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Effectiveness of Natural Irrigants Activated By Diode Laser on Elimination of E. Faecalis Biofilms- An in Vitro CLSM Study

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Abstract: ***Disinfecting the root canal is a crucial paradigm in the maintenance of teeth which have undergone root canal therapy.Though, a number of antibiotics and surfactants are being widely used in the treatment of biofilms however, the current trend is towards identification of natural products in disinfection. Aim: To investigate the impact of naturally occurring irrigants, activated by diode laser, on mature biofilms of Enterococcus faecalis in vitro. Materials & Methods: Thirty-six premolar teeth, each with a single root, underwent decoronation followed by determination of working length. The root canals were prepared utilizing Pro-Taper Gold rotary nickel titanium instruments and irrigated with 3% sodium hypochlorite and 17% EDTA solutions. Subsequently, the teeth were vertically sectioned along the midsagittal plane into two halves. The resulting prepared teeth were randomly assigned to one of six groups, each containing six teeth, and treated with laser activation. These groups included: Group 1 - 0.2% Chitosan, Group 2 - Neem Extract, Group 3 - Curcumin Extract, Group 4 - 0.2% Chitosan + Neem Extract (1:1 ratio), Group 5 - 0.2% Chitosan + Curcumin Extract (1:1 ratio), and Group 6, which served as the control and was treated with saline. The dentin specimens were then examined carefully, with random areas of the biofilm observed utilizing a confocal laser scanning microscope. Statistical Analysis: The results were subjected to one – way ANOVA followed by Bonferri test. Results: All groups had a significantly higher percentage of dead bacteria than the saline control. Neem Extract had a significantly higher percentage of dead bacteria than other groups. Conclusion: Within the limitations of this in vitro study it can be concluded that Neem extract has superior antibiofilm activity against the E.faecalis and there was no significant difference for the bacterial kill efficacy between activated and non-activated neem extract.***

Keywords: Enterococcus faecalis, Neem, Chitosan, irrigants, Confocal, diode laser

1. Introduction

Root canal disinfection represents a fundamental paradigm in the preservation of teeth that undergo root canal treatment for extended periods. The disinfection of root canals presents a primary hurdle in endodontic procedures. Although irrigants can reduce the microbial population in infected root canals, complete disinfection of entire root canal systems is not feasible. Lasers have emerged as a viable solution to this challenge by enabling access to tubular systems beyond the reach of irrigants.[1]

Medicinal plants have been an integral part of human life since the dawn of civilization. Pharmacological studies have acknowledged the value of medicinal plants as potential sources of bioactive compounds. Chitosan is a natural polysaccharide which is biocompatible, biodegradable, shows bio-adhesion and lacks toxicity. Chitosan is a cationic biopolymer that possesses lasting antibacterial properties and low production costs. Chitosan is obtained by the de-acetylation of chitin, which is found in crab and shrimp shells.[2]Curcumin (diferuloylmethane), is widely used as a spice, food preservative, and coloring material in India, China, and Southeast Asia. It has been traditionally used in medicine for treating various diseases. Curcumin, the main bioactive component of turmeric, has been found to possess antimicrobial, anti-inflammatory, and antioxidant activities[3,4,5]. These properties make curcumin a promising compound for potential therapeutic applications. It has been shown to have a wide range of biological actions, including the ability to inhibit the growth of bacteria, reduce inflammation, and scavenge free radicals. Azadirachta indica (neem) is well known in India and its neighboring countries as one of the most versatile medicinal plants with a wide spectrum of biological activity, and most commonly used traditional medicinal plant of India for household remedies against various human ailments, from antiquity[6,7,8]

The antimicrobial effect of lasers has been shown in previous studies. Diode laser irradiation destabilized E. faecalis biofilm and reduces the formation and adherence of the biofilm.[8] According to some studies, laser alone is not more effective than irrigants. Naturally occurring irrigants, such as herbal solutions, have been explored as alternatives to synthetic drugs in root canal treatments [10]. One innovative approach to activate these irrigants is by using a diode laser. Many studies on the effect of diode lasers in root canal bacterial disinfection treatments have been conducted, but studies on natural irrigants such as neem and chitosan alongside diode laser activation is not done which is experimented in the present study.

Aim

To investigate the impact of naturally occurring irrigants, activated by diode laser, on mature biofilms of Enterococcus faecal is in vitro.

