. The extracts were concentrated under partial vacuum at 80°C to dryness, leaving behind thick semi-solid residue. This extract was dissolved in dimethyl sulfoxide (DMSO) to get six different concentrations to be tested.

Procurement of microorganisms

The microbial strains investigated in the study were obtained from Imtech-Chandigarh, India. The strains are *E faecalis* (MTCC 439), *S. mutans* (MTCC 497), and *S. aureus* (MTCC 737).

Test for antibacterial assay

Agar diffusion test

The test microorganisms were subcultured on specific media procured by Himedia Laboratory Pvt. Ltd., Mumbai, India and incubated aerobically at 37°C for 24 h. A total of six wells were made into a nutrient agar plate using sterile cork borer (6 mm. in diameter) and inoculums containing 1×10^5 CFU/ml of bacteria were spread on the solid plate with the bacterial suspension. 100 µl of the working solution of different medicinal plant extract carrying different concentration of the medicine was filled in the wells with the help of micropipette. The plates were then incubated at 37°C for 24 h in an aerobic environment. After overnight incubation, the plates were observed for the zone of inhibition and the diameters of the inhibition zone in millimeters were measured using a scale. Each extract was tested three times and mean values were recorded.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the micro broth dilution method.

Statistical analysis

The collected data was analyzed using the following statistical test (SPSS version 17, Inc. Chicago, USA)

1. Mean value and standard deviation
2. One-way analysis of variance
3. Bonferroni *post-hoc* test to carry out multiple

comparisons in bacterial inhibition zones between the groups ($P < 0.05$).

RESULTS

The current study showed that all the plant extracts and 2% CHX exerted antibacterial activity against selected endodontic pathogens. The antimicrobial activities of the test agents were in direct proportion with the concentration used. The effect of different concentrations of Neem, Tulsi, Bakul, Giloy and 2% CHX on selected microorganisms is tabulated in Table 1. The MIC and MBC values are presented in Table 2.

It is interesting to note that even at the lowest concentration, all the tested agents showed significant antimicrobial activity. *O. sanctum* (Tulsi) showed highest zone of inhibition against *S. mutans* at 3 mg concentration. *M. elengi* (Bakul) was most effective against *E. faecalis* at 3 mg concentration, whereas *A. indica*, *O. sanctum* and *T. cardifolia* showed no inhibitory effect at lower concentration. CHX was most effective agent showing highest zone of inhibition at the highest concentration against *S. aureus*. At the same time it was most consistent of all the medicaments tested, showing inhibitory effect against all the three pathogens at all selected concentrations.

Table 1: Values of Zone of Inhibition of different concentrations of tested agents

Concentration	<i>A. Indica</i>	<i>O.Sanctum</i>	<i>M.Elelngi</i>	<i>T. Cardifolia</i>	Chlorhexidine (%)	ANOVA	P value
<i>Streptococcus mutans</i> (MTCC 497)							
3 mg	24.67±2.517	30.33±1.528	25.00±1.000	29.67±0.577	(2) 27.00±1.000	9.242	0.002*
2 mg	20.67±1.555	21.67±3.512	20.33±2.517	20.00±2.000	(1)-22.00±3.000	0.338	0.846
1 mg	15.67±0.577	20.33±1.528	17.33±1.528	19.67±2.517	(0.5)-22.33±0.577	8.743	0.003*
500 µgm	15.33±1.528	20.00±2.000	15.00±2.000	18.33±2.517	(0.25)-22.00±1.000	7.67	0.004*
250 µgm	14.00±1.000	15.33±0.577	15.00±1.000	11.67±0.577	(0.125)-20.67±1.528	32.833	0.001*
125 µgm	11.67±0.577	14.33±0.577	11.67±0.577	7.67±1.528	(0.0625)-12.40±3.247	30.059	0.001*
<i>Enterococcus faecalis</i> (MTCC 439)							
3 mg	18.00±1.000	18.33±0.577	20.33±0.577	18.33±3.512	(2) 17.00±2.000	1.222	0.361
2 mg	16.33±0.577	16.00±2.000	17.67±4.509	16.00±1.000	(1)-15.00±0.000	0.539	0.711
1 mg	15.33±0.577	15.33±5.508	18.00±0.000	14.33±2.517	14.67±0.577 (0.5)	0.839	0.531
500	11.67±0.577	R	16.33±1.528	12.00±3.000	(0.25) 15.33±0.577	53.014	0.001*
250	R	R	15.00±1.732	R	(0.125) 15.00±1.000	253.125	0.001*
125	R	R	5.00±8.660	R	(0.0625) 12.00±2.000	5.278	0.015*
<i>Staphylococcus aureus</i> (MTCC 737)							
3 mg	18.00±0.000	18.00±6.928	20.33±1.528	16.33±0.577	(2) 23.33±0.577	2.167	0.147
2 mg	15.67±0.577	20.00±1.000	18.33±0.577	15.33±0.577	(1) 20.00±1.000	25.722	0.001*
1 mg	14.33±0.577	16.67±0.577	16.00±1.000	14.67±0.577	(0.5) 18.00±0.000	16.833	0.001*
500 µgm	13.00±1.000	14.67±2.517	14.00±0.000	14.00±0.000	(0.25) 17.33±0.577	5.261	0.015*
250 µgm	15.00±0.000	14.00±0.000	13.67±1.528	R	(0.125) 17.33±0.577	264.688	0.001*
125 µgm	12.33±0.577	R	R	R	(0.0625) 16.00±0.000	2785	0.001*

