Research Article

Comparison of antibacterial efficacy of calcium hydroxide, neem, allangium, and *Piper longum* on *Enterococcus faecalis* biofilm formed on tooth substrate – An *in vitro* study

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ABSTRACT

Aim: To compare the anti-bacterial efficacy of Calcium Hydroxide, Neem, Alangium, *Piper longam* on *Enteroccous Faecalis* biofilm formed on tooth substrate. **Materials and Method:** 40 single-rooted human upper maxillary anterior teeth with fully formed apices were collected. The root canals were then enlarged with the use of k-files till size 50. All the teeth were treated in an ultrasonic bath of 17% ethylenediaminetetraacetic acid for 5 min followed by 3% NaOCl for 5 min to remove the organic and inorganic debris. Root canal surface is then infected with *E. faecalis* to form a biofilm for two week. At the end of two week, all groups were treated as follows: Group 1, treated with Calcium Hydroxide; group 2, treated with extracted Neem; group 3, treated with extracted aiangium; group 4, treated with extracted Piper longam; and treated for one week. The collected dentin shavings were transferred into 1 mL of sterile saline for five times and incubated for 24 h. Colonies were counted and readings were tabulated. Dentin present in the collection of 40 teeth was taken and sends it for CFU test. **Results:** In treated groups, neem has shown minimum bacterial count (850 CFU/ml). **Conclusion:** In treated groups, neem has shown minimum bacterial count (850 CFU/ml). Moreover, calcium hydroxide, neem, allangium, piperlongam are very good chelating agents and neem, in particular, contains fruits that are rich in citric acid that may aid in removal of the smear layer.

KEY WORDS: Allangium, Antibacterial efficacy, *Enterococcus faecalis*, Piper longam, Root canal treatment, Sodium hypochlorite

INTRODUCTION

Endodontic infection is the infection of the dental root canal system and the major etiologic agent of apical periodontitis.[1] Endodontic infection is polymicrobial. Persistence of infection due to residual microorganisms can lead to potential failures in endodontic therapy. Hence, the objective of successful endodontic therapy depends on effective debridement and disinfection of root canal system.[2] There are bacterial interactions and nutrient availability and there is low oxygen potential in root canals with necrotic pulp, leading to anaerobic microorganism. Persistence of microorganism in dentinal tubule is seen due to complexity of anatomy of canal and limitation of irrigant and difficulty in accessing the canal system by instruments.[3] The use of plant compounds for pharmaceutical purposes has gradually increased. According to the World Health Organization, medicinal plants would be the best source to obtain a variety of drugs.[4]

About 80% of individuals from developed countries use traditional medicine, which has compounds derived from medicinal plants. Therefore, such plants should be investigated to better understand their properties, safety, and efficiency.[5] Plant extracts have been used as irrigants and medicaments in endodontics due to reduced cytotoxicity of herbal extracts. Plant products are used in endodontics. Neem is one of the well-known Indian Ayurvedic herbal formulations consisting of dried and powdered leaves. Neem has also been proven to be safe, containing active constituents that have beneficial physiologic effect

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apart from its curative property such as antioxidant, anti-inflammatory, and radical scavenging activity[6] and may have an added advantage over the traditional root canal irrigants. It can be used as an effective antiplaque agent due to its antioxidant properties and it can effectively inhibit the biofilm formation.[7]

The word pepper is derived from the Sanskrit word for long pepper (pippali). Long pepper (*Piper longum*), sometimes called Javanese, Indian, or Indonesian long pepper, is a flowering vine in the family *Piperaceae* cultivated for its fruit, which is usually dried and used as a spice. Long pepper is a close relative of *Piper nigrum*, which gives black, green, and white pepper and has a similar but generally hotter flavor. The fruits contain the alkaloid piperine, which contributes to their pungency. Another species of long pepper, *Piper retrofractum*, is native to Java, Indonesia. When applied topically, it soothes and relieves muscular pains and inflammation. Piperine is the major plant alkaloid present in black pepper *P. nigrum* and long pepper *P. longum* is reported to have bioavailability enhancing activity for some drugs.[8] Piperine has good anticonvulsant and antimicrobial properties, it also acts as an antioxidant and anticancer agent by its numerous macromolecules associated with them.[9]

Alangium salviifolium Wang. belongs to the family Alangiaceae and is commonly known as sage leaved allangium. It is a well-known traditionally used medicinal plant in India and it is also one of the most versatile medicinal plants having a wide spectrum of biological activities such as antidiabetic, antiulcer, analgesic, anti-inflammatory, antimicrobial, antioxidant, antiarthritic, diuretic, antifertility, anthelmintic, antiepileptic, and antifungal.[10]

The root of *A. salvifolium* has been used in medicine as a diuretic, astringent, and antidote for several poisons. The fruit of plant is useful in treating burning sensation and hemorrhage. Studies show that they have antibacterial efficacy. The activity of plant extracts on bacteria and fungi has been studied by a very large number of researchers in different places of the world. The specific plants to be used and the methods of application for particular ailment were passed through oral tradition. Therefore, the present study investigates about the antimicrobial efficacy of calcium hydroxide, neem, allangium, and *P. longum* against *Enterococcus faecalis* biofilms formed on the tooth substrate.

