Real Time PCR Evaluation of the Antibacterial Efficacy of *Azadirachta indica*, *Commiphora myrrha*, *Glycyrrhiza glabra* against *Fusobacterium nucleatum*

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**Abstract**

**Aim**: To evaluate the antibacterial efficacy of 2% chlorhexidine (CHX) with *Commiphora myrrha*, *Azadirachta indica*, *Glycyrrhiza glabra* against *Fusobacterium nucleatum* by using Real Time PCR.

**Materials and Methods**: Fifty teeth specimens (n = 50) in total were inoculated with *Fusobacterium nucleatum* organism for 21 days. Specimens were categorized into five groups as follows (Group 1: Myrrh, Group 2: Neem, Group 3: Liquorice, Group 4: 2% CHX and Group 5: Saline (negative control). The tooth specimens were then packed with intracanal medicaments. The residual microbial load was assessed by using real time PCR, after 5 days.

**Results**: Threshold cycle (Ct) values of Myrrh extract, Neem extract, Liquorice Extract, 2% CHX and saline were found to be 30.06, 26.1, 16.45, 29.9 and 14.62 respectively.

**Conclusion**: Myrrh extract exhibited suppression of *Fusobacterium nucleatum* equivalent to that of 2% CHX subsequent by Neem, Liquorice and Saline.

**Keywords**: Real-Time Polymerase Chain Reaction; *Commiphora myrrha*; 2% Chx; Neem; Liquorice; Intracanal Medicament; Dentinal Tubules

**Introduction**

Bacteria plays a causative role in the patho-genesis of apical periodontitis [1]. Hence elimination of bacteria is a critical step in root canal therapy. Elimination of bacteria during root canal treatment is achieved by mechanical instrumentation, irrigation with antibacterial agents, and medication with intracanal dressing [2]. Ethylene diamino tetra acetic acid (EDTA), Mixture of Tetracycline, Acetic acid and Detergent (MTAD), Sodium Hypochlorite (NaOCl), 2% Chlorhexidine are commonly used as Antibacterial Irrigants to curtail residual root canal bacteria. Inspe of using antibacterial irrigants, certain bacteria like *Fusobacterium nucleatum* might survive and are frequently isolated from Retreatment and Flare-up cases.

*Fusobacterium nucleatum* is a gram-negative, non-spore forming, non-motile, obligate anaerobic rod. Studies have shown that this species is intimately associated with human periodontal diseases and has also been reported in endodontic canals [3]. It has been identified that *F. nucleatum* is responsible for development of the most severe forms of inter appointment endodontic flare-ups [3] indicating its potential role in endodontic infections [4].

Intracanal medicaments can help in reducing the bacteria remaining after chemo-mechanical instrumentation and can provide an environment, conducive to periapical tissue repair [3]. The quest for alternatives and complements to the common regimen continue to be investigated with the aim to improve bacterial elimination during the root canal treatment [2].

Natural herbal extracts have gained therapeutic importance in dental sciences because of their scientifically proved efficacy, and increased incidence of bacterial resistance to antibiotics [5,6]. Herbal extracts of neem and liquorice have gained the attention of Endodontists because of their unique anti-adherence activity thereby altering bacterial adhesion and ability of an organism to colonize [7].

Glycyrrhiza glabra also called as Liquorice, an herb, commonly used in Kampo medicines as a flavouring agent, and has antiviral [8], anti-carcinogenic [9,10] and anti-inflammatory properties [7].

Azadirachta indica popularly known as Indian neem has antibacterial [11,12], antiviral [12], antifungal [13], antioxidant [14], anti-inflammatory [15], antimalarial [16] properties Commiphora myrrha is a shrub known for its analgesic [17], antiseptic, anti-inflammatory [18], astringent, antispasmodic, antibacterial [19], antifungal [19] and anticancer [20] properties.

**Aim of the Study**

The aim of this study was to evaluate the antibacterial efficacy of 2% chlorhexidine (CHX) with natural herbal extracts of Commiphora myrrha, Azadirachta indica, Glycyrrhiza glabra against Fusobacterium nucleatum by using Real Time PCR.

**Materials and Method**

**Preparation of herbal extracts**

All herbal extracts were procured from the Faculty of Pharmacy, Sri Ramachandra University. Commiphora myrrha oleo-resin gum was finely grounded into powder and sequentially extracted with ethanol. Neem leaves were shade dried, powdered and stored in air-tight containers. Air dried Neem powder was repeatedly macerated with 500ml of 99% ethanol and filtered using what man filter paper [21]. Rotary flash evaporator was used to evaporate ethanol and concentrate the extracts which were stored at 4°C to be used in the assay [21]. The protocol for preparation of the Liquorice extract was as per the reports of Badr., et al. [22].

