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Antimicrobial efficacy of endodontic irrigants from *Azadirachta indica*: An *in vitro* study

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Abstract

Objective. This study analyzed the antimicrobial effect of five irrigants formulated from different parts of the tree *Azadirachta indica* (Neem) and compared with 2.5% sodium hypochlorite and 0.2% chlorhexidine gluconate through an agar diffusion test. ***Materials and methods.*** A clinical isolate of *Candida albicans* was innoculated on Sabourad Dextrose Agar and *Enterococcus faecalis* (ATCC 29212) on Sheep Blood Agar. Wells with 6 mm diameter were created in agar and 100 μL aliquiots of each irrigant were introduced to five different wells. After incubation, the largest uniform diameter of the inhibition zone was recorded. ***Results.*** The leaf extract of the tree and a mixture of the seed-bark powder dissolved in dimethyl sulfoxide were active against both organisms. The other neem-based irrigants, a leaf powder dissolved in dimethyl sulfoxide, aqueous bark decoction and neem oil, did not possess any antimicrobial efficacy. Sodium hypochlorite completely inhibited growth of *C. albicans* and the leaf extract had larger inhibition zones than chlorhexidine ($p = 0.011$) or the seed-bark irrigant ($p = 0.008$). Against *E. faecalis*, inhibition zones with chlorhexidine were the largest and differed significantly from sodium hypochlorite ($p = 0.039$), leaf extract ($p = 0.008$) and seed-bark irrigant ($p = 0.011$). ***Conclusions.*** Two neem irrigants displayed antimicrobial properties. The efficacy of the standard endodontic irrigants varied depending on the organisms tested. *Clinical relevance:* Neem-based endodontic irrigants may be formulated for clinical application.

Key Words: *Azadirachta indica, sodium hypochlorite, chlorhexidine gluconate, Candida albicans, Enterococcus faecalis*

Introduction

The conditions prevailing within the root canal of a tooth with periapical periodontitis provide an ecological niche for the establishment and propagation of a unique microbial population [1]. The survival of fungal strains and *Enterococcus faecalis* in root-filled teeth has been demonstrated [2,3]. The elimination of this microbial flora is a pre-requisite for successful treatment outcome. Hence, root canal irrigants must have antimicrobial properties. Since the introduction of sodium hypochlorite to endodontics in 1920, it has been the agent of choice for irrigation of the root canal system with recognized anti-microbial action [4]. Chlorhexidine gluconate has been recognized as an efficacious endodontic irrigant in the last 30 years and is known for its antimicrobial activity, substantivity and relative absence of toxicity [5,6].

Elimination of *E. faecalis* and *C. albicans* has been formidable with high recovery rates in failed cases [2,3]. The Neem tree (*Azadirachta indica* A. Juss) has been used in traditional Indian medicine with demonstrable anti-microbial properties [7–17]. It was therefore proposed to investigate extracts of the neem tree for their antimicrobial action against *E. faecalis* and *C. albicans* and to compare these with 2.5% sodium hypochlorite and 0.2% chlorhexidine gluconate through this initial screening study.

Materials and methods

Ten experimental groups were studied, each group defining either an irrigant or a solvent used for the experimental irrigant (Table I).

Preparation of the extracts

Group A: HND. Commercially-available neem capsules (Himalaya Drug Company, Bangalore, India) which contained pure neem powder of various parts of

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Table I. Experimental and control groups.

Group	Code	Description
A	HND	Himalaya Neem Powder in Dimethyl Sulfoxide (DMSO)
B	JLD	Dr Jain's Neem Leaf Powder in DMSO
C	NO	Neem Oil
D	NBD	Neem Bark Decoction
E	ELE	Ethanolic Leaf Extract
F	SHC	2.5% Sodium Hypochlorite
G	CHX	0.2% Chlorhexidine Gluconate
H	S	0.9% Saline
I	EA	Ethanol
J	DMSO	Dimethyl Sulfoxide

Groups A–E constituted the experimental irrigants, Groups F and G the positive controls and Groups H–J, the negative controls.

the tree were utilized. Two hundred and fifty milligrams of powder was obtained by breaking open the capsule while maintaining sterility and emptying it into a sterile test tube containing 5 ml of Dimethyl Sulfoxide (DMSO, Thomas Baker Chemicals Limited, Mumbai, India) as solvent and shaken in a vortex machine for 1 min and the powder allowed to soak in the solvent for 48 h at a temperature of 4°C. The extract was centrifuged at 2000 rpm for 10 min and the supernatants filtered through a Whatman's No. 1 filter paper and stored at 4°C until use.

