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Antimicrobial efficacy of Propolis, *Morinda citrifolia,* Azadirachta indica (neem) against *Actinomyces radicidentis*: An *in vitro* study.

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Abstract

Objective: The objective of this *in-vitro* study was to evaluate and compare the antimicrobial activity of three endodontic herbal irrigating agents, Propolis, *Azadirachtaindica* (neem) and *Morinda citrifiolia* (MCJ) against *Actinomyces adicidentis.*

Methods: Disk diffusion method was used. After the preparation of herbal medicine, few colonies were transferred in Trypticase Soy Broth (Hope Bio China) medium contained in a test tube and kept for overnight growth. Culture was centrifuged at 3000 rpm (Cence China) for 10 minutes and 0.5 McFarland standard prepared until optical density of 600 nm was achieved. The samples were collected and 1:10 serial dilution was prepared with it. 100 µL of the suspension was added to Columbia blood agar (Oxoid) plates by using streak method. Primary culture plates were incubated at 37°X in 5% CO_2 incubator for 24 hours. After 24 hour zone of inhibition was achieved, sample that were analyzed by counting the colony forming units. The ANOVA test was used for statistical analysis of the data. The level of significance was set at 0.05.

Result: A quantitative and qualitative analysis showed complete inhibition. Neem showed the highest zone of inhibition against Actinomyces radicidentis followed by sodium hypochlorite, Propolis and MCJ. Neem, propolis, MCJ and NaOCl showed a significant reduction ($p < 0.05$) in the mean CFU counts for bacteria reduction.

Conclusion: An *in vitro* observation of herbs seems promising and is highly satisfactory. It seems herbs have better efficacy, bio compatibility, economics and easily available. NaOCL have several undesirable characteristics.

Keywords: Efficacy of herbal irrigants, Microbita of teeth, Herbs, *Actinomyces radicidents*.

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Introduction

The success of endodontic therapy is dependent upon several factors, eradication of root canal (RC) infection is very important during endodontic treatment because residual infection is one of the chief factors leading to post-treatment failure [1].

Bacteria and their products have the main role in the initiation and exacerbation of pulp and periapical diseases [2]. Therefore, the main purpose of root-canal treatment (RCT) is to eliminate bacteria and their products from the pulp space. Studies have shown that the most probable cause of RCT failure is bacterial flora in the apical portion of the RC [3]. In research undertaken on necrotic human teeth, Sundqvist et al. concluded that apical periodontitis occurred only in teeth containing bacteria in RCs [4].

Use of chemical agents during instrumentation to clean all aspects of the RC system completely is central to successful endodontic treatment. Irrigation is complementary to instrumentation in facilitating removal of pulp tissue and/or microorganisms [5].

Recent advances in chemotaxonomic and molecular biology-based identification methods have clarified the taxonomy of the genus Actinomyces, and have led to the recognition of several new Actinomyces (and related) species. Actinomyces-like Gram-positive rods have been isolated increasingly from various clinical specimens [6]. Recently, Actinomyces radicidentis (AR) has been reported in the infected RCs of patients after repeat RCT failure [7,8].

A study by Nair et al. showed the ability of AR to build characteristic host-resistant actinomycotic colonies/ monospecies and biofilms in the vital tissues of rats. Their finding supports the hypothesis that AR is a potential etiologic agent of persistent apical periodontitis owing to its ability to survive in the inflamed periapical tissues of the host [9].

Elimination of bacteria can be achieved with mechanical preparation combined with use of antimicrobial irrigants [10].

The most commonly used irrigant is sodium hypochlorite (NaOCl), which was introduced by Dakin HD [11]. Some of the drawbacks of (NaOCl) are toxicity, unpleasant odour and taste, as well as an inability to remove microorganisms and smears completely from RCs [12,13]. Due to the side-effects of these agents and the development of resistant strains, research is on-going to find the "ideal" substance for RC disinfection. Herbal extracts have also been tested as RC irrigants [14,15].

NaOCl is the "gold standard" due to its efficacy against pathogenic organisms and pulp degeneration in endodontic treatment. Its concentration for use varies from 0.5% to 5.25%, and 2% be a common concentration because the risk of an iatrogenic incident is reduced [16].

Azadirachta indica (neem) has antifungal, antibacterial, antioxidant, antiviral, anti-inflammatory, analgesic, anti-pyretic and immunostimulatory activities [17]. The leaf extract of neem is a commonly used antibacterial agent. However, little information is available on its potential use in RC irrigation [18].

Propolis is a hard, resinous material derived by bees from plant juices and used to seal openings in hives. It contains pollen, resins, waxes and large amounts of flavonoids (benzopyrone derivatives found in all photosynthesizing cells). Flavonoids have several biologic effects in animal systems but have received relatively little attention from pharmacologists [19]. Flavonoids are potent antimicrobial, antioxidant and anti-inflammatory agents [20]. The antibacterial and antifungal properties of propolis have been investigated extensively [21-23].