**MIC determination of natural extracts**

Agar diffusion test was carried out on Mueller-Hinton agar by using the well diffusion method. 0.2 ml of brain heart infusion broth culture of Fusobacterium nucleatum (ATCC10953) was inoculated on the surface of freshly prepared Mueller-Hilton agar. A total of six wells were created, which constitutes of 5mm in diameter and 4 mm in depth were created in the agar, out of which three wells were filled with 50 percent ethanol as solvent with plant extracts. Followed by plain 50 percent ethanol without extract was filled in one well, which acted as negative control. In addition to that, remaining wells (two wells) were loaded with 2% CHX and saline, respectively. The same procedure was repeated in duplicate and incubated for 24 hours anaerobically at 37ºC. Then the presence of zone of inhibition were checked by reading the plates after 24 hours.

**Dentin specimen preparation**

Fifty single rooted human mandibular premolars were used in this study Teeth with resorption, caries and apical fractures were not included in the study. Teeth were decorated below cement enamel junction and apical part of root by using a rotary diamond disc. Thereby, standardising the remaining portion of the root by 6mm. In addition to that, removal of cementum was done from the root surface. Gates Glidden drills No 4 (Mani Inc, Tachigiken, Japan) were utilised to standardize the internal canal diameter. Subsequent to 17% ethylene diamine tetra acetic acid for 5 min (ultrasonic bath), 3% NaOCl for 5 min were used to remove the debris. ultrasonic bath of distilled water for 10 minutes was used to immerse the teeth, followed by autoclave of the specimens for 20 min at 121ºC.
Antimicrobial assessment

A suspension of 50 µl of 50 µl of *Fusobacterium nucleatum* (ATCC10953) was incubated in 5 ml of Trypticase Soy Agar broth (TSA) culture medium (Difco, Sparks, MD, USA) at 37°C anaerobically for 4 hours. The concentration of the inoculation was then adjusted for a degree of turbidity 1 according to the McFarland scale (Bio Merieux, Marcyl'Etoile, France), which corresponds to a bacterial load of 3 × 10^8 cells/ml referent to an optical density of 550 nm. The samples were recontaminated with fresh broth containing *Fusobacterium nucleatum* every second day under laminar flow. The tooth specimen was removed from the broth, rinsed with sterile saline and dried.

The blocks were segregated 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). For all the groups, methyl cellulose was used as a thickening agent, followed by packing of medications inside the root canal. Both the ends of the root canal were sealed using paraffin wax and proceeded with incubation at 37°C in an anaerobic environment. Dentin was harvested after 5 days of incubation, at 400 µm depth using a sterile Gates Glidden drill no 5 [23].

Bacterial genomic dna isolation from harvested dentin

Isolation of Bacterial Genomic DNA from Harvested dentin is achieved by adding 1ml of lysozyme stock solution to the dentin sample. After vortexing the mixture is incubated for 30 minutes at 37°C. 10% Sodium dodecyl sulphate (SDS) is added to the mixture, vortexed and incubated again for 30 minutes in a water bath at 37°C. An equal volume of Phenol chloroform is added and the mixture is spun at 10,000 rpm for ten minutes. The Supernatant is taken in Sterile new Eppendorf tubes, and mixed with an equal volume of Chloroform iso-amyl alcohol. The supernatant is transferred to another sterile new Eppendorf tube and sodium acetate and ethanol are added 1/10th by volume. This solution is blended by spinning it for 10 minutes at 10,000rpm, following which the supernatant is cast-off. The DNA which was set for Real-time PCR analysis was dissolved by adding 30 µl of sterile water to the dried pellet.

Real Time PCR - Detection of 16s rRNA Gene

A 96-well optical plate covered with an optical adhesive sheet was used to detect 16s rRNA while performing Real Time PCR in a final volume of 20 µl. A Thermal cycler (7900 HT Real-Time PCR system) was used to carry out the Real time PCR Assay. The reaction mix contained 16SrDNA primers, sterile water, template and SYBR Green master mix. Universal 16s rRNA primers (Forward primer 5'-ATTGG GCT AAA AAT TAT AGTT-3' and Reverse Primer Reverse primer 5' ACC CTC ACT TTG AGG ATT ATA G 3') for *Fusobacterium nucleatum* were used for quantification. The PCR parameters were as follows-the Sample volume was 5 µl and the Number of cycles were 40. Initial denaturation at 95°C for 10 minutes, Denaturation at 95°C for 1 minute, Annealing at 50°C for 15 seconds and Extension at 72°C for1 minute. Cycle threshold (CT) value was used to calculate the efficacy of the treatment against *Fusobacterium nucleatum* [24,25].

