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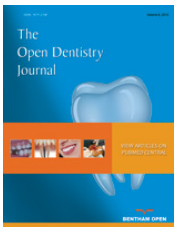

Evaluation of the Antibacterial Efficacy of *Azadirachta Indica, Commiphora Myrrha, Glycyrrhiza Glabra* Against *Enterococcus Faecalis* using Real Time PCR

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Abstract:

Aim:

To compare the antibacterial efficacy of Azadirachta indica (Neem), Commiphora myrrha (Myrrh), Glycyrrhiza glabra (Liquorice) with 2% Chlorhexidine (CHX) against *E. faecalis* by using Real Time PCR

Materials and Methods:

A total of fifty teeth specimens (n=50) were inoculated with *E. faecalis* for 21 days. Specimens were divided into five groups (Group 1: Myrrh, Group 2: Neem, Group 3: Liquorice, Group 4: 2% CHX and Group 5: Saline (negative control)). The intracanal medicaments were packed inside the tooth. After 5 days, the remaining microbial load was determined by using real time PCR

Results:

Threshold cycle (Ct) values of Myrrh extract, Neem extract, Liquorice Extract, 2% CHX and saline were found to be 30.94, 23.85, 21.38, 30.93 and 17.8 respectively

Conclusion:

Myrrh extract showed inhibition of E.faecalis equal to that of 2% CHX followed by Neem, Liquorice and Saline

Keywords: Commiphora myrrha, Neem, Liquorice, 2% CHX, Dentinal tubules, intracanal medicament, real- time polymerase chain reaction.

INTRODUCTION

Elimination of bacteria is a critical step in root canal therapy since it is primarily responsible for periapical disease [1]. Bacterial elimination is usually attempted by mechanical instrumentation, irrigation and use of intracanal medicaments [2, 3]. Irrigants like sodium hypocholorite (NaOCl), MTAD and 2% Chlorhexidine (CHX) are commonly

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used in root canal treatment [4]. Inspite of good efficacy of these irrigants, certain bacteria like *Enterococcus faecalis* might still survive inside the root canal system. *E.faecalis* plays a major role in persistent endodontic infections and is frequently isolated from failed root canal systems [5, 6]. Intracanal medicaments help in the reduction of residual bacteria present after instrumentation and irrigation [7].

Natural herbal extracts have gained therapeutic importance in dental science in the recent days.Liquorice is a very sweet, moist, soothing herb which is most commonly used as a flavoring agent in kampo medicines. It has anti-inflammatory [8] antiviral [9] and anticarcinogenic [10, 11] properties.

Azadirachta indica, also known as "Indian neem" extensively used in Ayurveda, Unani and Homoeopathic medicine and has become a cynosure of modern medicine [12]. It has antibacterial [13, 14] antiviral [14] antifungal [15] antioxidant [16] anti-inflammatory [17] antimalarial properties [18]. *Commiphora myrrha* are small trees or shrubs with short, thorny branches, a variable species found in southern Arabia and northeast Africa (chiefly Somalia) as far south as northeast Kenya [19]. Myrrh is a known stimulant [20] analgesic [21] anti-inflammatory [22] antibacterial [23] antifungal [23] anticancer properties [24].

Hence, the aim of the present study was to evaluate the antibacterial efficacy of naturally available herbal extracts of Myrrh, Liquorice, Neem and compare it with that of 2% CHX against *E. faecalis*, by using Real Time PCR.

MATERIALS AND METHOD

Preparation of Herbal Extracts

Herbal extract was obtained from the Faculty of Pharmacy, Sri Ramachandra University. Commiphora Myrrha, Neem leaves were shade dried, powdered and stored in air-tight containers. 1g powder of Myrrh was dissolved in 10ml of sterilized distilled water. They were soaked at room temperature for 24 hours, then filtered [25]. Air dried Neem powder was repeatedly macerated with 500ml of 99% ethanol and filtered using whatman filter paper [26]. The ethanol was evaporated and the extracts were concentrated using rotary flash evaporator and stored at 4°C until used in the assay [26]. Liquorice extract was prepared by the protocol mentioned in the reports of Badr *et al* [27].