MTCC: Microbial type culture collection, ANOVA: Analysis of variance, R: Resistant, * $P < 0.05$

Table 2: Minimum inhibitory and minimum bactericidal concentrations of the tested agents

Microorganism	Test agent	MIC	MBC
<i>Streptococcus mutans</i> (MTCC 497)	<i>A. Indica</i>	125 µg	250 µg
	<i>O. Sanctum</i>	125 µg	250 µg
	<i>T.Cardifolia</i>	250 µg	500 µg
	<i>M. Elengi</i>	500 µg	1 mg
	Chlorhexidine	<0.0625%	0.0625%
<i>Enterococcus faecalis</i> (MTCC 439)	<i>A. Indica</i>	500 µg	1 mg
	<i>O. Sanctum</i>	1000 µg	2 mg
	<i>T.Cardifolia</i>	250 µg	500 µg
	<i>M. Elengi</i>	500 µg	1 mg
	Chlorhexidine	<0.0625%	0.0625%
<i>Staphylococcus aureus</i> (MTCC 737)	<i>A. Indica</i>	250 µg	500 µg
	<i>O. Sanctum</i>	250 µg	500 µg
	<i>T.Cardifolia</i>	250 µg	500 µg
	<i>M. Elengi</i>	500 µg	1 mg
	Chlorhexidine	<0.0625%	0.0625%

MIC: Minimum inhibitory concentration, MTCC: Microbial type culture collection, MBC: Minimum bactericidal concentrations

DISCUSSION

In the current study, we explored four medicinal plants such as *A. Indica* (Neem), *O. sanctum* (Tulsi), *M. elengi* (Bakul) and *T. cardifolia* (Giloy) as well as 2% Chlorhexidine for their antimicrobial effectiveness against target endodontic pathogens like *E. faecalis*, *S. mutans* and *S. aureus*.

S. mutans is believed to be the most common bacteria associated with dental caries. It acts on available carbohydrate in the mouth, breaking it down with the production of lactic acid and subsequent demineralization of tooth structure. *S. mutans* along with *S. aureus* is frequently isolated from primary endodontic infection,^[13-15] as well as in root filled teeth.^[16] The presence of these microorganisms is also found to be more strongly associated with pre-operative symptoms and presence of swelling.^[17,18]

E. faecalis remains to be the most frequently identified species in canals of root filled teeth with periapical lesions as established by different molecular methods from time to time.^[19-23] This may be due to its ability to survive the effects of a wide range of antimicrobial solutions and intracanal medicaments used during endodontic treatment procedures. It also endures prolonged periods of nutritional deprivation. The root canal is hardly a nutrient rich medium, but *E. faecalis* may survive on serum components from the dentinal fluid. Therefore, even in a well debrided and coronally well-sealed root canal, remaining or surviving cells of

E. faecalis may still grow and utilize local sources of energy and nutrients.^[24]

The selected plant extracts have shown significant antimicrobial effect against these endodontic pathogens. Tulsi showed highest zone of inhibition against *S. mutans* at 3 mg concentration. The difference in antimicrobial activity at this concentration was statistically significant when compared with other agents like Neem and Bakul ($P < 0.05$). It has also shown its antimicrobial property against *S. aureus* and *E. faecalis*. However, in lower concentrations, *E. faecalis* showed resistance against Tulsi with MIC value of 1 mg. The results of our study are in agreement with previous studies where different concentrations of Tulsi have been used against all three tested microorganisms.^[25-29] The biological properties of the plant has been attributed to the presence of active compounds like Ursolic acid, flavonoids (epigenin, orientin and vicenin),^[30] and phenolic compounds (cirsilineol, circimaritin, isothymusin, eugenol).^[31] The leaves of Tulsi contain 0.7% volatile oil comprising about 71% eugenol and 20% methyl eugenol.^[32] Eugenol is the most prominent phytoconstituents present in this plant which may be responsible for antimicrobial activity.^[33]

