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Original Article

In vivo antimicrobial efficacy of 6% *Morinda citrifolia, Azadirachta indica,* and 3% sodium hypochlorite as root canal irrigants

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ABSTRACT

Objective: To evaluate and compare the antimicrobial efficacy of 6% *Morinda citrifolia*, *Azadirachta indica*, and 3% sodium hypochlorite (NaOCl) as root canal irrigants. **Materials and Methods:** Thirty nonvital maxillary anteriors were randomly assigned to one of the three groups corresponding to the irrigant to be tested; 6% *Morinda citrifolia* juice (MCJ) (n = 10), *A. indica* (n = 10) and 3% NaOCl (n = 10). After the root canal access opening a root canal culture sample was taken with two paper points and cultured under aerobic and anaerobic conditions. Cleaning and shaping were completed with irrigation by 10 mL of respective irrigants and 5 mL of final rinse. The patients were recalled after 3 days and canals were rinsed again with 5 mL of the test irrigants. This was followed by obtaining a posttreatment root canal culture sample and culturing and analyzed by counting the colony forming units (CFUs). **Results:** Six percentage MCJ, *A. indica,* and 3% NaOCl showed a significant reduction ($P < 0.05$) in the mean CFU counts for aerobic and anaerobic bacteria between baseline and 3 days. **Conclusion:** There was no difference in the antimicrobial efficacy of 6% *M. citrifolia*, *A. indica*, and 3% NaOCl as root canal irrigants.

Key words: *Azadirachta indica*, herbal, *Morinda citrifolia*, root canal irrigants, sodium hypochlorite

INTRODUCTION

The main objective of root canal treatment is complete elimination of microorganisms from the infected root canals. Although cleaning and shaping reduce microorganisms, the use of irrigants is complimentary to instrumentation in facilitating their removal. Several chemicals and therapeutic agents are used to achieve this goal. The gold standard and the most effective among these is sodium hypochlorite (NaOCl).[1] However, owing to the potential side effects, constant increase in antibiotic resistant strains,[2,3] safety concerns,[4,5] and cytotoxic reactions of synthetic irrigants,[6] usage of various herbal agents has increased over the last few decades,[7,8] and the search is on for a root canal irrigant that can match or better the gold standard. Herbal products have been used, since ancient times in dental and medical practice, and the trend is growing now due to their high antimicrobial activity, excellent biocompatibility, anti-inflammatory, and antioxidant properties.[9]

Morinda citrifolia juice (MCJ) appears to be the first juice to be identified as a possible alternative to the use of NaOCl. Commercially, called as Noni, it is also known as Nono, Nonu, great morinda, Indian mulberry, Ba Ti Tian, dog dumpling (Barbados), mengkudu (Malaysia and Indonesia), Kumudu (Balinese), pace (Javanese), beach mulberry, Nhan, cheese fruit, and nunaakai (Tamil Nadu, India) across various cultures throughout the world.[3,5,8,10] It contains antibacterial compounds L-asperuloside with alizarin[11] and also exhibits, antiviral, antifungal,

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antihelminthic, antitumor,[12] anti-inflammatory, analgesic, hypotensive,[11] and immune-enhancing effect,[13] suggesting its potential to be used as an endodontic irrigant.

Azadirachta indica (neem) is known for its Indian medicinal value. Several parts of the plant such as fruits, seeds, leaves, and bark are used to isolate more than 140 compounds. The isoprenoid group of neem leaf and its constituents have demonstrated anti-inflammatory,[14,15] immune-modulatory,[16] antibacterial,[17,18] antifungal,[18-20] antiviral, antioxidant, and anti-carcinogenic properties.[21] Although initial scientific reports have demonstrated favorable *in vitro* results, there is no *in vivo* study that has compared the antibacterial efficacy of these three root canal irrigants against aerobic and anaerobic bacteria.

