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In Vitro Evaluation of Smear Layer Removal Ability of Azadirachta Indica and Morinda Citrifolia

[1]Dr. Vamsee Krishna. N, M.D.S, Associate Professor, Department of Conservative Dentistry & Endodontics, CKS Theja Institute of Dental Sciences & Research, Tirupati, Andhra Pradesh, India.

[2]Dr. Lenin Babu.T, M.D.S, Associate Professor, Department of Conservative Dentistry & Endodontics, Vananchal Dental College, Garhwa, Jharkhand, India.

[3] Dr. Arunodaya Reddy EC, M.D.S, Clinical Practitioner, Cuddapah, Andhra Pradesh, India.

[4]Dr. Swapnika. P, M.D.S, Senior Resident, AIIMS Mangalagiri, Andhra Pradesh, India.

[5]Dr. Rakesh. G, M.D.S, Senior Lecturer, Department of Conservative Dentistry & Endodontics, CKS Theja Institute of Dental Sciences & Research, Tirupati, Andhra Pradesh, India.

[6]Dr. Madhuri Nadhamuni, M.D.S, Clinical Practitioner, Tirupati, Andhra Pradesh, India.

Corresponding Author: Dr. Vamsee Krishna. N, M.D.S, Associate Professor, Department of Conservative Dentistry & Endodontics, CKS Theja Institute of Dental Sciences & Research, Tirupati, Andhra Pradesh, India.

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Abstract

Objective: To evaluate the invitro effectiveness of smear layer removal of Azadirachta indica extract with Sodium hypochlorite and Morinda citrifolia extract when used as an endodontic irrigant.

Methods: 50 teeth with a single canal were selected and were inoculated with Enterococcus faecalis for 4 weeks. The teeth were randomly allocated to 5 groups; the pulp chamber was accessed, cleaned, and shaped by using ProTaper Universal. During instrumentation the irrigation was provided by Azadirachta indica extract, Morinda citrifolia extract, Sodium hypochlorite and normal saline. The teeth were then processed for scanning electron microscopy, and the removal of smear layer was examined. Statistical analysis was done using one way ANOVA followed by Bonferroni's Multiple Comparison test.

Results: Results were found to be statistically significant ($p < 0.0001$). There was a significant difference in the ability of 8% Azadirachta indica extract with 17% EDTA, 8% Azadirachta indica extract with Saline, 12% Morinda citrifolia extract with 17% EDTA and 3% Sodium hypochlorite with 17% EDTA to remove the smear layer when compared with Normal Saline ($p < 0.001$). None of the irrigation treatments completely removed the smear layer from all of the instrumented root canals.

Conclusion: Smear layer removal efficiency for Sodium hypochlorite, Morinda citrifolia extract and Azadirachta indica extract were more or less equal when used in conjunction with EDTA followed by Azadirachta indica extract with saline.

Clinical Significance: The efficacy of Azadirachta indica extract was similar to NaOCl in conjunction with EDTA as an intracanal irrigant. Azadirachta indica extract appears to be a possible alternative to the use of NaOCl as an intracanal irrigant.

Keywords: Azadirachta, Morinda, Smear layer, Sodium hypochlorite, EDTA.

Introduction

Debridement of the root canal by instrumentation and irrigation is considered the most important factor in the prevention and treatment of endodontic diseases. In cases of infected root canals, residual debris and smear layer harbor microorganisms and their by-products. A smear layer is formed on instrumented root canal walls comprising inorganic and organic material such as dentin filings and pulp tissue remnants.[1-3] Bacteria can survive and multiply in the smear layer and can also penetrate into dentinal tubules. Since the smear layer itself is contaminated and has the potential to harbour bacteria within the dentinal tubules, it is sensible to remove the smear layer and allow disinfection of the entire root canal system.[4,5] The goal of instrumentation and irrigation is to remove all necrotic tissue, microorganisms and their components present in the smear layer from the root canal system.[6,7]