Bakul was most effective agent against *E. faecalis* at 3 mg. concentration. However, the difference with other agents was statistically insignificant ($P < 0.05$). This plant extract has been tested for its antimicrobial effects against dental pathogens. The extract was found to be effective against Streptococci isolated from tooth tartar of dental patients, thus confirming the traditional claim.^[34] In a similar study, a concentration of 450 µg was found to be inhibitory for the growth of most of the tested salivary microflora.^[35] Our study has shown that the extract is effective at even lower concentration with MIC value of 250 µg. This may be due to solvent used for the preparation of extract. Methanolic extracts may show greater activity because more phytoconstituents are leached in it when compared to other extracts. Different types of glycosides, alkaloids, phenols, tannins and saponins have been screened in the methanolic extract of this plant.^[36]

Neem extract has shown antimicrobial activity against *E. faecalis* and *S. mutans* in previous *in vitro* studies.^[37-39] Prashant *et al.*, demonstrated that Neem stick extract produced maximum zone of inhibition against *S. mutans* at 50% concentration. Even at 5% concentration, Neem extract was effective against all four species of microorganisms tested in their study.^[39]

Bohora *et al.*, concluded that Neem leaf extract has a significant antimicrobial effect against *E. faecalis*, *Candida albicans* and mixed culture.^[37] Our study has shown the leaf extract of Neem is very effective against *S. mutans* and *S. aureus* with MIC value of 125 µg. The maximum antimicrobial activity was observed on *S. mutans* at 3 mg. concentration with zone of inhibition of (24.67 ± 2.517) mm. However, at lower concentrations, it was ineffective on *E. faecalis*. This is in agreement with the study by Dhanya Kumar *et al.* where 10% concentration at the highest volume of 75 µl was not effective on *E. faecalis*. It was effective only at the highest concentration of 50% and at the highest volume of 75 µl.^[38] Neem contains different active phytoconstituents such as alkaloids, glycosides, terpenoids, steroids and tannins.^[36] Neem has been found to be highly effective in the treatment of periodontal diseases, thus exhibiting its biocompatibility with human PDL fibroblasts. The use of Neem as an endodontic irrigant might be recommended because it is biocompatible antioxidant and thus not likely to cause severe injuries to patients.^[40,41] Bitter test associated with this plant can be altered by the addition of sweeteners and flavors to increase the patient compliance and acceptability.^[40]

Nearly 2% CHX showed the most consistent antimicrobial activity against all three pathogens with MIC value of <0.0625%. In our study we have used six different concentrations of CHX with two fold reduction (2%, 1%, 0.5%, 0.25%, 0.125% and 0.0625%). The concentration often used in endodontic therapy is 2%. This has been found to be more effective in the least time when compared with other concentrations of CHX ranging from 0.002% to 2%.^[42] CHX is active against a wide range of micro-organisms, such as Gram-positive and Gram-negative bacteria, bacterial spores, lipophilic virus, yeast and dermatophytes being bacteriostatic at lower concentrations and bactericidal at high concentration.^[43] It shows substantial antimicrobial effect against common endodontic pathogens like *S. aureus*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis*, *Prevotella intermedia*, *E. faecalis*, *C. albicans* and *S. mutans*.^[42,44,45] The antimicrobial effect is because of reaction of CHX molecule with negatively charged groups on the cell surface, causing an irreversible loss of cytoplasmic constituents, membrane damage and enzyme inhibition. At higher concentrations CHX results in extensive cell damage, coagulation of cytoplasm and precipitation of proteins and nucleic acids.^[46]

Giloy also exerted considerable antimicrobial effectiveness against tested pathogens. However,

it is ineffective against *E. faecalis* and *S. aureus* at lower concentrations with MIC value of 500 µg. This plant has been subjected to chemical investigations extensively and a number of chemical constituents belonging to different groups such as terpenoids, alkaloids, lignans and flavonoids, tannins, cardiac glycosides and steroids have been reported.^[47] which may account for the antimicrobial property of this agent. Similar results have been observed in previous studies where this plant extract has shown promising results against the pathogens used in our study.^[47-49]

CONCLUSION

Under the limitations of this study, it can be concluded that *A. Indica*, *O. sanctum*, *T. cardifolia*, *M. elengi* and Chlorhexidine have antimicrobial effects against the endodontic pathogens like *S. mutans*, *S. aureus* and *E. faecalis*. However, further preclinical and clinical trials are required to evaluate the cytotoxicity and safety issues of these plant extracts before they can be recommended as an endodontic irrigant or intracanal medicament. Furthermore, looking at the polymicrobial nature of endodontic infections these agents needs to be tested for their antimicrobial effectiveness against a wide range of microorganisms including strict anaerobes.

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Source of Support: Nil.
Conflict of Interest: None declared