ECRONICON OPEN ACCESS

EC DENTAL SCIENCE
Research Article

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

Anand Suresh*, Shyamala Nagendran, Senthilkumar Ganapathy, Subin Jacob and Jalison Jacob

Department of Conservative Dentistry and Endodontics University, Penang International Dental College, Malaysia

***Corresponding Author**: Anand Suresh, Department of Conservative Dentistry and Endodontics University, Penang International Dental College, Malaysia.

Received: August 01, 2019; **Published:** October 05, 2019

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 14.62respectively

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. Inspite 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 37ºC 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 medicaments inside the root canal. Both the ends of the root canal were sealed using paraffin wax and proceeded with incubation at 37ºC 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 37ºC. 10% Sodium dodecyl sulphate (SDS) is added to the mixture, vortexed and incubated again for 30 minutes in a water bath at 37ºC. An equal volume of Phenol chloroform is added and the mixture is spinned at 10,000 rpm for ten minutes. The Supernatant is taken in Sterile new Eppendorf tubes, and mixed with an equal volume of Chloroform isoamyl alcohol. The supernatant is transferred to another sterile new Eppendorf tube and sodium acetate and ethanol are added $1/10^{th}$ 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	17.38
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.

Bibliography

1. Sundqvist G. "Bacteriological studies of necrotic dental pulps [Diss]". Umeå (1976).
2. Sathorn C., *et al*. "Antibacterial efficacy of calcium hydroxide intracanal dressing: a systematic review and meta-analysis". *International Endodontic Journal* 40.1 (2007): 2-10.
3. Chavez de Paz Villanueva LE. "Fusobacterium nucleatum in endodontic flare-ups". *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology* 93.2 (2002): 179-183.
4. Johnson EM., *et al*. "Coaggregation interactions between oral and endodontic Enterococcus faecalis and bacterial species isolated from persistent apical periodontitis". *Journal of Endodontics* 32.10 (2006): 946-950.
5. Graf J. "Herbal anti-inflammatory agents for skin disease". *Skin Therapy Letter* 5.4 (2000): 3-5.
6. Walsh FM and Amyes SG. "Microbiology and drug resistance mechanisms of fully resistant pathogens". *Current Opinion in Microbiology* 7.5 (2004): 439-444.
7. Hanus L., *et al*. "A Moussaieff Myrrh-Commiphora chemistry". *Biomedical Papers* 149.1 (2005): 1- 28.
8. Fukai T., *et al*. "Anti-Helicobacter pylori flavonoids from licorice extract". *Life Sciences* 71.12 (2002): 1449-1463.
9. Van Gelderen C., *et al*. "Glycyrrhizic acid: the assessment of a no effect level". *Human and Experimental Toxicology* 19.8 (2000): 434-439.
10. Shibata S. "A drug over the millennia: Pharmacognosy, chemistry, and pharmacology of Licorice". *Yakugaku Zasshi* 120.10 (2000): 849-862.
11. Rao DS., *et al*. "Antibacterial activity of aqueous extracts of Indian chewing sticks on dental plaque: An in vitro study". *Journal of Pharmacy and Bioallied Sciences* 6.1 (2014): S140-S145.
12. Bharitkar YP., *et al*. "Antibacterial and antiviral evaluation of sulfonoquinovosyl diacylglyceride: a glycolipid isolated from Azadirachta indica leaves". *Letters in Applied Microbiology* 58.2 (2014): 184-189.

13. Mahilrajan S., *et al*. "Screening the antifungal activity of essential oils against decay fungi from palmyrah leaf handicrafts". *Biological Research* 47 (2014): 35.

14. Rao A., *et al*. "Ayurtox for body Detoxification". *Journal of Enzyme Inhibition* 14 (1998): 856.

15. Tidjani M., *et al*. "Anti inflammatory activity of Azadirachta indica". *Planta Medica* 23 (1989): 259-266.

16. Dhar R., *et al*. "Inhibition of the growth and development of asexual and sexual stages of drug-sensitive and resistant strains of the human malaria parasite Plasmodium falciparum by Neem (Azadirachta indica) fractions". *Journal of Ethnopharmacology* 61.1 (1998): 31-39.