The purpose of irrigation is twofold: one is to remove the organic component, the debris originating from pulp tissue and microorganisms, and the other is to remove the smear layer. Countless compounds have been suggested as root canal irrigants, including inert substances such as sodium chloride or highly toxic and allergenic biocides such as formaldehyde.[2,3] The most frequently used irrigants in endodontic treatment are sodium hypochlorite (NaOCl), hydrogen peroxide, the combined use of both, chlorhexidine, citric acid, iodine-potassium-iodide, alcohol and Ethylenediaminetetraacetic acid (EDTA) solutions. More recently, several new solutions such as deionized water, bioglass, MTAD, and some more have been advocated for disinfection.[6,8] Since there is no single solution that can dissolve organic tissues and demineralize the smear layer, the sequential use of organic and inorganic solvents has been recommended. The most widely used irrigating regimen is by the alternate use of NaOCl and EDTA.[3,6,7,9,10]

Sodium hypochlorite has been criticized for its unpleasant taste, relative cytotoxicity and caustic effects on healthy tissue. The biocompatibility problems associated with the use of concentrated Sodium hypochlorite prompted researchers to look for herbal alternatives.[11,12] Currently, no natural herbal extracts that might be used as an alternative to Sodium hypochlorite as an irrigant has been identified. Endodontic literature has shown that Morinda citrifolia extract in an invitro study has antimicrobial and therapeutic effects when used as an endodontic irrigant.[13] Azadirachta indica, commonly called Neem, is a plant that has found varied use in ecological, medicinal and agricultural sectors. The purpose of this study is to evaluate the smear layer removal of Azadirachta indica extract with Sodium hypochlorite and Morinda citrifolia extract.

Materials & Methods

A total of 50 maxillary and mandibular, single-rooted, noncarious, extracted human teeth with fully developed apices and root lengths ranging from 12 to 16 mm were included in this study. Teeth with coronal restorations or root filling were excluded. The teeth were soaked in 5.25% NaOCl for 30 minutes to remove residual loose tissue and debris from the root surface. A rotary Diamond disc was used to decoronate the teeth below cementoenamel junction. An access opening was prepared and the pulp was removed with a barbed broach and the teeth were sterilized. A 24-hour pure culture of Enterococcus faecalis (ATCC # 29212) was grown in Brain Heart Infusion (BHI) broth. Fifty 10 ml test tubes were taken with BHI broth. Each tooth was placed in each test tube. Later 5 ml of Enterococcus faecalis (ATCC # 29212) that was grown in BHI broth was added to each test tube. These test tubes were placed in a incubator and

incubated for 4 weeks under aerobic conditions at 37°C. Fresh media was added every seventh day.[14]

Determination of Minimum Inhibitory Concentration (MIC) of Azadirachta indica extract:

Minimum Inhibitory Concentration (MIC) of Azadirachta indica extract (Sri Vishnu Biotec Formulations, Guntur, Andhra Pradesh, India) is found against Enterococcus faecalis by agar plating dilution method. MIC was performed by serial dilution method at concentration ranging from 1% to 20%. Azadirachta indica extract was first diluted to the highest concentration (20%) to be tested, and then serial dilution was made. The BHI plates were seeded with 1×10^8 CFU/ml and allowed to solidify and thereafter punched with a sterile cork borer (6.0mm diameter) to cut uniform wells. The open wells were filled with 0.1ml of Azadirachta indica extract with concentrations ranging from 1% to 20%. The plates were then incubated at 37^0C for 24h.[15] The concentration of the Azadirachta indica extract that showed no growth of the organisms is considered as Minimum Inhibitory Concentration. In the present study 8% concentration of Azadirachta indica extract showed no growth which was considered as MIC.