Group B: JLD. Commercially-available neem leaf powder (Dr. Jain's Forest Herbals Pvt Ltd., Vasai, India) was used to prepare an irrigant using a similar protocol.

Group C: NO. This represented the oil derived from the seed of the neem tree. It was obtained locally from an ayurvedic pharmacy (Jogappa Shenoy, Udupi, India).

Group D: NBD. An aqueous Neem Bark extract was also tested. Sun-dried bark was procured from a tree in Udupi, India and coarse ground in a mechanized mill. The Department of Ayurveda, Kasturba Hospital, Manipal, India produced the decoction in a concentration of 1:4, as per description in classical texts [18]. One part of the bark was boiled with eight parts of water, till the entire volume was reduced to a fourth. A fresh preparation had a shelf life of 24 h.

Group E: ELE. An Ethanolic Leaf Extract of the tree was prepared by the Manipal College of Pharmaceutical Sciences, Manipal. Fresh leaves were procured from the neem tree, washed and dried in the shade. The leaves were powdered and extraction was performed by maceration in rectified spirit.

Micro-organisms. One clinical isolate of *Candida albicans* and a standard strain of *Enterococcus faecalis* (ATCC 29212) were obtained from the Department of Microbiology, Kasturba Medical College, Manipal, India.

Anti-microbial susceptibility testing

The agar diffusion method was used to test the antimicrobial activity. The micro-organisms were suspended in 2 mL of sterile distilled water to achieve a concentration of 1.5×10^8 colony forming units per milliliter, as confirmed with a 0.5 McFarland standard. The innoculum was used to prepare lawn cultures on Sabourad Dextrose Agar (SDA) for *C. albicans* and Sheep Blood Agar (SBA) culture plates for *E. faecalis*. Five wells with 6 mm diameters were created in the agar media using a hollow metal borer in each petridish; 100 µl aliquots of experimental or control formulations were pipetted into each well. All extracts or controls were tested on at least one petridish of SBA or SDA. The plates were transferred to an incubator and maintained at 37°C. They were observed after an interval of 24 h (for *E. faecalis*) and 48 h (for *C. albicans*) and the size of each inhibition zone (in millimeters) recorded along its most uniform diameter.

Results were subjected to statistical analysis using Mann Whitney U-test.

Results

The results of inhibition zones produced with the drugs and the control groups are represented in Table II. HND produced smaller zones than

Table II. Inhibition zones obtained.

	Candida albicans		Enterococcus faecalis	
Irrigant	Mean (in mm)	SD	Mean (in mm)	SD
HND	13.0*	0.07	13.1*	0.65
JLD	0	0	0	0
NO	0	0	0	0
NBK	0	0	0	0
ELE	17.8**	0.57	14.6**	0.42
SHC	C	C	17.2***	0.57
CHX	15.8***	0.84	18.1****	0.55
S	0	0	0	0
EA	0	0	0	0
DMSO	0	0	0	0

$n = 5$ for each substance; SD, Standard deviation; C, Complete inhibition with no microbial growth, not amenable to statistical analysis; *, **, ***, **** indicate statistical significance for each micro-organism separately between different rows.

ELE against *C. albicans* ($p = 0.008$) and *E. faecalis* ($p = 0.011$). Inhibition with HND was also significantly less than CHX and SHC with both organisms ($p = 0.008$). ELE had significantly larger zones than CHX against *C. albicans* ($p = 0.011$), but smaller than SHC against *E. faecalis* ($p = 0.009$). Other neem-based formulations failed to produce any inhibition of microbial growth. No microbial growth was found in the *C. albicans* culture plates inoculated with SHC. This result was reproduced over three sets of experiments (each with a sample size of five) to confirm the anti-microbial efficacy of this irrigant. Since no measurable values of inhibition zones could be obtained, SHC could not be included for statistical analysis and was the outstanding drug tested against *C. albicans*. This irrigant also produced a mean inhibition zone of 17.2 ± 0.57 mm against *E. faecalis*. CHX was efficacious against both organisms, with significantly larger zones against *E. faecalis* than SHC ($p = 0.039$), HND ($p = 0.008$) and ELE ($p = 0.008$). The irrigant solvents and control groups failed to prevent microbial growth.