The aim of the present *in vivo* study, therefore, was to evaluate and compare the antimicrobial efficacy of 6% MCJ, *A. indica*, and 3% NaOCl as root canal irrigants. The null hypothesis to be tested was that there was a difference in the antimicrobial efficacy of 6% MCJ, *A. indica*, and 3% NaOCl as root canal irrigants.

MATERIALS AND METHODS

This prospective study was carried out over a period of 6 weeks. A set protocol with informed consent from the patient and ethical committee approval by Institutional Review Board – Ethics Committee of Terna Dental College and Hospital for the procedure was followed strictly. A total of 32 mature permanent maxillary central incisors, lateral incisors and canines with pulp necrosis, and asymptomatic apical periodontitis were included in the study, whereas those with calcified pulp chamber, periapical cyst, and contributory medical history were excluded from this study. The teeth were analyzed by preoperative radiographs and electric pulp test for identification of their pulpal and periapical status. Diagnosis of periapical cyst was done on intraoral periapical radiographs. Teeth having well-defined large periapical radiolucency with corticated border suggestive of a radicular cyst were excluded from the study. Antibiotics were not prescribed before or during the course of treatment. Thirty teeth were randomly assigned to 1 of the 3 groups corresponding to the irrigant to be tested; 6% MCJ (n = 10), *A. indica* (n = 10), and 3% NaOCl (n = 10). The remaining two teeth served as control and were irrigated with normal saline. The clinical procedures in all the teeth were carried out by a single operator.

Preparation of access cavity

After administering local anesthesia with lignocaine containing 1:80000 adrenaline (Lignox, Warren, Mumbai, India) the tooth was polished with pumice and isolated with rubber dam. Surfaces of the tooth, rubber dam, and clamp were cleaned with 30% hydrogen peroxide followed by swabbing with 5% iodine tincture as described by Moller.[22] An autoclaved high speed air turbine with a round diamond point (BR 40: Mani, Inc., Tochigi, Japan) was used to initiate root canal access opening. The operating area was again swabbed with 5% iodine tincture and then a low-speed engine without water coolant was used to gain final access. The cavity walls were modified using safe end cutting diamond point (EX 24: Mani, Inc., Tochigi, Japan). The root canal was accessed with size 10 K-file (Mani, Inc., Tochigi, Japan) and the contents were debrided from the canal walls by push pull motion. Sterile water was then deposited in the canal and agitated using size 10 K-file (Mani, Inc., Tochigi, Japan).

Method for collection of sample

Under aseptic conditions, initial pretreatment root canal culture sample was taken with sterile paper points. Two paper points were placed in the canal for 60 s and then transferred into two separate tubes of 2 mL brain heart infusion broth (BHI broth); each marked as aerobic and anaerobic sample. These were designated as sample no. 1. The tubes were transferred in 10 min for culturing under aerobic and anaerobic conditions. After the first sample, working length was determined using electronic apex locator and confirmed by radiograph. Cleaning and shaping were done by step back technique with traditional stainless steel 0.02 taper K-files (Mani, Inc., Tochigi, Japan). Master apical size was kept three sizes larger than the initial binding instrument and a 1 mm step back was done until a file size that would no longer bind tightly in the canal. A total of 10 mL of solution of the respective test group, namely 6% MCJ (Lilvera, Noni juice, Shivral Foods, India), *A. indica* (GMP Krishna, India), 3% NaOCl (Prime Dental Products, Mumbai, India), and saline were used during instrumentation of the canals. After the root canal preparation was complete 5 mL of the respective irrigant was used as a final rinse. A sterile disposable 27 gauge needle with a beveled tip was used for all irrigation procedures and the needle was kept 1 mm short of the working length. The canals were dried and the access cavity was sealed with Cavit™ G (3M ESPE AG, Germany). The patient was recalled after 3 days for obtaining post

irrigation sample. After application and disinfection of the rubber dam the canals were accessed again and rinsed by 5 mL of the respective test solutions. In the NaOCl group, the canal was flushed with 5 mL of 5% sodium thiosulfate to neutralize the effect of NaOCl. The canals were dried with paper points and sterile water was deposited in the canal to obtain a sample no. 2 as described earlier.