2. Materials and Methods

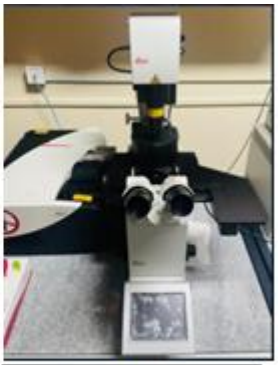
Tooth samples

0.2% Chitosan

Curcumin Extract

Neem Extract

Denlase Laser



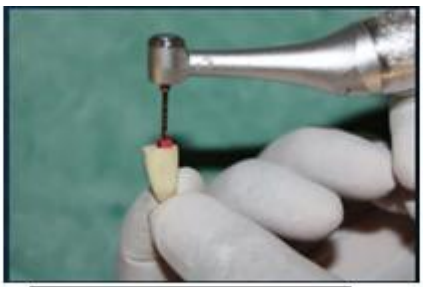
Confocal Microscope

Methodology

1) Sample Preparation

Thirty six single rooted premolar teeth were collected and maintained in phosphate - buffered saline (PBS) solution. The teeth were sterilized using a steam autoclave at 121 degree C for 20 min. The crowns were decoronated to obtain approximately 15 mm uniform root lengths and the working length wasdetermined as 1 mm short of the apical foramen at 14 mm. The root canals werepreprared with Pro-Taper gold rotary nickel titanium instruments (Dentsply, Maillefer) to an apical size of 25, 0.06 taper using 3% sodium hypochlorite and 17% EDTA as the irrigants. The tooth specimens werevertically sectioned along the midsagittal plane into 2 halves (mesial and distal). The split halves werethen re-approximated using utility wax placed over the root tip, and a dental stone encasing were fabricated.

Decoronation



Tooth preparation

Utility wax

2) Preparation and Inoculation of E. Faecalis Biofilm:

This is a laboratory experimental study with one colony of E.faecalis American Type Culture Collection (ATCC 29212). The suspension of E. faecalis ATCC strains (29212) in 2 ml BHI broth was prepared. The concentration of inoculation was adjusted to 1 McFarland scale. Root canals was inoculated with 30 µL of E. faecalis suspension and were incubated for 15 days at 37 degree Celsius and will be washed with 1 ml phosphate buffered saline to remove non-adherent bacteria of the root canal wall.

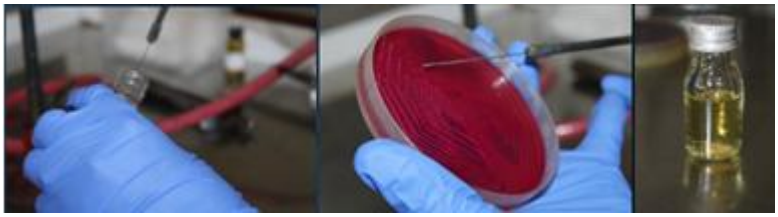


Figure E: faecalis inoculation in laboratory

3) Irrigation Procedure

The prepared teeth (n=36) were divided randomly into six groups of 6 teeth each and subjected to laser activation. ¬**Group 1** -0.2 % Chitosan ¬**Group 2** – Neem Extract ¬**Group 3** – Curcumin extract ¬**Group 4**- 0.2% Chitosan + Neem Extract (1:1 ratio) ¬**Group 5**- 0.2% Chitosan + Curcumin Extract (1:1 ratio) &Saline served as the control **(Group 6).** Samples were further divided into two

subgroups (n = 3) based on the activation method: **Subgroup A**- No Activation; **Subgroup B**- Diode Laser

Laser activation
The Subgroup B was irradiated with the Diode laser (810nm) with fibre size of 200µm set at 1.2W 10 sec each for 40 sec in continous mode.

4) Confocal Laser Scanning Microscopic Examination
The dentin specimens were carefully spread onto a microscope slide and stained with BacLight and examined in a CLSM which were set to monitor fluorescein isothiocyanate and propidiumiodide. The BacLight stain had two fluorescent dyes, fluorescein isothiocyanate and propidium iodide with emission of 480/500 nm and 490/635 nm respectively. Two to three random areas of the biofilm on each dentine section were scanned with a 2- mm step size. Only bacteria in focus of each optical section were counted and carried out carefully by one operator using a manual digital counter. The number of viable and non-viable bacteria were calculated. Charting of the data was done on Excel sheet

Statistical Analysis Method:

Sample Size Estimation
Using the formula,
$n = 2(SD)2(Z1-\alpha/2 + Z\beta)2\ (d)2$

Where,
SD = STANDARD DEVIATION
$Z1-\alpha/2$ = 2.58 AT 99% CONFIDENCE INTERVAL $Z\beta$ = 1.23 AT 90% power
d = MEAN DIFFERNCE Substituting the values, we get
n =36

Therefore the total sample size is 36.