Discussion

This initial study was undertaken to formulate an extract with anti-microbial potential. Several neem tree parts have been described with beneficial properties and, hence, five different products were chosen. HND is a mixture of components from the seed and bark of the tree and recommended for oral consumption. JLD is a powder derived from the leaf. The method of formulation of the extract with these two powders was a modification of the method followed by Almas [19]. Both powders were insoluble in water. The ideal properties of a suitable vehicle were identified to include the solubilization of the active ingredients of the drug, biocompatibility, with a known history of being used internally in the body and low viscosity to be used within a root canal. Dimethyl Sulfoxide (DMSO) met these parameters and was thus chosen as the vehicle. DMSO is a clear, colorless, highly polar, hygroscopic organic liquid with viscosity comparable to water (1.19 gm/mL as compared to water at 1 gm/mL). It is used as a vehicle for topical application of pharmaceuticals and has analgesic, anti-inflammatory and anti-oxidant properties [20].

After a 48-h soak, both HND and JLD were partially soluble in DMSO. We thus centrifuged the test tube to affect a complete separation of the dissolved drug and the sediment. The supernatant was decanted, filtered and used. A pilot study was conducted to confirm the presence of the active component of the drug within the supernatant. This showed anti-microbial action from the solubilized portion of the drug against both the test organisms used and validated DMSO as the chosen vehicle.

NO is derived from the seed and was included in this study because of antibacterial effects against *Pseudomonas aeruginosa*, *Staphylococcus pyogenes*, *Escherichia coli*, *Klebsiella aerugenes* and the *Proteus* organisms [11]. Anti-microbial and immunomodulatory properties of the oil have been demonstrated [21–23]. NBD was chosen for investigation because of its reported usage and recommendation in Ayurvedic (traditional Indian medicine) texts [24]. The bark has been described as a very useful part of the tree that is freely available and can be harvested at any point of the year.

ELE was derived by alcohol extraction from shade-dried leaves of the neem tree and has previously demonstrated antibacterial effects against *Streptococcus mutans* and *Lactobacillus* species [14]. Leaf-based extracts have also been used in other studies and shown to have action against *Streptococcus faecalis* [10], *Streptococcus sanguis* [12], several dermatophytes [7,8] and Dengue virus type-2 [25].

The *in vitro* study was performed by using the agar diffusion test. This method has been used by other researchers as well to test the anti-microbial capability of irrigants [4,5,26]. The size of the inhibition zones obtained depends upon the diffusibility of the test substance through the agar. This methodology demonstrates action against the planktonic forms of the micro-organisms which is important for an initial screening study.

HND and ELE had antimicrobial action, but other neem formulations lacked potency. Lack of inhibition of micro-organisms with NO may be explained by its viscosity and failure to diffuse through agar. Our results with the oil differ from those obtained by Rao et al. [11], who reported anti-bacterial activity against four different organisms using the disc diffusion method which relies on surface contact area. Patel and Trivedi [21] used a neem oil emulsion (with 96% alcohol) and also achieved anti-bacterial efficacy. We attempted an emulsion preparation using the oil with Tween 80 detergent. The two liquids were vortexed but found immiscible in the pilot study. Hence, we used neem oil alone and found the absence of inhibition zones with both organisms. Additionally, its high viscosity and oily nature precludes use within the root canal.

Even though NBD and JLD diffused through the agar medium (evidenced by color-change of agar), they did not produce inhibition zones. This could indicate resistant micro-organisms or the absence of anti-microbial potency.

HND and ELE demonstrated positive results against both the organisms. Inhibition zones measuring 17.8 mm and 14.6 mm were obtained with ELE against *C. albicans* and *E. faecalis*. HND showed inhibition zones measuring 13 mm and 13.1 mm against *C. albicans* and *E. faecalis*. In contrast, an extract derived from the chewing sticks of *Azadirachta indica* was found ineffective against *Streptococcus faecalis*, *Streptococcus mutans*, *Staphylococcus aureus* and *Candida albicans* [19]. This could be because

of the tree-part used. Our study also found the bark-derived extract (NBK) lacked anti-microbial potential. In the present investigation, the extracts that demonstrated inhibition zones were derived from the seed/bark combination (HND) or the leaf (ELE) and the differences between the formulations depends on the source and method of manufacture.

