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EVALUATION OF ANTI-BACTERIAL EFFICACY OF HERBAL IRRIGANTS AGAINST ENTEROCOCCUS FAECALIS USING RT-PCR

Dr. Gowtham S.P [1], Dr. Vandana J *[2], Dr. Charanya C [3], Dr. Balagopal S [4] and Dr. Sunil CV [5]

[1] Assistant Professor, Department of Dental Surgery, P.S.G Institute of Medical Science, Peelamedu, Coimbatore, Tamil Nadu, India
[2] Professor, Department of Conservative Dentistry and Endodontics, Tagore Dental College and Hospital, Rathinamangalam, Chennai, Tamil Nadu, India.
*Corresponding Author Email: vandnajames@yahoo.com
[3] Reader, Department of Conservative Dentistry and Endodontics, Tagore Dental College and Hospital, Rathinamangalam, Chennai, Tamil Nadu, India.
[4] Professor and Head, Department of Conservative Dentistry and Endodontics, Tagore Dental College and Hospital, Rathinamangalam, Chennai, Tamil Nadu, India.
[5] Professor, Department of Orthodontics, Tagore Dental College and Hospital, Rathinamangalam, Chennai, Tamil Nadu, India.

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Abstract

Introduction: A wide variety of synthetic antimicrobial agents have been used over the years as endodontic irrigants. Because of the increased antibiotic resistance to these antimicrobial agents, toxic and harmful side effects of few common antibacterial agents, there is a need for an alternative agents which are affordable, non-toxic and effective. Research shows that natural plant extracts could be used as effective endodontic irrigants. **Aim :** To compare and evaluate antimicrobial efficacy of herbal extracts Azadirachta indica (Neem), Aloe barbadensis miller (Aloe vera) and Curcuma longa (Turmeric) with 2.5% sodium hypochlorite (NaoCl)against the *Enterococcus faecalis* using real time polymerase chain reaction technique. **Statistical Analysis:** Statistical analysis was performed using One way Anova/Kruskal-Wallis test with post-hoc Tukey's HSD and was statistically significant ($P < 0.05$). **Results:** It was observed that Turmeric was highly efficient in reducing *Enterococcus faecalis* within the root canals when compared with other extract. **Conclusions:** Turmeric extract has a significant antimicrobial efficacy against *Enterococcus faecalis* similar to 2.5% sodium hypochlorite than Neem and Aloe vera.

Keywords: Endodontic İrrigant, Enterococcus Faecalis, Herbal Extracts, Microbiology, Polymerized Chain.

INTRODUCTION

One of the primary objectives of endodontic therapy is the microbial reduction or their elimination, to promote the normal healing and reestablishment of the health of the periradicular tissues. Mechanical instrumentation cannot sufficiently disinfect root canals and hence irrigating solutions and intracanal medicaments are required to eradicate microorganisms. Over a period of time, a variety of chemicals have been introduced. [1] An endodontic irrigant should ideally exhibit powerful antimicrobial activity, disinfect the root canal space, and have no cytotoxic effects on periradicular tissues.

Some of the gold standard irrigating solutions are sodium hypochlorite and chlorhexidine. However, constant increase in antibiotic resistant strains and side effects of chemical irrigants has led to the search for alternative herbal medicaments. Herbal extracts with their superior properties like ease of availability, cost effectiveness, low toxicity, anti-bacterial and anti-inflammatory effects can be a potential alternative. Various herbal extracts, such as *Aloe barbadensis miller,*

Azadirachta indica, Morinda citrifolia, and *Curcuma longa* are having antimicrobial, anti-inflammatory, and therapeutic effects. These are promising organic/natural products that can be used as endodontic irrigants against potent microorganism such as E.feacalis.[2]

E. faecalis possess different virulence factors that enable them to adhere to dentin and invade dentinal tubules.[3] *Enterococci* also express factors that aid their adhesion to host cells and extracellular matrix, which in turn facilitates tissue invasion, causes immunomodulation and produces toxin mediated damage.[4] The *E. faecalis* collagen binding protein, ACE and a serine protease, targets the extracellular matrix proteins of host cells and allows adherence to type I collagen.[5]

Sodium hypochlorite (NaOCl) is one of the most commonly used endodontic irrigant because of its ability to destroy a broad spectrum of microbes,[6] but it has some undesirable characteristics such as tissue toxicity, allergic potential, and disagreeable taste, which has prompted researchers to look for other alternatives.[7] The literature has shown that various natural plant extracts, a source of bioactive compounds has antimicrobial and therapeutic effects suggesting its potential to be used as an endodontic irrigant.[6,7]

Hence, the present study was performed to compare and evaluate antimicrobial effect of herbal extracts that can be used as root canal irrigating agents such as Azadirachta indica (Neem), Aloe barbadensis miller (Aloe vera) and Curcuma longa (Turmeric) with sodium hypochlorite using real time polymerase chain reaction technique.