The results were subjected to one – way ANOVA followed by Bonferri test

3. Results

Confocal Laser Scanning Microscopic (CLSM) analysis of biovolume and viable/dead cells in biofilm structure
The data obtained from the CLSM is tabulated (Table 1). Fig.1 shows biofilm in all the specimen groups. Disregarding the activation criteria, all groups had a significantly higher percentage of dead bacteria than the saline control (P< 0.05). Groups 2: Subgroup A & B had a significantly higher percentage of dead bacteria than other groups (P< 0.05). There was no significant difference between subgroup A and B (P> 0.05). Groups 5 had also shown an increased dead bacteria after group 2 followed by the other groups .

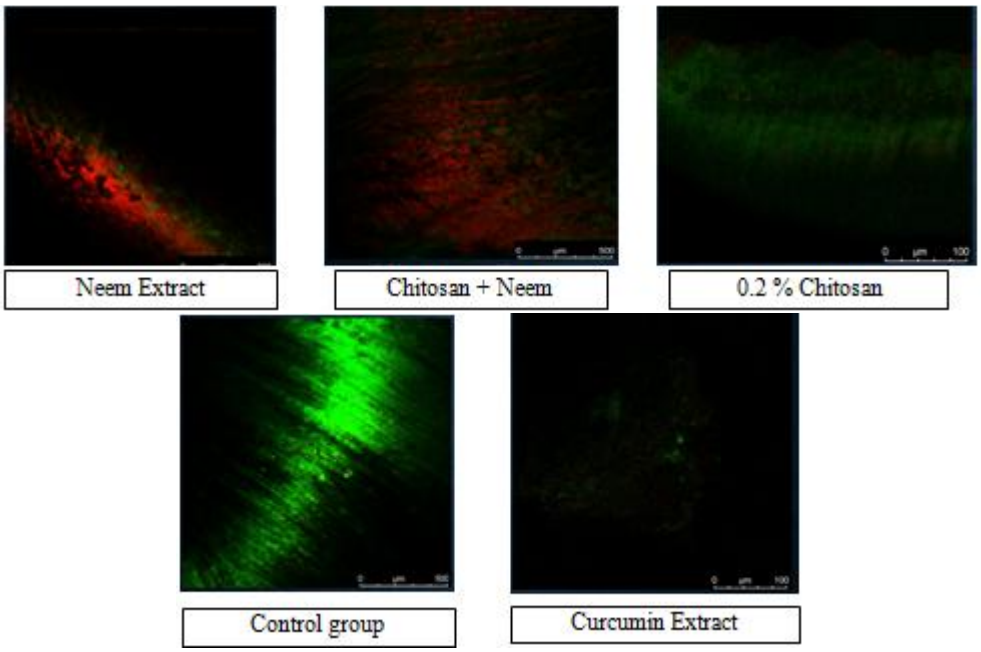


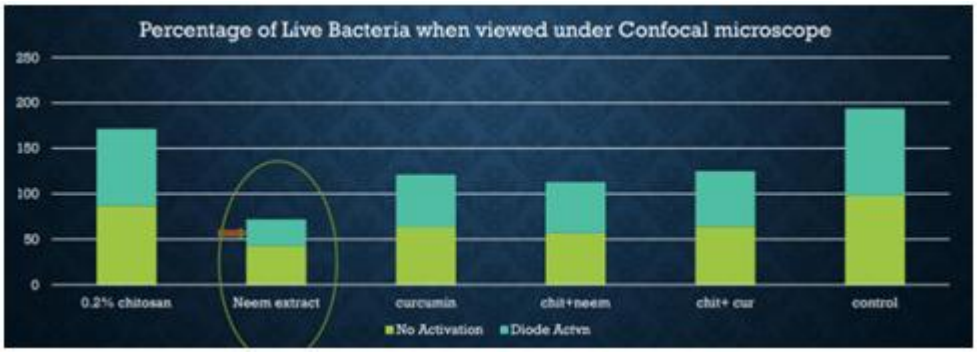
Figure 1: biofilms

Table 1

Groups	Live bacteria (per field)	Dead bacteria (per field)	Bonferri Value	Significance (p)
Chitosan- NA	107	34	0.88	0.1654
Neem-NA	32	136	0.43	0.00487
Curcumin-NA	77	84	0.78	0.0082
Chit + Neem -NA	72	86	0.791	0.03276
Chit + Curcumin- NA	76	56	0.752	0.02317
Control - NA	206	8	0.97	0.3961
Chitosan- D	94	54	0.79	0.0754
Neem-D	26	193	0.33	0.002947
Curcumin-D	68	79	0.61	0.0482
Chit + Neem -D	60	74	0.76	0.0219
Chit + Curcumin- D	53	90	0.72	0.03321
Control - D	193	32	0.95	0.7632

P<0.05 - significant

Table 2



4. Discussion

Reduction of intra-canal bacteria is an imperative step in attaining a successful outcome of root canal treatment. Microbiota in the root canal system are found in highly organized and complex entities known as biofilms. The complexity, variability of root canal system along with the nature of biofilm makes the root canal disinfection extremely challenging. Within a biofilm, a wide variety of bacteria are found forming a multi-species community however, E. faecalis has been one of the most persistent intraradicular infections compared with untreated chronic infections. Though, a number of antibiotics and surfactants are being widely used in the treatment of biofilms however, the current trend is towards identification of natural products in disinfection.[11]

Medicinal plants have been an integral part of human life since the dawn of civilization. Pharmacological studies have acknowledged the value of medicinal plants as potential sources of bioactive compounds. Siddiqui in 1942 isolated nimbin, the first bitter compound to be isolated from neem oil. More than 135 compounds have been isolated from different parts of neem. Arindam, Dutta et al. concluded that the leaf extracts of A. indica exhibited significant antibacterial activity against the test microorganisms has anti-microbial efficacy. The use of neem as an endodontic irrigant may be advantageous because it is not likely to cause severe injuries to patients such as might occur via standard NaOCl accidents.[12]In present study, neem extract **(Group 2: Subgroup A)** at a concentration of 0.94% against E. faecalis showed significant antibacterial activity when compared with other irrigants such as curcumin and chitosan.