After the second sample collection, the teeth were treated using standard operating protocol with a week of calcium hydroxide intracanal medicament followed by obturation using the lateral compaction technique with gutta-percha (Dentsply Maillefer, Ballaigues, Switzerland) and zinc oxide eugenol (DPI, Mumbai, Maharashtra, India) as sealer. The final restoration was done with composite resin Tetric N-Ceram (Ivoclar Vivadent, Schaan, Liechtenstein).

Microbial analysis

The samples were subjected to microbiological analysis to ascertain their individual microbiological load. This was analyzed by counting the colony forming units (CFUs).

Aerobic culturing technique

The paper points were transferred immediately to the sterile test tubes. Within 10 minutes, the BHI broth was transferred to the microbiological lab. Ten microliters of the BHI broth were inoculated using micropipette on 5% Columbia sheep blood agar plates (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The inoculation loop was heated on the blue flame of Bunsen burner, till it became red hot. The loop was allowed to cool at room temperature and streaking was done on the agar plates.[23] The agar plates were placed in the incubator at 37°C for 24 h. After 24 h, the bacterial growth was counted as CFUs using manual counting technique.

Anaerobic culturing technique

The lids of the test tubes were opened slightly, and paper points were inserted immediately in the BHI broth. This was done to maintain the anaerobic environment. Within 10 minutes, the BHI broth was transferred to microbiological lab. Ten microliters of BHI broth were inoculated using micropipette on 5% Columbia sheep blood agar plates (HiMedia Laboratories Pvt. Ltd., Mumbai, India). The inoculation loop was heated on the blue flame of Bunsen burner and then allowed to cool down up to room temperature before streaking was done on the agar plates. The culture plates were placed in the anaerobox chamber with its accessories (anaerogas pouch and anaerogas indicator tablet) and then incubated for 24 h at 37°C. Anaerogas pouch and indicator tablet maintained anaerobic environment in anaerobox.[24]

After 24 h, the bacterial growth was counted as CFUs using the manual counting technique. Microbial counting was done for both aerobic and anaerobic culture in the form of CFUs. The data were analyzed using SPSS version 17 software (SPSS Inc, Chicago, IL, USA). Kruskal–Wallis test was performed to check for statistical significance.

RESULTS

Mean CFU counts for aerobic and anaerobic bacteria preoperatively and after a 3 day recall visit following irrigation by MCJ, *A. indica*, and NaOCl are summarized in Table 1 and Figure 1. Mean and percentage change between baseline and 3 day recall CFU counts for aerobic and anaerobic bacteria following irrigation by MCJ, *A. indica*, and NaOCl are summarized in Table 2. MCJ, Neem, and NaOCl showed significant reduction ($P < 0.05$) in the mean CFU counts for aerobic and anaerobic bacteria

Table 1: Mean (SD) CFU for aerobic and anaerobic bacteria at baseline and after irrigation (posttreatment)

	n	Mean (SD)
Preaerobic		
MCJ	10	59.0 (49.25)
Neem	10	74.0 (48.24)
NaOCl	10	105.8 (26.74)
Control	2	128.00 (8.48)
Total	32	82.63 (45.82)
Preanaerobic		
MCJ	10	58.90 (42.99)
Neem	10	62.40 (27.49)
NaOCl	10	84.70 (54.77)
Control	2	86.00 (16.97)
Total	32	69.75 (42.19)
Postaerobic		
MCJ	10	18.60 (25.34)
Neem	10	43.00 (42.87)
NaOCl	10	81.60 (25.50)
Control	2	124.50 (7.77)
Total	32	52.53 (43.80)
Postanaerobic		
MCJ	10	14.00 (19.50)
Neem	10	24.20 (17.89)
NaOCl	10	57.30 (35.92)
Control	2	82.00 (16.97)
Total	32	34.97 (32.71)