MATERIALS AND METHODS

The study was presented and approved by Institutional review board of Tagore Dental College and Hospital (IEC/TDCH/110/2021)

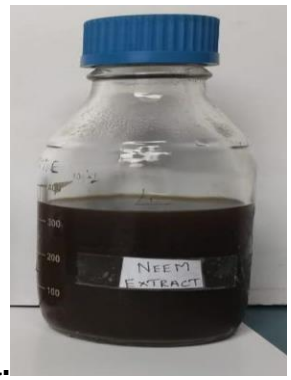
PREPARATION OF EXTRACTS

***Preparation of Neem extract(figure 1)*:** Mature fresh Neem leaves were collected taxonomic identification of the plant was performed.100 g of neem leaves were tied in a muslin cloth and socked in 800 ml of distilled water in a beaker. This beaker was boiled under low flame till the extract reduced to 400 ml to obtain 25% concentration of aqueous neem extract. After the extract cooled, it was filtered using a filter paper and stored for usage.[8]

***Preparation of Aloe vera extract (figure 1)*:** Leaves of Aloe Vera were collected. Pulp was removed from fresh 100g of Aloe Vera leaves and converted into a liquid form using a mixer. This mix was diluted by mixing with distilled water in a 1:5 ratio. The mix was then placed in a crucible on water bath for dehydration. Precipitate of extract was dissolved in methanol for use as an irrigating agent.[9]

***Preparation of Turmeric extract (figure 1)*:** Turmeric rhizomes were washed with distilled water and patted dry. They were then cut into pieces and completely dried in an oven by a tray drying process at a temperature of 40± 5 °C for a period of about 7-10 days till they are moisture-free. The pieces were ground to form a coarse powder which was then placed in a large glass chamber into which 80ml of sterile distilled water were added to prepare the aqueous extract. The glass chamber are closed with a glass lid to prevent evaporation of the menstruum and the chamber were allowed to stand for seven days with occasional stirring of the contents. The strained and

expressed liquids thus obtained were mixed and clarified by filtration. Pure turmeric extract are taken and mixed with distilled water. Turmeric preparation are of 6.4gms in 80ml of distilled water.[10]

a. 

b. 

c. 

Figure 1 shows the prepared herbal extracts of a. Neem, b. Aloe vera and c. Turmeric

Preparation Of Teeth Sample: Forty single rooted premolars which were extracted due to orthodontic reason were taken for the study. The tooth were decoronated to standardize the root length to 14 mm which was measured using vernier caliper. The canals were instrumented up to F3 Protaper Gold (DENTSPLY International Inc.,India) and teeth were autoclaved at 121°C for 20 minutes at 15 psi. 1 mL E*. faecalis* (ATCC 29212) suspension was injected into the root canals and the samples were incubated at 37°C and 100% humidity for 21 days. The samples were randomly divided into four groups [10].

Irrigating procedure: The samples were randomly irrigated according to the following groups and then evaluated for RT-PCR and CLSM.

Group 1 – Neem, GROUP 2 – Aloe vera , GROUP 3 - Turmeric , GROUP 4 - 2.5% NaOCl

Each irrigant (2ml) was used for irrigation respectively after instrumentation by each file. Irrigation was done using 29 gauge needle (Pvc Disposable Syringe 5cc,Soni Surgicals ,Hariyana, India) and final irrigant volume used was 10 ml per canal. Post instrumentation and irrigation again bacterial samples were taken using paper points and were cultured and subjected to real time PCR.[11]