MATERIALS AND METHODS

Preparation of the Blocks

Fourthy single-rooted human upper maxillary anterior teeth with fully formed apices were collected and used in this study. Then, all the teeth were cleaned of superficial debris, calculus, and tissue tags and stored in normal saline to prevent dehydration before use. Access opening was done and the apices were sealed. The root canals were then enlarged with the use of K-files till size 50. The tooth was treated in an ultrasonic bath of 17% ethylenediaminetetraacetic acid for 5 min followed by 3% NaOCl for 5 min to remove the organic and inorganic debris. The traces of chemicals used were removed by immersing the blocks in an ultrasonic bath containing distilled water for 5 min. All the blocks were sterilized in an autoclave for two cycles.

Contamination of Block

The test organism used for this study was *E. faecalis* which is a Gram-positive facultative anaerobic bacterium that is most common in endodontically failed cases. *E. faecalis* (ATCC 29212) was grown. A pure culture of *E. faecalis* was grown on Mueller-Hinton (MH) agar, inoculated into MH broth, incubated at 37°C overnight, and adjusted to an optical density of 1 with sterile MH broth. Each dentin block was placed in pre-sterilized microcentrifuge tubes containing 1 mL of the MH broth 50 µL of the inoculum containing *E. faecalis* and was transferred into each of the microcentrifuge tubes. At the end of 24 h, the dentin blocks were transferred into fresh broth containing *E. faecalis*. All procedures were carried out under laminar flow. Purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentin blocks. Contamination of the dentin blocks was carried out for a period of 21 days.

Antimicrobial Assessment

These teeth were divided into four groups. Root canal surface is then infected with *E. faecalis* to form a biofilm for 2 weeks. At the end of 2 weeks, all groups were treated as follows: Group 1, treated with calcium hydroxide; Group 2, treated with extracted neem; Group 3, treated with allangium; and Group 4, treated with *P. longum* and treated for 1 week. Harvesting of the dentin was carried out with gates Glidden drills no. 4 and 5, respectively. The collected dentin shavings were transferred into 1 mL of sterile broth and incubated in an anaerobic environment at 37°C for 24 h. After 24 h, the contents of each tube were serially diluted, 100 µL of the broth in 100 µL of sterile saline for 5 times and incubated for 24 h. Colonies were counted and readings were tabulated. Dentin present in the collection of 40 teeth was taken and sends it for colony-forming unit (CFU) test.

Groups where $n = 10$ for each group and the result were analyzed quantitatively.

RESULTS

Qualitative assay with the 2-week biofilm on the canal portion showed growth when treated with calcium hydroxide, neem, allangium, and *P. longum* shown

minimum inhibition. Table 1 shows the bacterial population in the quantitative assay with the 2-week biofilm for calcium hydroxide, neem, allangium, *P. longum*, and saline-treated tooth samples. All treated groups have shown a significant reduction of bacterial population. In treated groups, Triphala has shown minimum bacterial count (920 CFU/ml) [Table 2].

DISCUSSION

The prime objective of root canal treatment is to clean the root canal system thoroughly, free of microbiota and debris so that it can be sealed with a microbial-tight filling.[11]

E. faecalis is the most common *Enterococcus* sp. persisting in treated root canals and is resistant to traditional antibiotics. When *E. faecalis* grows as a biofilm, the altered genetic and metabolic processes of bacteria along with its complex matrix prevent the entry and action of several antimicrobial agents. *E. faecalis* was chosen as a test organism because it is a facultative organism that is non-fastidious, easy-to-grow, and efficiently and rapidly colonized tubules. It has been used extensively in endodontic research because it has been found to be present in 63% of teeth with failed endodontic treatment and previously root-filled teeth.[12]

Bacteria-induced dissolution of the dentin surface and the ability of *E. faecalis* to form calcified biofilm on root canal dentin may be a factor that contributes to their persistence after endodontic treatment.[13] It is established that the biofilm-forming capacity and its structural organization are influenced by the chemical nature of the substrate. Biofilm experiments conducted on polycarbonate or glass substrate will not provide a true indication of the bacteria-substrate interaction.[14] Hence, *E. faecalis* biofilm was formed on a tooth substrate in this study in accordance with the methodology done by Kimura *et al*. All the groups were tested in direct contact with the biofilm formed on tooth substrate at different durations (2 weeks and 6 weeks).