Determination of MIC of Natural Extracts

Agar diffusion test was performed on Mueller-Hinton agar using the well diffusion method. Mueller-Hinton agar was freshly prepared after which, the surface was inoculated with 0.2 mL of brain heart infusion broth culture of *E. faecalis* strain ATCC 29212. The six wells (5 mm in diameter and 4 mm in depth) were created in the agar, of which three wells were filled with the plant extracts with 50% ethanol as the solvent. Then, one well was filled with plain 50% ethanol without the extract which acted as the negative control. Further, two wells were filled with 2% CHX and saline, respectively. The same procedure was carried out in duplicate and incubated for 24 hours anaerobically at 37ºC. Plates were read after 24 hours for the presence of zone of inhibition.

Preparation of Dentin Specimens

A total of fifty human mandibular premolars with single roots were used. Teeth with caries, apical fractures and resorption were excluded from the study. A rotary diamond disc was used to decoronate the teeth below the cemento enamel junction and the apical part of the root.Hence remaining portion of the root has been standardised (6mm). Cementum was removed from the root surface. The internal canal diameter was standardized by using Gates Glidden Drills No 4 (Mani Inc, Tachigiken, Japan). The debris was removed using ultrasonic bath of 17% ethylene diamine tetra acetic acid for 5 min, followed by 3% NaOCl for 5 min. The teeth were immersed in ultrasonic bath of distilled water for 10 min. The specimens were autoclaved for 20 min at 121ºC.

Antimicrobial Assessment

A suspension of 50µl of *E. faecalis* (ATCC 29212) strain was incubated in 5ml of Trypticase Soy Agar broth (TSA) culture medium (Difco, Sparks, MD, USA) at 37ºC, anaerobically for 4 hours. The concentration of the inoculation was then adjusted for a degree of turbidity of 1, according to the McFarland scale. This corresponds to a bacterial load of 3×10^8 cells/ml referent to an optical density of 550nm. The samples were recontaminated with fresh broth containing the *E.faecalis* every second day under laminar flow.The tooth specimen were removed from the broth , rinsed with sterile saline and dried .

The blocks were divided into 5 groups (n=10 each): Group 1: Myrrh; Group 2: Neem; Group 3: Liquorice; Group 4: 2% CHX and Group 5: saline (negative control). Methyl cellulose was used as the thickening agent for all the groups and the medicament was packed inside the root canal. The canals were then sealed at both the ends with paraffin wax and incubated at 37ºC in an anerobic environment. After 5 days, the harvesting of dentin was carried out at 400 µm depth with a sterile Gates-Gliden drill no. 5 [28].

Bacterial Genomic DNA Isolation from Harvested Dentin

To the dentin sample, 1ml of lysozyme stock solution was added and vortexed, which was then incubated at 37ºC for 30 minutes. To this mixture, 10% sodium dodecylesulphate (SDS) was added and vortexed. The samples were again incubated at 37ºC for 30 min in a water bath. Following the above step, equal volume of Phenol Chloroform was added and mixed by spinning at 10,000rpm for 10 min. The supernatant was then transferred to new sterile Eppendorf tubes, to which an equal volume of Chloroform iso-amyl alcohol was added. Again, the supernatant was collected from this tube and transferred to a new Eppendorf tube, to which $1/10^{th}$ by volume sodium acetate and ethanol were added. Then, the solution was mixed by spinning at 10,000rpm for 10 min and the supernatant was discarded. The pellet was dried and to this 30µl of sterile water was added to dissolve the DNA, which was ready for the Real-time PCR analysis