RT-PCR PROCEDURE

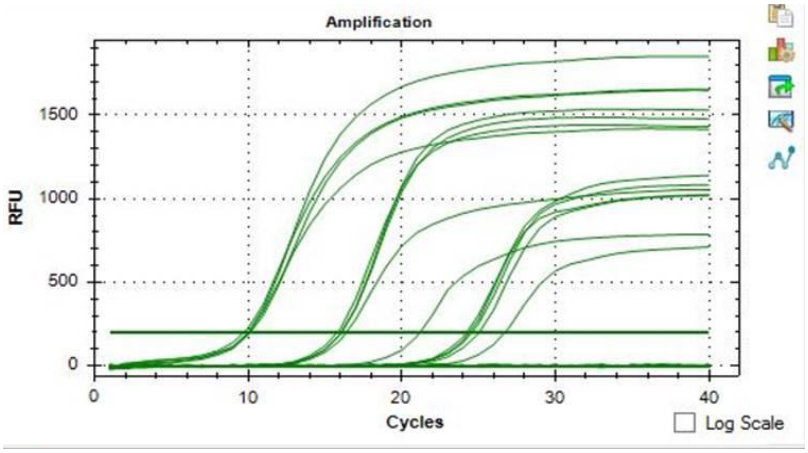
Real-Time quantitative polymerase chain reaction:

The PCR reaction was performed in a final volume of 20 µl and loaded in an optical 96-well plate, which was then covered with an optical adhesive sheet. The primers used amplified enterococcal DNA sequences in the tuf gene. The PCR conditions were as follows: The initial denaturation was at 94°C for 15 seconds, annealing at a temperature of 55°C and extension at 72°C for 45 seconds. The final extension was at 72 for 5 minutes and then cooled to 4°C until removed. All PCR experiments had positive and negative controls. The rtPCR assay was carried out in a thermal cycler (7900 HT Real-time PCR system). The reaction mix contained 16Sr DNA primers, sterile water, template and SYBR Green master mix.[12] Data obtained will be statistically analyzed using one-way ANOVA, followed by post hoc Tukey's at $p < 0.05$.

RESULTS

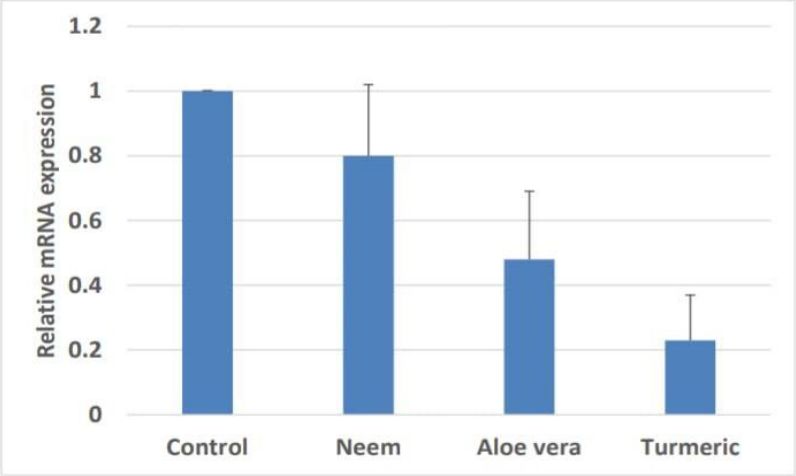
On evaluation of RT-PCR results it was observed that group 3 (Turmeric) showed better antibacterial efficacy compared to other groups (Figure – 2). Rt-PCR results from figure 2 shows that Group 3 (Turmeric- 0.25) has the higher significant expression of virulence gene in *E.faecalis* than control group sodium hypochlorite(1.0) when compared with Neem(0.8) and Aloe vera(0.5).

Amplification plot Relative :



Relative mRNA expression of the virulence gene of *Enterococcus faecalis* as observed in different groups , Figure 2:

Effect of different bioactive material on the expression of virulence gene in *Enterococcus faecalis*



DISCUSSION

Sodium hypochlorite (NaOCl) is one of the most commonly used endodontic irrigant because of its ability to destroy a broad spectrum of microbes, but it has some undesirable characteristics such as tissue toxicity, allergic potential, and disagreeable taste, which has prompted researchers to look for other alternatives. Literature has shown that various natural plant extracts, a source of bioactive compounds has antimicrobial and therapeutic effects suggesting its potential to be used as an

endodontic irrigant. The available scientific evidence suggests that irrigating solution must be effective against *E.faecalis* to become successful in clinical endodontic practice.[14,15] In rtPCR the release of the fluorescent dye during each amplification round allows the products to be detected and measured in real-time when the amplification is first detected.[16]

In the past decade a major shift has occurred in oral microbiology from studies based on culturing to one that utilize molecular techniques. Among the most popular molecular techniques to detect bacteria are those based on PCR amplification of the 16S or other ribosomal DNA sequences.