Table 1: Qualitative analysis of 2-week *Enterococcus faecalis* biofilm formed on tooth substrate for different groups

Group	Number of bacteria in colony-forming unit/ml
Calcium hydroxide	920
Neem	850
Allangium	3020
Piper longum	2248

Table 2: Qualitative analysis of antibacterial efficacy of medicament against *Enterococcus faecalis* biofilm formed on tooth substrate for different groups in percentage

Group	Antibacterial efficacy in %
Calcium hydroxide	85
Neem	73
Allangium	61.2
Piper longum	63.68

In this study, 40 single-rooted human upper maxillary anterior teeth with fully formed apices were collected. Then, all the teeth were cleaned of superficial debris, calculus, and tissue tags and stored in normal saline to prevent dehydration before use. Access opening was done and the apices were sealed. The root canals were then enlarged with the use of K-files till size 50. The tooth was treated in an ultrasonic bath of 17% ethylenediaminetetraacetic acid for 5 min followed by 3% NaOCl for 5 min to remove the organic and inorganic debris. The traces of chemicals used were removed by immersing the blocks in an ultrasonic bath containing distilled water for 5 min. All the blocks were sterilized in an autoclave for two cycles. A pure culture of *E. faecalis* was grown on MH agar, inoculated into MH broth, incubated at 37°C overnight, and adjusted to an optical density of 1 with sterile MH broth. Each dentin block was placed in pre-sterilized microcentrifuge tubes containing 1 mL of the MH broth 50 µL of the inoculum containing *E. faecalis* and was transferred into each of the microcentrifuge tubes. At the end of 24 h, the dentin blocks were transferred into fresh broth containing *E. faecalis*. All procedures were carried out under laminar flow. Purity of the culture was checked by subculturing 5 µL of the broth from the incubated dentin blocks. Contamination of the dentin blocks was carried out for a period of 21 days.

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Previous studies have showed neem having good antibacterial efficacy.[15,16] Evaluation of antimicrobial efficacy of herbal alternatives (neem and green tea polyphenols), MTAD, and 5% sodium hypochlorite against *E. faecalis* biofilm was checked, 5% sodium

hypochlorite showed maximum antibacterial activity against *E. faecalis* biofilm formed on tooth substrate. Neem, green tea polyphenols, and MTAD showed statistically significant antibacterial activity.[17] *P. longum* is an alkaloid[18] having good antibacterial efficacy which has been used in this study.

Herbal alternatives showed promising antibacterial efficacy on 2-week biofilm along with calcium hydroxide, neem, allangium, and *P. longum*. From above result, neem shows minimum bacterial count, which is considered to be highly sensitive against a particular organism. Neem exhibited good antibacterial efficacy on *E. faecalis* biofilm. This may be attributed to its formulation, which contains three different medicinal plants in equal proportions. In such formulations, different compounds may be of help in enhancing the potency of the active compounds resulting in an additive or synergistic positive effect.

Allangium and *P. longum* are shown to have fairly less antibacterial efficacy when compared to neem. *P. longum* is marginally better than allangium, they containing active constituents that have beneficial physiologic effect apart from its curative property such as antioxidant, anti-inflammatory, and radical scavenging activity and may have an added advantage over the traditional root canal irrigants. Moreover, neem, allangium, and *P. longum* are very good chelating agents and neem, in particular, contains fruits that are rich in citric acid that may aid in removal of the smear layer. The major advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far.

A. salviifolium Linn. (Alangiaceae) is a small deciduous tree or shrub, which grows in the wild throughout the hotter parts of India.[19] The major phytochemical constitutes of the plant are alangine A and B, alangicine, markindine, lamarckinine, and emetine.[20] The root of *A. salviifolium* has been used in the Indian system of medicine as an acrid, diuretic, astringent, and antidote for several poisons. The fruits (mucosa) of the plant are useful in treating burning sensation and hemorrhages.[21]

However, no scientific evidence is available regarding its antimicrobial activity. An investigation of *A. salviifolium* as an anti-infective agent is the objective of our present study.

One study showed administration of 6% neem and 0.2% chlorhexidine, there was a significant reduction in the CFUs/ml at 48 h and 7 days. The control group showed no decrease, but on the contrary showed a slight increase in the CFUs/ml. The neem group showed 17% and 44% reduction, while the chlorhexidine group showed 16% and 45% reduction.[22]

In another study, neem showed a maximum zone of inhibition measuring 22 mm at a concentration of 6% against lactobacilli. Maximum zone of inhibition for *Candida* species was 20 mm at 9% concentration. Increasing the concentration further above these percentages did not produce any increase in the zone of inhibition. Both species were found to be resistant to the action of neem at lower concentrations.[23]

A recent study showed that neem was as effective as NaOCl and a doxycycline-based irrigant on root canal biofilms that were 3 weeks old. It brought about 8-log reduction in *E. faecalis* counts when compared to saline. Moreover, neem is also a very good chelating agent due to the fruits that are rich in citric acid and holds promise in the removal of smear layer.[24]

CONCLUSION

Qualitative assay with the 2-week biofilm on the canal portion showed growth when treated with calcium hydroxide, neem, allangium, and *P. longum* shown minimum inhibition. All treated groups have shown a significant reduction of bacterial population. In treated groups, neem has shown minimum bacterial count (920 CFU/ml). Moreover, calcium hydroxide, neem, allangium, and *P. longum* are very good chelating agents and neem, in particular, contains fruits that are rich in citric acid that may aid in removal of the smear layer. The major advantages of using herbal alternatives are easy availability, cost-effectiveness, increased shelf life, low toxicity, and lack of microbial resistance reported so far.

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