SD: Standard deviation, CFUs: Colony forming units, MCJ: *Morinda citrifolia* juice, NaOCl: Sodium hypochlorite

between baseline and 3 days. Intergroup comparison for the percentage reduction in aerobic and anaerobic bacteria was statistically insignificant ($P > 0.05$). There was no difference in the antimicrobial efficacy of MCJ, *A. indica*, and NaOCl for aerobic, as well as anaerobic bacteria. When compared with the control group MCJ showed a significant reduction of aerobic and anaerobic bacteria ($P < 0.05$), while neem showed a significant reduction of anaerobic bacteria ($P < 0.05$), and NaOCl showed a significant reduction of only aerobic bacteria ($P < 0.05$).

DISCUSSION

Hitherto several animal and human studies have been done to study toxicity,[25,26] cell-mediated immunity,[27] and effectiveness of topical preparations[28] of *M. citrifolia* in its different forms. Similar animal and human investigations to study the antimicrobial efficacy,[29,30] toxicity,[31-33] and antisecretory property[29,34,35] of neem have been published in literature. Hence, this *in vivo* investigation to study the antimicrobial efficacy of herbal irrigants in an endodontic model was planned. Maxillary central incisors, lateral incisors, and canines were chosen for this study as they have a single large, wide, and straight canal in which disinfection protocol can be evaluated without procedural errors. Smith *et al*.[36] have reported that for achieving comparable results and to avoid bacterial contamination from other canals in multirooted teeth and uniradicular teeth must be selected. Rubber dam isolation during endodontic treatment provides protection against salivary contamination of the pulp chamber.[37] In the present study, iodine tincture was used for decontamination of surfaces before and after initial access cavity preparation with a high speed air turbine.[22] This was done to disinfect the tooth, rubber dam, and retainer from contamination by water spray of air turbine.

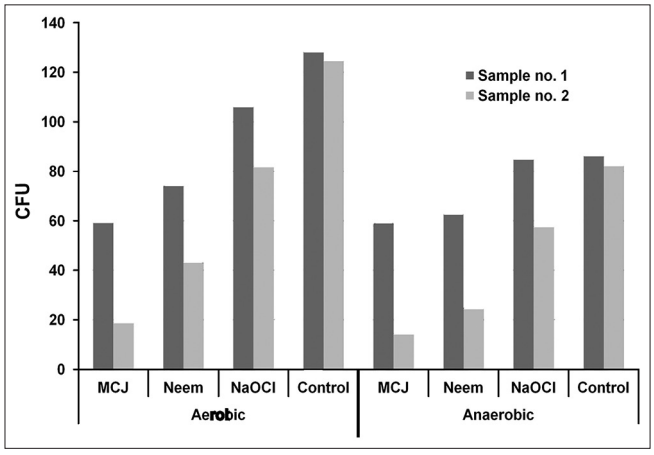

Figure 1: Reduction in mean (standard deviation) colony forming units for aerobic and anaerobic bacteria between sample no. 1 and sample no. 2 for various irrigants

BHI broth was used as a transport medium as it propagates the growth of fastidious pathogenic cocci and other organisms. This contaminated broth was inoculated on 5% Columbia sheep's blood agar culture plates as it contains peptone which supports rapid and luxuriant growth of fastidious and nonfastidious organisms. These culture plates were used for growth of both aerobic and anaerobic microorganisms, as plates can be incubated in an atmosphere containing approximately 3–10% CO2. In the present study, anaerobic conditions were maintained by placing the agar plates in an anaerobox, a transparent polycarbonate box which contains anaerobic gas pack for consuming the residual oxygen. Simultaneously, an anaerobic indicator tablet was placed keeping the transparent cover intact and the box was sealed with a lid. This box was incubated at 37°C for 24 h. The color of anaerobic indicator tablet changed to pink indicating anaerobic conditions and no leakage in the system. Microbiological research techniques often rely on accurate determination of colony forming units. This is routinely done by aliquoting a small amount of broth on culture plates. After incubation the colonies are counted to determine the number of CFUs. This is done by manual counting of colonies on plates illuminated by transmitted light. The concentration of bacteria in the original culture can then be calculated based on the assumption that each colony has raised from one single bacterium.[38] In the current study, manual counting technique was used for determination of CFUs.