Morinda citrifolia is a fruit-bearing tree in the coffee family Rubiaceae. *Morinda citrifolia* juice (MCJ) has antibacterial, antiviral, antifungal, anti-tumor, antihelminthic, analgesic, hypotensive, anti-inflammatory, and immune-enhancing effects [24].

Several studies using a tooth model based on the agar disk diffusion method have been published, but few *in vitro* studies have been done. The antimicrobial efficacy of irrigants *in vitro* should be tested using biofilm models that closely resemble clinical situations. Developing poly microbial biofilms obtained from clinical isolates of disease processes is important [25].

The objective of this *in vitro* study was to evaluate and compare the antimicrobial activity of three endodontic herbal irrigating agents (propolis, neem and MCJ) against AR.

Growth of bacteria

AR (CCUG 36733T; DSMZ, Germany) was inoculated from Columbia blood agar (Oxoid, China) and trypticase soy broth (TSB; Hope Bio, China).

Preparation of media

Prepared agar plates of Columbia blood agar were purchased from Thermo Fisher (USA). TSB was obtained from Qingdao Hope Biotechnology (China). Media powder was weighed on a digital weighing machine (Toledo; Mettler, Switzerland) and prepared according to manufacturer instructions. It was autoclaved at 121 for 20 minutes, immediately after autoclaving, was allowed to cool in a water bath at 45-50℃.

Before inoculation, media and broth were prepared according to manufacturer instructions. Briefly, a double-vial glass ampule sealed in a plastic box was opened. Precautions were undertaken to break the glass ampule safely (protective spectacles). A Bunsen burner was used to heat the ampule tip. A few drops of distilled water were poured onto the hot ampule tip to crack the glass. Forceps were used to carefully stroke-off the glass tip, remove the insulation material, and take out the inner vial. The cotton plug was lifted and removed with the help of forceps. The vial was kept under sterile conditions, and the top of the inner vial was heated to prevent contamination. After addition of 0.5 mL of broth, the plug was replaced and pellet allowed rehydrating for 30 minutes. Subsequently, the contents were mixed gently with a sterile pipette, and the entire mixture transferred to a 5 mL test tube containing TSB. Then, 100 μL of the suspension was transferred to an agar plate for subculture.

The "streak" technique was used for the disk diffusion method and inoculation in Columbia blood agar. The inoculated primary culture plates were incubated immediately at 37℃ in a 5% CO_2 incubator for 24 hours in petri dishes.

Samples were divided into four experimental groups: propolis (in distilled water, dimethyl sulfoxide (DMSO) or ethylene); MCJ; Neem; 3% NaOCl; distilled water.

Preparation of irrigants

Neem: Neem powder (100% purity) was purchased from Alka Ayurvedic Pharmacy (India). Neem powder was used in 15 g of 70% ethanol (150 mg/mL) as reported by Nayak Aarati et al. [26] Neem powder was weighed accurately and mixed with solvent. The mixture was kept in amber bottles, closed tightly, taped with aluminium foil, and stored at 4℃. The mixture was stirred twice-daily for 2 weeks, after which it was stored at room temperature before filtration. Then, the mixture was filtered with muslin cloth (folded four times). The supernatant was filtered twice with filter paper #1 (Whatman, USA). After the final filtration, it was stored in the dark at room temperature.

Propolis: Propolis extract in powder form (100% purity) was purchased from Guangzhou Jiehe Bees (China). Propolis was prepared at different concentrations in 100 mL. Propolis solution was prepared 70% ethanol propolis 150 mg/mL, (15 g), propolis 30% in 10% DMSO (150 mg) was adopted from Shveta Gupta [27]. Propolis was prepared in the same manner as mentioned above for neem. Finally, the filtered dark-brown mixture was stored in the dark at room temperature.

MCJ: MCJ powder (100% purity) was purchased from Guangzhou Jiehe Bees. A 100 mL mixture was prepared. MCJ 6% (6 g), DMSO 10% MCJ in distilled water. The mixture was prepared in the same way as that for neem and propolis solutions were prepared. The target concentration was prepared according to the work of Barani et al. [28].

NaOCl: 5% NaOCl was used in this *in vitro* study.

Distilled water: Distilled water was used as a control. Its activity was checked before experimentation, and a zone of inhibition (ZoI) against cultures was not observed.