Determination of Minimum Inhibitory Concentration (MIC) of Morinda citrifolia extract

Broth dilution method was performed to determine the minimum inhibitory concentration of Morinda citrifolia extract (Sri Vishnu Biotec Formulations, Guntur, Andhra Pradesh, India) against Enterococcus faecalis. Morinda citrifolia extract was taken in different test tubes with concentrations ranging from 1% to 20%. Then 0.1 ml of standardized suspension of bacteria (1×10^8 CFU/ml) was added to these test tubes. The lowest concentration of the test tube that did not show any visible growth was considered as the MIC and it is 12% for Morinda citrifolia extract in this study.[13]

Cleaning and Shaping of the root canals

After 30 days of incubation, contaminated teeth were removed from the broth, and were randomly divided into five experimental groups of 10 teeth each. The working length was established for each tooth using #10 K-file (DentsplyMaillefer, Ballaigues, Switzerland). The root canals were instrumented 1 mm short of the apical foramen by using ProTaper Universal (DentsplyMaillefer, Ballaigues, Switzerland) rotary instruments according to manufacturer's recommendations. For each tooth, canal irrigation was performed with 1.5 ml of experimental irrigant using 30-gauge, 25mm irrigation needle that has a close-end tip and side port opening (Canal Clean, Biodent, Korea). The needle is passively placed without binding to canal walls and moved in coronal-apical direction inside the canal. The irrigant was left inside the canal for 20 sec. At the end of instrumentation, the canal was irrigated for 3 min with 3 ml of the experimental irrigant as a final flush.[13]

Briefly, the irrigation regimen used for the five groups was as follows:

Group I: 8% AIE with a flush of 17% EDTA Solution (Meta Biomed Co LTD. Korea) followed by a final flush of 8% AIE

Group II: 8% AIE with a flush of Normal Saline (Nirma limited, Sachana, Gujarat, India) followed by a final flush of 8% AIE

Group III: 12% MCE with a flush of 17% EDTA Solution followed by a final flush of 12% MCE

Group IV: 3% Sodium hypochlorite (Vishal Dentocare PVT.LTD. Gujrat, India) with a flush of 17% EDTA Solution followed by a final flush of 3% Sodium hypochlorite – Positive control group

Group V: Normal Saline (0.9% sodium chloride) – negative control group

From each group, the teeth were taken for evaluation of smear layer removal of the experimental irrigants.

Evaluation of Smear layer removal of experimental irrigants

The presence or absence of Smear layer in five groups that contain 10 teeth each was checked by examining the root canals under scanning electron microscope (SEM). For scanning electron microscopy the teeth were fixed by submerging them in a 10% neutral buffered formalin solution at 18°C for 24 hours. The teeth were dehydrated in a graded series of alcohol solutions and fixed in hexamethyldisilazane. The teeth were prepared for visualization in SEM by fracturing them into 2 halves along the longitudinal axis with a chisel. The fractured teeth were dried on filter paper for 15 minutes each. The dried tooth specimens were mounted on aluminum Stereoscan stubs with carbon tape. The dried mounted specimens were gold Sputtered and viewed in a Scanning Electron Microscope.[13] SEM micrographs were taken of the coronal, middle, and apical surface topography of each tooth specimen. In total, 150 SEM micrographs were taken from the 50 teeth.

Evaluation of SEM Images

The root canal surfaces were assessed for the presence of smear layer by using a modified semiquantitative visual criterion, with the scale 0–2 as follows:

(0) No removal of smear layer and no dentinal tubules visible

(1) Some removal of smear layer and some dentinal tubules visible

(2) Complete removal of smear layer and all dentinal tubules visible.

Results

Data were analyzed using Statistical Package for Social Science (SPSS version 12.0). The level of significance was set at $P<0.05$ for the tests. One way ANOVA was done between five irrigant groups and found that the median scores of smear layer removal (Table 1) on SEM by experimental irrigants vary significantly($p < 0.0001$). Bonferroni's Multiple Comparison Test was the post hoc analysis used to compare the median scores of smear layer removal on SEM by irrigants between two irrigants (Table 2). On a semiquantitative scale, the most to least effective intracanal irrigants in Smear layer removal were NaOCl and EDTA, MCE and EDTA, AIE and EDTA, AIE and Saline.