Table 2: Mean change and percentage change of aerobic, anaerobic, and total CFUs

Name of group	Mean change (aerobic)	Percentage change (aerobic)	Mean change (anaerobic)	Percentage change (anaerobic)	Mean change (total)	Percentage change (total)
MCJ	40.4*	68.47	44.9*	76.23	85.3	72.34
Neem	31*	41.8	38.2*	61.21	69.2	51.50
NaOCl	24.2*	23.04	27.4*	32.34	51.6	27.69
Control	3.5	2.70	4	4.60	7.5	3.65

*Statistically significant ($P<0.05$). MCJ: *Morinda citrifolia* juice, NaOCl: Sodium hypochlorite, CFUs: Colony forming units

Satisfactory antimicrobial efficacy of NaOCl as a root canal irrigant has been shown at concentrations of 3%[39-41] or even lower.[42,43] Therefore, a 3% concentration was chosen for comparison. The *in vivo* antimicrobial efficacy of neem against a mixed flora of aerobic and facultative endodontic pathogens of primary endodontic disease was tested in the present study. Several *in vitro* investigations in the past have illustrated that *A. indica* can be used as a root canal irrigant owing to its desirable antimicrobial property.[7,44-47] Agar diffusion method to study antimicrobial efficacy of neem implies that it has significant antimicrobial activity against endodontic pathogens.[7,45,46] Tyagi *et al*.[44] have found neem to have a lower antimicrobial efficacy than 5% NaOCl against *C. Albicans*. Although we found neem to have significant antimicrobial activity against both anaerobic and aerobic bacteria, our findings cannot be directly when compared with those of Ghonmode *et al*.[7] and Hegde *et al*.[45] as their study was carried out on *Enterococcus faecalis* and *C. Albicans*, which are commonly found in endodontic reinfection cases. Our results are in partial agreement with Dutta *et al*.[30] who found no difference in antimicrobial efficacy of neem and 2.5% NaOCl against anaerobic bacteria. Our findings suggest that there is no difference in the efficacy against facultative, as well as anaerobic bacteria. Our findings also clinically validate those of Mistry *et al*.[46] who found neem to be very effective against *Streptococcus mutans* and *Staphylococcus aureus* in an agar diffusion model.

The ability of MCJ to remove smear layer has been established by Murray *et al*.[48] and Saghiri *et al*.[10] However, endodontic literature regarding the antimicrobial efficacy of MCJ is sparse and contradictory with Kandaswamy *et al*.[49] showing MCJ to be effective against *E. faecalis* in dentine of extracted teeth. A study by Bhardwaj *et al*.[50] revealed ultrasonic irrigation of MCJ to be ineffective in completely removing *E. faecalis* biofilm. Our findings showed a significant reduction of CFUs of aerobic, as well as anaerobic bacteria and support the use of MCJ as an endodontic irrigant.

Under the limitations of this study, it was concluded that all the test irrigants caused a significant reduction in the mean CFU counts of aerobic and anaerobic bacteria. Six percentage MCJ and *A. indica* were as antibacterial as 3% NaOCl in root canal irrigation. The null hypothesis was rejected as it was concluded that there was no difference in the antimicrobial efficacy of 6% MCJ, *A. indica*, and 3% NaOCl as root canal irrigants. Future *in vivo* studies with combinations of irrigants and the role of ultrasonic activation will best help exploit the maximum efficacy of herbal irrigants.

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Conflicts of interest
There are no conflicts of interest.

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