Disk diffusion method

The Kirby–Bauer method was used as recommended by the National Committee for Clinical Laboratory Standards (USA). In this well-known procedure, agar plates were inoculated with a standardized inoculum of the test microorganism. Then, filter-paper disks 6 mm in diameter and prepared with Whatman filter paper using a hole-punch) containing the test compound at the desired concentration were placed on the agar surface. Using sterile forceps, the test solution-impregnated disks were placed in the medium. In each experiment, test solutions were evaluated in triplicate. Petri dishes were incubated under suitable conditions. One milliliter of the bacterial culture (which was equivalent to the 0.5 McFarland standard) was taken for serial dilution and mixed with 9 mL of distilled water contained within tubes number 1-10. Subsequently, a series of dilutions was made to maintain a ratio of 1:10. From each dilution, 100 µL was collected and disseminated on agar plates using the streak technique. These preparations were incubated for 24 hours at 37℃. In general, antimicrobial agents diffuse into agar after 24 hours of incubation at 37℃, and then the diameters of ZoIs were measured. A ZoI is the area on an agar plate where the growth of bacteria is prevented in the presence of antibiotics or other microbial compounds. The dimension of a ZoI can be measured, and is an indicator of the efficacy of an antibiotic. For ZoI measurement, we used a digital vernier caliper and ruler.

Turbidity standard for inoculum preparation (0.5 McFarland standard)

To standardize the inoculum density, a suspension containing approximately 1.2 × 108 CFU/mL of AR was prepared. Adequate light was used to compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black lines (Wickerham turbidity card). The density of the turbidity standard was verified using a eppendorf bio photometer plus (germaney) with a 1-cm light path and matching cuvette to determine the absorbance. The absorbance at 620 nm was 0.008–0.10 for the 0.5 McFarland standards.

Statistical analysis

Experiments were n=5 and done in triplicate. A single operator undertook experiments. Data were checked for completeness, and then analysed using statistical package for social sciences (SPSS v20) (IBM, USA). Descriptive statistics (frequencies, percentages) for categorical variables and mean ± standard deviation for numerical variables were obtained for the mean ZoI (in mm). The difference between the effect of NaOCl and herbal irrigants were analyzed by analysis of variance (ANOVA). $p<0.05$ was considered significant.

Results

ZoI values were highest for NaOCl, followed by neem, propolis and MCJ. The lowest ZoI was found for MCJ in distilled water (Table 1).

Table 1. *Antimicrobial activity of the test materials (N=5) (ANOVA)*

95% Confidence Interval for Mean								
	N	Mean	Std. Deviation	Std Error	Upper border	Lower border	F	Sig
10% DMSO Propolis	5	17	1.581	0.707	1.581	18.96	11.478	0.002
Distilled Water Propolis	5	19.2	4.97	2.223	13.03	25.37	3.876	0.05
70% Ethanol Propolis	5	14.6	1.817	0.812	12.34	16.86	0.087	0.918
Distilled Water Neem	5	22.6	7.301	3.265	13.54	31.66	4.69	0.031
70% Ethanol Neem	5	19.4	3.847	1.72	14.62	24.18	0.539	0.597
Distilled Water	5	12.4	2.074	0.927	9.83	14.97	0.519	0.608
10% DSMO MCJ	5	15	2.55	1.14	11.83	18.17	10.072	0.003
3% Sodium Hypochlorite	5	24.2	2.28	1.02	21.37	27.03	3.646	0.058

The ZoI (in mm) for propolis was 19 in 10% DMSO, 23 in distilled water, and 23 in 70% ethanol. The ZoI for neem was 17 mm in distilled water and 25 mm in 70% ethanol. The ZoI

for MCJ was 15 mm in distilled water and 17 mm in 10% DMSO. The ZoI for NaOCl was 27 mm (Table 2).

Table 2. Antimicrobial activity of test material, Zone of inhibition (ZOI) in mm (N=5)

Medicine	N=5	ZOI (mm)	Mean	Std Deviation
10% DMSO Propolis	5	19.1	17	1.581
Distilled Water Propolis	5	23	20	2.236
70% Ethanol Propolis	5	23	14.6	2.236
Distilled water (Neem)	5	17	14.6	2.236
Ethanol Neem	5	25	19.4	3.847
MCJ distilled water	5	15	12.4	2.074
MCJ 10% DMSO	5	17	15	2.55
3% Sodium Hypochlorite	5	27	27	2.28

Each colony-forming unit (CFU) count was done to normalize data before statistical evaluation due to the high variance of bacterial numbers. The reduction in CFU/mL for bacteria was significant (Table 3).

Table 3. Colony forming unit (CFU) bacterial reduction after test experiment.