The utilization of chitosan is justified as alternative option of an irrigating agent with antimicrobial potency. Chitosan's antibacterial nature is due to the interaction between positively charged chitosan and a negatively charged bacterial cell which transmutes the bacterial cell permeability, leading to the leakage of intercellular components and cell death[13]. Chitosanwas used as an irrigant alone (**Group 1: subgroups A &B)** alongside in combination of neem and curcumin (**Group 4&Group 5-subgroups A &B:)** in the present study. Though the study demonstrated least effectiveness of chitosan against the E.faeaclis biofilm in both activated and non-activated forms (**Group 1: subgroups A &B)**, the combination of chitosan with other effective irrrigant such as curcumin (**Group 5 – Subgroup B)** extract have shown relatively increased bacterial reduction.

Several techniques have been proposed to improve the efficacy of irrigants. These include changes in concentration, temperature, addition of surfactants, and activation (Stojicic et al. 2010).Laser Activation of irrigants appears to be an important method of increasing antibacterial and antibiofilm activity of root canal irrigants, not only within the root canal, but also within the anatomical complexities of the root canal system and dentinal tubules.[14]This study revealed that there was difference though not significant in subgroups with combination of diode lasers and irrigants as it results in the greater bactericidal effect ,possibly as the diode lasers brings about an increase in the kinetics & thus subsequent increase in the temperature of the irrigants. Hence, neem extract

activated diode laser (**Group 2: Subgroup B)** have shown increased dead bacteria per field as compared to the other subgroups.Therefore, the diode laser is considered as an adjunct to enhance the bactericidal effect of endodontic irrigants[15]. A recent report suggested that curcumin in aqueous preparations exhibits a phototoxic effect against gram positive and gram negative bacteria. Thus the use of Diode laser to enhance the activity of curcumin **(Group 3: Subgroup B)** was used in this study which demonstrated a significant kill of bacteria but less than the activated neem extract.

5. Conclusion

Within the limitations of this in vitro study it can be concluded that Neem extract has superior antibiofilm activity against the E.faecalis and there was no significant difference for the bacterial kill efficacy between activated and non-activated neem extract. Chitosan used alone or with activation showed the least bacterial kill efficiency.

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