Statistical analysis

Statistical Analysis of the data was done with one-way ANOVA. To compare the differences in bacterial inhibition between the Groups, Tukey’s HSD multiple comparison of means was used. Statistically significant P value was obtained- p < 0.05.

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. The result in Cycle Threshold value (CT) was determined by Polymerase chain reaction. The antibacterial efficacy of herbal extracts, in descending order is as follows Myrrh, 2%CHX, Neem, Liquorice and Saline (Table 1). The results after treatment with Myrrh and 2% Chlorhexidine didn’t show any statistically significant difference. After treatment with Myrrh extract, Neem extract, Liquorice Extract, and 2% CHX, the percentage reduction of *Fusobacterium nucleatum* was calculated and recorded. These values were compared with Saline (Table 2).
Groups (n=10 each) | Ct values (Mean ± SD)
--- | ---
Myrrh | 29.56 ± 0.73
Neem | 26.14 ± 0.89
Liquorice | 16.95 ± 0.75
2% Chlorhexidine | 30.04 ± 0.74
Saline | 14.62 ± 1.09

Table 1: Ct value (Mean ±SD) for Myrrh, Neem, Liquorice, 2% CHX and saline.
*p < 0.05 was considered to be statistically significant.

Groups (n=10 each) | Percentage (%) reduction in bacterial load in comparison to Saline
--- | ---
Myrrh | 73.5
Neem | 35.1
Liquorice | 17.3
2% Chlorhexidine | 73.8

Table 2: Percentage change in bacterial load.

Discussion

The primary aim of endodontic treatment is to remove as many bacteria as possible from the root canal system and then to create an environment unsuitable for survival of any remaining organisms [26]. The use of a biocompatible intracanal medication possessing antimicrobial properties between appointments may reduce or eliminate bacteria in the root canal system and significantly increase the success of root canal therapy [27]. *Fusobacterium nucleatum* is a key organism in endodontic flare-ups and has been found to be present in 31% of teeth that presented with the most severe symptoms [28].

The results of the present study showed that myrrh extract caused inhibition of *Fusobacterium nucleatum* equal to that of 2% Chlorhexidine myrrh is composed of a volatile (essential) oil, including sesquiterpenes, an alcohol-soluble resin containing commiphoric acids and a water-soluble gum [29]. Sesquiterpene is bactericidal and it interacts with the cell envelopes causing disruption of cell membrane thereby leading to bacteriolysis and subsequent fatal loss of intracellular material [30].

This study displayed the anti-bacterial efficacy of 2% Chlorhexidine against *Fusobacterium nucleatum*. Chlorhexidine belongs to the group of bis-guanide [31]. It causes leakage of the intracellular components by adsorbing onto the cell wall of the microorganisms and causing changes in the outer cell membrane. It has a broad-spectrum antimicrobial activity [32], targeting both gram positive and gram-negative microbes and biocompatible [33].

Next to Myrrh and 2% Chlorhexidine gel, Neem was found to possess effective antibacterial activity, which can be attributed to the presence of constituents like nimbidin, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, margolone and cyclic trisulphide [34]. The respiratory chain in the bacterial cell is inhibited because the oxidative phosphorylation in the mitochondria is uncoupled by these active constituents [35]. It also decreases intramitochondrial levels of acetyl CoA and acid-soluble CoA esters and reduces the mitochondrial ATP content [34].

The antimicrobial effect of Liquorice is due to its isoflavonoid components [36]. Rather than simply altering the surface tension of the extracellular medium, the antibacterial effects of saponins involve membranolytic properties [37]. The flavonoid content of Liquorice extract is also a strong inhibitor of oxygen consumption in bacterial cells [38].

**Conclusion**

Based upon the results and considering the limitations of this study, it can be concluded that Myrrh can be used as a supplement to 2% chlorhexidine gel as an intracanal medicament because of its higher antibacterial efficacy. Further researches are needed to ascertain the exact mechanism of action of myrrh against *Fusobacterium nucleatum*.

**Conflict of Interest**

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

**Acknowledgement**

Declared none.

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