None of the irrigation treatments completely removed the smear layer from all of the instrumented root canals (Table 3). There was a significant difference in the ability to remove smear layer from the root canals by 8% AIE with 17% EDTA, 8% AIE with Saline, 12% MCE with 17% EDTA and 3% Sodium hypochlorite with 17% EDTA when compared with Normal Saline ($P<0.001$). It indicates that AIE, MCE and Sodium hypochlorite with a rinse of EDTA was efficiently removing Smear layer from the root canal when compared to Normal Saline.

4. Discussion

During biomechanical preparation, formation of the smear layer composed of debris, organic material, and microorganisms that adhere to the root canal walls, obstructs the openings of dentinal tubules. This layer must be removed with auxiliary chemicals for the better diffusion of intracanal medicaments and for proper adaptation of sealers to the dentinal walls. Root canal irrigation plays an important role in the debridement and disinfection of the root canal system and is an integral part of root canal preparation procedures. Irrigation facilitates in removal of necrotic tissue and dentine chips from the root canal and prevents packing of the infected hard and soft tissue apically in the root canal and into the periapical area.

Root canal irrigants[3] ideally should:

- Have a broad antimicrobial spectrum and high efficacy against anaerobic and facultative microorganisms organized in biofilms
- Prevent the formation of a smear layer during instrumentation or dissolve the latter once it has formed
- Dissolve necrotic pulp tissue remnants
- Inactivate endotoxin
- Be Biocompatible

The use of irrigating solutions is an important part of effective chemomechanical preparation. A large number of substances have been used as root canal irrigants, including acids (citric and phosphoric), chelating agent (EDTA), proteolytic enzymes, alkaline solutions (sodium hypochlorite, sodium hydroxide, urea, and potassium hydroxide), oxidative agents (hydrogen peroxide and Gly-Oxide), local anesthetic solutions, and normal saline.[11,12]

Although endodontic irrigants are generally considered to be safe, severe complications can occur during or as a consequence of root canal irrigation.[6,8] These complications can be hemolysis of red blood cells, dissolution of vital tissue, hypersensitivity reactions, skin ulceration, corrosion to metals, cytotoxic to human periodontal ligament (PDL) cells and human fibroblasts via inhibition of protein synthesis, staining dentin, damage to the patient's clothing, splashing the irrigant into the patient's or operator's eye, to injection through the apical foramen, or air emphysema and allergic reactions to the irrigant.[8,16,17] In order to avoid the undesirable effects of endodontic irrigants, the need for an alternative irrigant solution with smear layer removal property has increased the demand for testing the natural herbal extracts as endodontic irrigants. The first step towards this goal is invitro testing of the natural extracts for their smear layer removal efficiency.

Azadirachta indica, a Meliaceae family tree, commonly called as "Neem", has been used in India for many years in the treatment of several diseases in medicine and is being tested in dentistry as a herbal drug. Biological activity of Azadirachta indica is reported with the crude extracts and their different fractions from leaf, bark, root, seed and oil. Various pharmacological activities attributed by Azadirachta indica compounds are Anti-inflammatory, Antifungal, Antibacterial, Antiviral, Antipyretic, Antiarthritic, Antimalarial, Hypoglycaemic, Antigastric ulcer and Spermicidal. In dentistry, Azadirachta indica has been investigated, due to its antimicrobial potential against oral microorganisms.[18,19] Imran Khan et al. (2010) through their study concluded that Azadirachta indica leaf extract has antimicrobial activity against dental pathogens.[20] Literature has shown that Azadirachta indica has antimicrobial and therapeutic effects suggesting its potential to be used as an endodontic irrigant. Aarti Bohora et al. (2010) compared that the antimicrobial efficacy of Azadirachta indica leaf extract as irrigant with the standard irrigant sodium hypochlorite against Enterococcus faecalis and found that Azadirachta indica leaf extract has a significant antimicrobial effect against Enterococcus faecalis and Candida albicans and mixed state.[21] Rajesh et.al. (2015) compared the antimicrobial efficacy of Azadirachta indica, Morinda citrifolia, with sodium hypochlorite and concluded that all are equally effective when used as root canal irrigants.[22]

The intent of the present study was to find out the smear layer removal property of natural plant extract 8% Azadirachta indica extract with EDTA in comparison to that of commonly used endodontic irrigant 3% Sodium hypochlorite with EDTA and with 12% Morinda citrifolia extract with EDTA.