17. Dolara P., *et al*. "Analgesic effects of myrrh". *Nature* 379.6560 (1996): 29.

18. Su S., *et al*. "Anti-inflammatory and analgesic activity of different extracts of Commiphora myrrha". *Journal of Ethnopharmacology* 134.2 (2011): 251-258.

19. Dolara., *et al*. "Local anaesthetic, antibacterial and antifungal properties of sesquiterpenes from myrrh". *Planta Medica* 66.4 (2000): 356-358.

20. Shoemaker M., *et al*. "In vitro anticancer activity of twelve Chinese medicinal herbs". *Phytotherapy Research* 19.7 (2005): 649-651.

21. Vinothkumar TS., *et al*. "In vitro evaluation of five different herbal extracts as an antimicrobial endodontic irrigant using real time quantitative polymerase chain reaction". *Journal of Conservative Dentistry* 16.2 (2013): 167-170.

22. Badr AE., *et al*. "A laboratory evaluation of the antibacterial and cytotoxic effect of Liquorice when used as root canal medicament". *International Endodontic Journal* 44.1 (2011): 51-58.

23. Kandaswamy D., *et al*. "Dentinal tubule disinfection with 2% chlorhexidine gel, propolis, morinda citrifolia juice, 2% povidone iodine, and calcium hydroxide". *International Endodontic Journal* 43.5 (2010): 419-423.

24. Endo MS., *et al*. "Quantitative and qualitative analysis of microorganisms in root-filled teeth with persistent infection: Monitoring of the endodontic retreatment". *European Journal of Dental* 7.3 (2013): 302-309.

25. Winfred SB., *et al*. "Antimicrobial activity of cationic peptides in endodontic procedures". *European Journal of Dental* 8.2 (2014): 254-260.

26. Siqueira JF., *et al*. "Patterns of microbial colonization in primary root canal infections". *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology and Endodontology* 93.2 (2002): 174-178.

27. Bystrom A., *et al*. "The antibacterial effect of camphorated paramonochlorophenol, camphorated phenol and calcium hydroxide in the treatment of infected root canals". *Endodontics and Dental Traumatology* 1.5 (1985): 170-175.

28. Moraes SR., *et al*. "Comparison of the effectiveness of bacterial culture, 16S rDNA directed polymerase chain reaction, and checkerboard DNA-dNA hybridization for detection of Fusobacterium nucleatum in endodontic infections". *Journal of Endodontics* 28.2 (2002): 86-89.

29. Omar SA., *et al*. "Antimicrobial Activity of Commiphora myrrha Against Some Bacteria and Candida albicans Isolated from Gazelles at King Khalid Wildlife Research Centre". *Research Journal of Medicinal Plant* 5.1 (2011): 65-71.

30. Claeson P., *et al*. "Bactericidal effect of the sesquiterpene T-cadinol on Staphylococcus aureus". *Phytotherapy Research* 6.2 (1992): 94-98.

31. Carlo Ceschel G., *et al*. "A Design and evaluation in vitro of controlled release mucoadhesive tablets containing chlorhexidine". *Drug Development and Industrial Pharmacy* 32.1 (2006): 53-61.

32. Delany GM., *et al*. "The effect of chlorhexidine gluconate irrigation on the root canal flora of freshly extracted necrotic teeth". *Oral Surgery, Oral Medicine, Oral Pathology* 53.5 (1982): 518-523.

33. Yesilsoy C., *et al*. "Antimicrobial and toxic effects of established and potential root canal irrigants". *Journal of Endodontics* 21.10 (1995): 513-515.

34. Biswas K., *et al*. "Biological activities and medicinal properties of neem (Azadirachtaindica)". *Current Science* 82.11 (2002): 1336-1345.

35. Bohora A., *et al*. "Comparison of antibacterial efficacy of neem leaf extract and 2% sodium hypochlorite against E. faecalis, C. albicans and mixed culture- an in vitro study". *Endodontology* 22 (2010): 8-12.

36. Murray MT. "The Healing Power of Herbs". Prima publishing (1995).

37. Fouad AF and Barry J. "The effect of antibiotics and endodontic antimicrobials on the polymerase chain reaction". *Journal of Endodontics* 31.7 (2005): 510-513.

38. Haraguchi H., *et al*. "Mode of antibacterial action of retrochalcones from Glycyrrhiza inflata". *Phyto Chemistry* 48.1(1998): 125-129.

Volume 18 Issue 11 November 2019