The negative control groups, NS, EA and DMSO, did not show any inhibition zones. This indicated that none of the solvents used prevented microbial growth. Thus, anti-microbial action achieved with HND or ELE cannot be attributed to their vehicles and was achieved exclusively by the action of the neem drug.

The positive controls (SHC and CHX) showed inhibition zones against both organisms. SHC inhibited growth of *C. albicans* completely on the SDA plates and demonstrated superior action against the fungus compared with all other drugs. Estrela et al. [26] found inhibition zones measuring 12 mm each against *C. albicans* and *E. faecalis* in an agar diffusion test. Larger zones were obtained in our study with *C. albicans*, possibly because of difference in microbial strains used. Even though both studies used the same standard strain of *E. faecalis* (ATCC 29212), the results of our study differ owing to a higher concentration of SHC. Siqueira Jr et al. [4] reported good anti-bacterial action of 2.5% SHC against *E. faecalis* with inhibition zones measuring 21.5 mm that are much larger than those obtained by us, probably because of different methodology. The anti-fungal action of SHC could be attributable to the oxidation of the beta (1–3)-D glucan component of the yeast cell [27]. Against *E. faecalis*, SHC possesses a high pH which interferes with the integrity of the cytoplasmic membrane, produces irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation in lipid peroxidation [28].

Our results with CHX are in close conformity to inhibition zones obtained by Estrela et al. [26], who reported values of 16 mm against *C. albicans* and 18 mm against *E. faecalis*, even though the concentration of CHX used was 2%. CHX has been tested for anti-fungal properties, but the mechanism of action against the yeast is not well explained [29]. The bacteriostatic and bactericidal action of the drug have been well researched and relate to the adsorption of the drug molecule onto the cell wall, causing leakage of the intracellular components and coagulation of the cytoplasm [30].

This *in vitro* study was an initial indicator test. It identified the neem drugs with anti-microbial properties. Even though only two micro-organisms were included, the *in vitro* study provided a platform to screen the neem irrigants. Amongst the neem extracts, ELE was significantly better than HND. The anti-microbial efficacy of ELE was further tested within the root canal against a mixed flora in an *in vivo* phase of the project. The further scope of this study includes testing the tissue-dissolution properties of ELE, its substantivity within the root canal and determining whether the irrigant possesses microbistatic or cidal action.

From this study, we concluded that, against *Candida albicans*, sodium hypochlorite was the best antimicrobial irrigant among the experimental groups and followed in descending order by the neem ethanolic leaf extract, chlorhexidine gluconate and neem powder in DMSO (HND). Among the experimental irrigants, chlorhexidine gluconate showed the best anti-microbial property against *E. faecalis*, followed in descending order by sodium hypochlorite, neem ethanolic leaf extract and neem powder in DMSO (HND).

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References

[1] Jung IY, Choi B, Kum KY, Roh BD, Lee SJ, Lee CY, et al. Molecular epidemiology and association of putative pathogens in root canal infection. J Endod 2000;26:599–604.

[2] Molander A, Reit C, Dahlen G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J 1998;31:1–7.

[3] Waltimo TM, Siren EK, Torkko HL, Olsen I, Haapasalo MP. Fungi in therapy-resistant apical periodontitis. Int Endod J 1997;30:96–101.

[4] Siqueira Junior JF, Rocas IN, Favieri A, Lima KC. Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. J Endod 2000;26:331–4.

[5] Yesilsoy C, Whitaker E, Cleveland D, Phillips E, Trope M. Antimicrobial and toxic effects of established and potential root canal irrigants. J Endod 1995;21:513–15.

[6] Ercan E, Ozekinci T, Atakul F, Gul K. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: *in vivo* study. J Endod 2004;30:84–7.

[7] Venugopal PV, Venugopal TV. Antidermatophytic activity of neem (*Azadirachta indica*) leaves *in vitro*. Indian J Pharma 1994;26:141–3.

[8] Natarajan V, Pushkala S, Karuppiah VP, Prasad PV. Anti dermatophytic activity of *Azardirachta indica* (neem) by *in vitro* study. Indian J Pathol Microbiol 2002;45:311–13.

[9] Fabry W, Okemo P, Ansorg R. Fungistatic and fungicidal activity of east African medicinal plants. Mycoses 1996;39: 67–70.