Morinda citrifolia, a Rubiaceae family plant has been used for centuries in folk medicine. Morinda citrifolia is

commonly known as “Indian mulberry,”“cheese fruit,” or “noni.” Morinda citrifolia plant is reported to have a broad range of therapeutic effects, including anti-inflammatory, antibacterial, antifungal, antiviral, analgesic, antitumor, antihelminthic, hypotensive and immune enhancing effects. All of the parts of Morinda citrifolia (root, bark, leaf, bud, and fruit) have been used to treat a wide range of health problems.[23,24] A study has been done by Murray et al. (2008) in comparing the effectiveness of Morinda citrifolia with sodium hypochlorite and chlorhexidine gluconate to remove the smear layer from the walls of instrumented root canals and came to a conclusion that Morinda citrifolia when used with a rinse of EDTA was more effective in removing smear layer.[13] Another study was done on Morinda citrifolia extract by D. Kandaswamy et al. (2010) on dentinal tubule disinfection comparing with 2% chlorhexidine gel, propolis, 2% povidone iodine, and calcium hydroxide and concluded that morinda citrifolia extract can be used for dentinal tubule disinfection against E. faecalis.[25] Hussain Mookhtiar et al. (2018) through their literature review stated that MCJ can be used as an endodontic irrigant and medicament due to its better smear layer removal properties and antimicrobial activity.[26]

Enterococcus faecalis is the most common and, occasionally, the only single isolated bacteria from root canals of teeth with persistent periapical periodontitis. Its inherent antimicrobial resistance, ability to adapt to harsh environmental changes, and its growth in root canal walls as biofilm make it responsible for many endodontic failures.[27-32] For these reasons, Enterococcus faecalis was used as testing organism in the present study.

In the present study Minimum Inhibitory Concentration (MIC) was found for Azadirachta indica extract as 8% and for Morinda citrifolia extract as 12% against Enterococcus faecalis. Minimum inhibitory concentration (MIC) is defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation.[33]

Saline is taken as negative control to compare the antimicrobial efficacy and smear layer removal properties with the experimental irrigant groups. In the present study, all the experimental irrigant groups were effective in smear layer removal property than Saline. Studies of Murray et al.[13], M.A. Saghiri et al.[34], M. S. Sadr Lahijani et al.[35] have shown the same result that Saline is least effective as an irrigant. The smear layer removal effect of all the irrigants was more pronounced in the coronal and middle thirds than in the apical parts of the root canals. This finding concurs with other studies Costa et al.[36], Hulsmann et al.[37] and Bilge Hakan Sen et al.[38] The smaller diameter of the root canal and the consequent decrease in the flow of the irrigant was the most probable explanation.

Azadirachta indica extract with EDTA and Azadirachta indica extract with Saline, both of these two groups were able to remove smear layer from the root canals. The smear layer removal efficacy of Azadirachta indica extract was due to its active constituents such as Nimbin, nimbidin and nimbidol.[39] On considering the numerical values, it was shown that Azadirachta indica extract with EDTA was more effective than Azadirachta indica extract with Saline, but no statistical significant difference was found between the two groups. This property was due to the presence of EDTA which helps in removing the inorganic portion of the smear layer. This was in accordance with the previous studies done by Zehnder et al.[3], Vadachkoria et al.[40], Deari et al.[41] and Sedigheh Khedmat et al.[42]