Test Agents	Total bacteria +ve Group (ml)	Test experiment/ml	Bacteria killed by medicine
Distilled Water Propolis	2.28×10^5	1.74×10^5	5.40×10^4
70% Ethanol Propolis	6.72×10^6	6.54×10^6	1.80×10^5
Distilled water (NEEM)	1.08×10^5	7.50×10^4	3.30×10^4
70% Ethanol Neem	8.70×10^4	6.60×10^4	2.10×10^4
MCJ distilled water	2.52×10^5	2.43×10^5	9.00×10^4
MCJ 10% DMSO	8.70×10^4	2.25×10^5	2.70×10^4
3% Sodium Hypochlorite	2.25×10^5	2.07×10^5	1.80×10^4

Discussion

NaOCl is the gold-standard RC irrigant used in endodontic practice [29]. Nevertheless, the drawbacks of NaOCl include an unpleasant taste, toxicity and iatrogenic accidents. To overcome these side-effects and to meet the requirements of ideal irrigants, researchers have focused on phytomedicines. These agents possess several pharmacologic activities and medicinal applications. Interest in these substances is based on their antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antipyretic, and analgesic properties.

We employed the agar diffusion method. This study design is in accordance with other studies testing the antimicrobial actions of drugs [30].

Propolis contains pollen, resins, waxes and large amounts of flavonoids. The latter have many biologic effects in animal systems, but have received relatively little attention from pharmacologists [31].

We wished to investigate the beneficial effect of propolis as an endodontic intracanal irrigant. A 30% solution of propolis was very effective in eliminating AR. Our results are similar to Sinha DJ and co-workers in which propolis were efficacious against other RC pathogens.

AR was selected for the present study because it is a relatively newly described bacterial species. AR was isolated first from the infected RCs of human teeth in 1996. AR has been found in repeat RCT failures (~37%) [7,8]. We used a particular AR strain (CCUG 36733T) for the first time in an *in vitro* study in China. Also, we tested, for the first time, the antimicrobial efficacy of herbal RC irrigants and compared them with the antimicrobial efficacy of a conventional irrigant: NaOCl [32].

Propolis has been reported to kill 7.33% of *Enterococcus faecalis* and 8.33% of Candida albicans colonies. Akca et al. reported that 20 g of propolis dissolved in 100 mL of 80% ethanol could kill 8.8% of *E. faecalis* colonies, 16.16% of *Actinomyces israelii* colonies, and 16.16% of *C. albicans* colonies. An ethanol extract of propolis has been shown to be more efficacious against Gram-positive bacteria and *C. albicans* than against Gram-negative bacteria [33]. While our study results 70% Ethanol 33% Propolis 15 g (14.6%), 10% DMSO propolis (17%) and 36 g propolis in distilled water were on AR (20%), which strongly supports our stud. Rathore JP and co-workers claimed that the pH and concentration of propolis solutions might be altered due to solvents, and that acidic propolis solutions were more effective against bacteria [34]. Those data may suggest that biofilms of test microorganisms may respond in an identical way to the concentrations of all test and control agents used in the present study.

Extracts of neem leaves have been shown to possess equivalent broad-spectrum antimicrobial activities against endodontic pathogens and, most importantly, to exert biocompatibility to oral and periapical tissues. Extracts of neem leaves seem to be efficacious against biofilms due to their anti-adherence properties [35].

In the present study, neem in distilled water could kill 22.6% of AR colonies and NaOCl could kill 24.2% of AR colonies. Neem in ethanol could kill 19.4% of AR colonies. Also, 3% NaOCl was shown to be the optimal concentration for use as an irrigant. NaOCl at 2-4% concentration showed an antimicrobial effect with minimum irritation. NaOCl at 2% concentration has antimicrobial properties but it is ineffective against most resistant strains in biofilms. Therefore, 3% NaOCl was selected due to its broad-spectrum efficacy against most resistant species in chronic periapical infections, especially endodontic pathogens and AR.

Ambhore et al., using ZoI values, stated that 5% NaOCl and a 10% extract of neem leaves were very efficacious against *E. faecalis* and *C. albicans* [35]. Srinidhi and co-workers detected no significant difference in ZoI values between neem leaf extracts and 3% NaOCl. We found that neem in ethanol had a very satisfactory antimicrobial effect as compared with neem in distilled water.

Conclusion

MCJ has a broad range of therapeutic effects and was tested in the present study. Chandwani and co-workers showed that 1% NaOCl and MCJ, when used as irrigants, were very effective in reducing the mean CFU/mL value after irrigation. However, intergroup comparison revealed no significant difference in antimicrobial efficacy between 1% NaOCl and MCJ. In our study, MCJ in distilled water could kill 12.4%, MCJ in 10% DMSO could kill 15%, and NaOCl could kill 24% of AR colonies. Our study showed a significant reduction in CFU/mL of AR, and supports the use of MCJ as an endodontic irrigant.

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Conflict of Interest

The authors declare no conflict of interest.

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