[10] Almas K. The antimicrobial effects of extracts of *Azadirachta indica* (Neem) and *Salvadora Persica* (Arak) chewing sticks. Indian J Dent Res 1999;10:23–6.

[11] Rao DK, Singh I, Chopra P, Chhabra PC, Ramanujalu G. *In vitro* antibacterial activity of neem oil. Indian J Med Res 1986;84:314–16.
[12] Wolinsky LE, Mania S, Nachnani S, Ling S. The inhibiting effect of aqueous *Azadirachta indica* (Neem) extract upon bacterial properties influencing *in vitro* plaque formation. J Dent Res 1996;75:816–22.
[13] Fabry W, Okemo PO, Ansorg R. Antibacterial activity of East African medicinal plants. J Ethnopharmacol 1998;60:79–84.
[14] Vanka A, Tandon S, Rao SR, Udupa N, Ramkumar P. The effect of indigenous Neem *Azadirachta indica* mouth wash on Streptococcus mutans and lactobacilli growth. Indian J Dent Res 2001;12:133–44.
[15] Pai MR, Acharya LD, Udupa N. Evaluation of antiplaque activity of *Azadirachta indica* leaf extract gel-a 6-week clinical study. J Ethnopharmacol 2004;90:99–103.
[16] Balappanavar AY, Nagesh L, Ankola AV, Tangade PS, Kakodkar P, Varun S. Antimicrobial efficacy of various disinfecting solutions in reducing the contamination of the toothbrush - a comparative study. Oral Health Prev Dent 2009;7:137–45.
[17] Jagannath JH, Radhika M. Antimicrobial emulsion (coating) based on biopolymer containing neem (*Melia azardichta*) and turmeric (*Curcuma longa*) extract for wound covering. Biomed Mater Eng 2006;16:329–36.
[18] Nadkarni AK. Appendices. Dr. K. M. Nadkarni's Indian Materia Medica. 3rd ed. Mumbai, India: Popular Prakashan Pvt. Ltd; 2000. p 495–7.
[19] Almas K. The antimicrobial effects of seven different types of Asian chewing sticks. Odontostomatol Trop 2001;96:17–20.
[20] Jacob SW, Herschler R. Pharmacology of DMSO. Portland, Oregon: Oregon Health Sciences University. Available online at http://www.dmso.org/articles/information/herschler.htm. [accessed 22 May 2012].
[21] Patel RP, Trivedi BM. The *in vitro* antibacterial activity of some medicinal oils. Indian J Med Res 1962;50: 218–22.
[22] Zhang YQ, Xu J, Yin ZQ, Jia RY, Lu Y, Yang F, et al. Isolation and identification of the antibacterial active compound from petroleum ether extract of neem oil. Fitoterapia 2010;81:747–50.
[23] Upadhyay SN, Dhawan S, Garg S, Talwar GP. Immunomodulatory effects of neem (*Azadirachta indica*) oil. Int J Immunopharmacol 1992;14:1187–93.
[24] Nadkarni AK. Appendices. Dr. K. M. Nadkarni's Indian Materia Medica. 3rd ed. Popular Prakashan Pvt. Ltd; 2000. p 784–5.
[25] Parida MM, Upadhyaya C, Pandya G, Jana AM. Inhibitory potential of neem (*Azadirachta indica* A. Juss) leaves on the dengue virus type-2 replication. J Ethnopharmocol 2002;79: 273–8.
[26] Estrela C, Ribeiro RG, Estrela CA, Pecora JD, Sousa-Neto MD. Antimicrobial effect of 2% sodium hypochlorite and 2% chlorhexidine tested by different methods. Braz Dent J 2003;14:58–62.
[27] Miura NN, Miura T, Ohno N, Adachi Y, Watanabe M, Tamura H, et al. Gradual solubilization of Candida cell wall beta-glucan by oxidative degradation in mice. FEMS Immunol Med Microbiol 1998;21:123–9.
[28] Estrela C, Estrela CA, Barbin EL, Spano JE, Marchesan MA, Pecora JD. Mechanism of action of sodium hypochlorite. Braz Dent J 2002;13:113–17.
[29] Sen BK, Safavi KE, Spangberg LW. Antifungal effects of sodium hypochlorite and chlorhexidine in root canals. J Endod 1999;25:235–8.
[30] Hennessey TD. Some antibacterial properties of Chlorhexidine. J Periodont Res 1973;8:61–7.