Azadirachta indica extract, Morinda citrifolia extract and NaOCl when used in conjunction with EDTA were able to remove smear layer from the root canals. On statistical

analysis, there was no significant difference between the three groups. More or less all the three groups were equally effective in removing smear layer from the root canals. But on considering the numerical values, it was shown that NaOCl with EDTA was more effective than Morinda citrifolia extract with EDTA and Azadirachta indica extract with EDTA. In case of NaOCl with EDTA group, both NaOCl and EDTA play a role in removing smear layer. NaOCl dissolves the organic portion and EDTA removes the inorganic portion by chelation, thereby smear layer is effectively removed. This action of NaOCl and EDTA was in accordance with the previous studies of Zehnder [3], M. S. Sadr Lahijani et al.[35], Bilge Hakan Sen et al.[38] and Cardoso et al.[43]

On statistical analysis, there was a significant difference between NaOCl with EDTA and Azadirachta indica extract with Saline in removing the smear layer from root canals. NaOCl with EDTA was more effective in removing the smear layer than Azadirachta indica extract with Saline. The possible reason can be due to the combined action of NaOCl with EDTA which was mentioned earlier.

On statistical analysis, Morinda citrifolia extract with EDTA has showed more or less equal efficiency in removing smear layer from root canals with Azadirachta indica extract with Saline. On considering the numerical values, it was shown that Morinda citrifolia extract with EDTA was more effective than Azadirachta indica extract with Saline. This was in accordance with the previous studies of M. S. Sadr Lahijani et al.[35], Jeremy S. Rees et al.[44] and D. R. Violich et al.[45] As the components of the smear layer are small particles with a large surface/mass ratio, they are highly soluble in acids. The reason for more effective smear layer removal by Morinda citrifolia than Azadirachta indica can be due to the presence of organic acids in it which act in conjunction with EDTA.[26,39]

5. Conclusion

The present findings are revolutionary because they suggest that intracanal irrigants and dental disinfecting solutions can be formulated from herbal extracts. Research in this area into the millions of compounds contained in fruit juices, seeds, roots, bark, and flowers will identify further compounds with the potential to be used in dentistry and endodontics. Within the limitations of this in vitro study, 8% Azadirachta indica extract is more or less equally efficient with 12% Morinda citrifolia extract and 3% NaOCl in removing the smear layer from the instrumented root canals when used in conjunction with 17% EDTA. However, none of the experimental irrigants completely removed smear layer from the dentinal walls. Further investigations on their antimicrobial efficiency, on sealing ability and combinations of irrigants will best help exploit the maximum efficacy of herbal irrigants.

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Table 1: Mean, Median, Standard Deviation of SEM Scores for five irrigant groups

Group	I	II	III	IV	V
n	30	30	30	30	30
Median	1	1	2	2	0
Minimum	0	0	0	0	0
Maximum	2	2	2	2	2
Mean ± SD	1.27± 0.69	0.97± 0.67	1.43± 0.68	1.67± 0.61	0.4± 0.56

Table 2: Comparision of Smear layer removal by five irrigant groups

Irrigant group	Irrigant group	P value
I	**II**	P > 0.05
	III	P > 0.05
	IV	P > 0.05
	V	P < 0.001*
II	**III**	P > 0.05
	IV	P < 0.001*
	V	P < 0.01*
III	**IV**	P > 0.05
	V	P < 0.001*
IV	**V**	P < 0.001*

* Significant P value < 0.05

Table 3: Effectiveness of Smear Layer removal in Apical, Middle, and Coronal aspects of root canals.

GROUP	Root canal aspect	No. of SEM micrographs analyzed	Category of smear layer removal criteria		
			0	1	2
			No removal of smear layer (%)	Some removal of smear layer (%)	Complete removal of smear layer (%)
I	Apical	10	20	60	20
	Middle	10	20	40	40
	Coronal	10	0	40	60
II	Apical	10	40	50	10
	Middle	10	30	50	20
	Coronal	10	0	70	30
III	Apical	10	20	50	30
	Middle	10	10	30	60
	Coronal	10	0	30	70
IV	Apical	10	20	30	50
	Middle	10	0	20	80
	Coronal	10	0	10	90
V	Apical	10	70	30	0
	Middle	10	60	40	0
	Coronal	10	60	30	10