Real Time PCR - Detection of 16s rRNA Gene

The Real Time PCR reaction was performed in a final volume of 20 µl using a 96-well optical plate covered with an optical adhesive sheet. The Real-time PCR assay was carried out using thermal cycler (7900 HT Real-Time PCR system) with SYBR green .Universal 16s rRNA primers (forward primer 5'GATTAGATACCCTGGTAGTCC 3' and reverse primer 5' CCCGGGAACGTATTCACCG 3') for *E. faecalis* were used for quantification. The PCR conditions used were, 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 35 cycles. The efficacy of the treatment against *E. faecalis* was calculated based on the cycle threshold (CT) value [29, 30]

Table 1. Ct value (Mean ±SD) for Myrrh, Neem, Liquorice, 2% CHX and saline.

Groups (n=10 each)	Ct values (Mean ± SD)
Myrrh	30.94 ± 1.41
Neem	23.85 ± 0.76
Liquorice	21.38 ± 0.89
2% CHX	30.93 ± 1.58
Saline	17.8 ± 0.31

* $p < 0.05$ was considered to be statistically significant.

Table 2. Percentage change in bacterial load.

Groups (n = 10 each)	Percentage (%) reduction in bacterial load in comparison to Saline
Myrrh	73.8
Neem	33.9
Liquorice	20.1
2% CHX	73.7

STATISTICAL ANALYSIS

Data were statistically analysed using one-way ANOVA, followed by post hoc Tukey's HSD multiple comparison of means to compare the differences in bacterial inhibition between the groups. $p < 0.05$ was considered to be statistically significant.

RESULTS

The minimum inhibitory concentration was determined to be 2.5 µg/ml,4 µg/ml,4 µg/ml,2 µg/ml for Myrrh, Neem , Liquorice and 2% CHX respectively. Polymerase chain reaction determines the result in threshold cycle (C_T). The extracts in descending order of efficacy is as follows: Myrhh, 2% CHX, Neem, Liquorice and saline (Table **1**). Also, no statistically significant difference was observed seen between Myrrh and 2% CHX. The percentage reduction of E faecalis after treatment with Myrhh, Neem, Liquorice, 2% CHX was compared with saline (Table **2**).

DISCUSSION

The results of the present study showed that myrrh extract caused inhibition of E.faecalis equal to that of 2% CHX. This might be attributed to the anti-bacterial compounds sesquiterpene and T. cadinol present in Myrrh [31]. Sesquiterpene interacts with the cell envelopes which in turn leads to the disruption of cell membranes and thereafter, bacteriolysis [31].

In the present study, 2% CHX was also shown to cause reduction of E.faecalis. Chlorhexidine is a bis-guanide that acts by adsorbing onto the cell wall of the microorganisms, causing changes in the outer cell membrane, resulting in leakage of intracellular components [32]. It is biocompatible and has broad-spectrum antimicrobial activity [33] targeting both the gram positive and gram-negative microbes [34].

Neem followed Myrrh extract and 2% CHX in causing inhibition of bacterial growth. The antibacterial activity of neem could be due to the presence of several active constituents like nimbidin, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, margolone and cyclictrisulphide [35]. The respiratory chain is inhibited by these active constituents which inhibits the mitochondrial oxidative phosphorylation. The mitochondrial ATP content and intra mitochondrial levels of acetyl CoA, acid soluble COA and acid soluble CoA esters are also reduced [35].

The antimicrobial efficacy of Liquorice extract against *E. faecalis*, might be attributed to the Glycyrrhizin [36]. Unlike simply altering the surface tension of the extracellular medium, the antibacterial effects of saponins involve membranolytic properties [37]. Inhibition of oxygen consumption in bacterial cells is also due to the flavanoid content of the liquorice extract [38].

CONCLUSION

Myrrh extract had higher antibacterial efficacy compared to Neem and Liquorice. Further studies need to be conducted to ascertain the exact mechanisms of action of Myrrh extract against *E. faecalis*.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Declared none.

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