In contrast to endpoint PCR methods that essentially provide qualitative data, quantitative real-time PCR detects both the specific gene targets in bacteria and allows quantification of bacteria in samples.[17] *E. faecalis* was taken in the present study because it has been identified as the most common species in root canal diseases.[18]

These 3 herbal extracts such as Neem (Group 1), Aloe vera(Group 2) and Turmeric (Group 3) used in this study are proven to be safe, containing active constituents that have beneficial property such as antimicrobial, antioxidant and anti-inflammatory activity.

The Azadirachita indica extracts has undergone extensive pharmacological screening and found to have several pharmacological activities due to the presence of several active constituents like nimbidin, nimbin, nimbolide, gedunin, azadirachtin, mahmoodin, margolone and cyclictrisulphide responsible for its antibacterial action.[19]

Its anti-adherence activity by altering bacterial adhesion and the ability of organism to colonize has resulted in Azadirachita indica having the maximum reduction in adherence of *E. Faecalis* to dentin.[20]

Aloe vera is a naturally occurring herbal medicament having antibacterial properties. [21]

It has anti-inflammatory, antibacterial, antifungal, and antiviral properties.[22]

Because it contains anthrax quinine, it inhibits *E. faecalis* and *Streptococcus pyogenes.* Karkare *et al* [23] concluded that Aloe vera showed the highest zone of inhibition against *E. faecalis* similar to NaOCl.

Curcumin, a yellow bioactive pigment, is the major constituent of turmeric which has a wide spectrum of biological actions such as anti-inflammatory, antioxidant, antifungal and antibacterial activities.[24]Components of tumeric are named curcuminoids, which include mainly curcumin (diferuloyl methane), demethoxycurcumin, and bisdemethoxycurcumin. Curcumin (diferuloylmethane) is a polyphenol derived from *Curcuma longa* plant, commonly known as turmeric.

The active constituents of turmeric are the flavonoid curcumin (diferuloylmethane) and various volatile oils including tumerone, atlantone, and zingiberone. Other constituents include sugars, proteins, and resins. The best-researched active constituent is curcumin, which comprises 0.3-5.4% of raw turmeric. Curcumin has been used extensively in ayurvedic medicine for centuries, as it is nontoxic and has a variety of therapeutic properties including antioxidant, analgesic, anti-inflammatory, antiseptic activity, and anticarcinogenic activity. The in vitro studies have shown that curcumin

inhibits the polymerization of FtsZ protofilaments and disturbs the GTPase activity in cytoskeleton of B. subtilis, E. coli, and S. aureus.

Through this mechanism, it can influence the cell division and proliferation of bacteria. Other investigations have exhibited that curcumin stimulates an apoptosis-like response in E. coli.[25].

In our study, it was observed that Turmeric leaf extract has a significant expression of virulence gene when compare to other herbal irrigants (Neem, Aloe vera) i.e. antimicrobial efficacy against *E. Faecalis* .

The results of our study have shown Turmeric herbal extract(0.25) has the higher significant expression of virulence gene in *E.faecalis* similar to control group sodium hypochlorite(1.0) when compared with Aloe vera(0.5) and Neem(0.8)Curcumin and aloe vera also has shown better results which is statistically significant.

With the results of our study, it would appear prudent to replace the traditional root canal irrigants with these potential natural extracts. The limitation of the study is that it is an *in vitro* study with a limited sample size. Major disadvantage with herbal extracts is the need of fresh preparation and modification in taste for acceptability.

CONCLUSION

Within the limitation of this invitro experiment we conclude that Turmeric herbal extract has better antibacterial efficacy against E.faecalis than Neem and Aloe vera confirming the great potential of bioactive compounds and are useful for rationalizing the use of this plant as an endodontic irrigant.

Conflicts of interest

There are no conflicts of interest

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