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Evaluation of antimicrobial activity of Neem and black pepper extracts and its efficacy in decontaminating Gutta percha cones

Niranjan Nandkumar Patil* **and Shweta Bhise Patel**
Department of Microbiology, Patkar-Varde College, S.V. Road, Goregaon, Mumbai, India
niranjanpatil1992@gmail.com

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Abstract

Dental caries is the disease very common worldwide, after periodontitis. If left untreated, the entire tooth demineralizes. Root canal treatment is the solution to the untreated decays. GP cones are used to fill in the abscess cavity of the tooth. Various irrigants are used to sterilize the GP cones and cavity is like sodium hypochlorite, ethanol etc., but these chemicals come with their own disadvantages. Thus, different parts of plants are tested for their antimicrobial activity to decontaminate GP cones. In this study, neem and black pepper was studied for their antimicrobial activity. The ethanolic extracts of neem leaves, neem bark and kernels, and black pepper along with mixture of all three materials were used to check for their antimicrobial activity against Streptococcus mutans, Enterococcus faecalis, Escherichia coli and Staphylococcus aureus. Further GP cones were dipped in different concentration of the extracts and tested against S. mutans, which is the main causative oral pathogen. The MIC was also determined to check the minimum inhibitory concentration of these extracts. Results showed that neem and black pepper both had good anti-microbial activity against the organisms used in the study but the mixture extract showed excellent cumulative anti-microbial activity.

Keywords: Gutta percha Cones, Root canal treatment, *Streptococcus mutans, Enterococcus faecalis*, Dental caries, Neem leaves extract, Black pepper extract, Endodontic Treatment.

Introduction

Oral micro biome is a complex ecological system where up to 700 species of micro-organisms have been identified. The oral cavity includes various microbial habitats, such as teeth, tongue, cheek, lip, hard palate, soft palate, gums etc. The environment of the mouth which provides a source of water, nutrients, as well as a moderate temperature supports the growth of microorganisms found there. Both the hard and the soft oral tissues tend to possess biofilms on it. This leads to two major dental diseases: dental caries and periodontal disease. Periodontitis is caused by bacterial species which live in polymicrobial biofilms at or below the gingival margin and progress largely as a result of the inflammation initiated by specific sub gingival species[1]. Inspite various preventive measures available, tooth decay is yet one of the most prevalent diseases[2]. The major critical cause of dental caries is external and is relating to dietary intake of fermentable carbohydrates and in particular sucrose, which is often found in high concentrations in variety of eatables like sweets, biscuits, snacks, sweet drinks, etc.[3]. Dental caries causes pain and anxiety, unfocused mind in work and school leading to unproductive output, and in young children untreated decay is common cause of irritation, loss of appetite and hospitalization[4]. Root canal or endodontic treatment is the treatment done to save the caries affected tooth. In this treatment, the gum of that tooth is anaesthetized. Further, the pulp which is infected is completely removed and cleaned; along with the abscess if any. This makes the tooth "dead" as it does not contain any nerves in it. The tooth is then filled and sealed with a gutta-percha cones and temporary cement. Afterwards, the tooth is built with the filling matching to the color of the tooth or with a crown/cap[5-6]. The principal bacterial genera found in the oral cavity are *Abiotrophia, Enterococcus, Finegoldia, Gemella, Granulicatella, Peptostreptococcus, Streptococcus, Corynebacterium, Bifidobacterium, Lactobacillus, Propionibacterium, Veillonella, Campylobacter etc.* Genera of fungi that are frequently found in the mouth include *Candida, Cladosporium, Aspergillus, Fusarium, Glomus, Alternaria, Penicillium and Cryptococcus*[7].

Gutta percha (GP) is a dried coagulated extract particularly obtained from Palaquium gutta but also Isonandra gutta and Dichopsis gutta[8]. The inertness of the GP material has made it suitable in dentistry because it does not react with the human body as a pathogenic foreign material. It is used in variety of surgical devices and during the root canal treatment. Its physical and chemical properties include flexibility, malleability etc. GP cones, also known as GP points are used in endodontics. These are thin cone like structures which can be used to replace the area of the pulp in a tooth. GP cones supplied commercially are packed in boxes according to different sizes of cones. They are not usually sterilized or decontaminated before obturation. Sterilization by moist or dry heat is not possible as it carries a risk of physical deformation. GP decontamination has been tried by chemicals such as, hydrogen peroxide, chlorhexidine, ethyl

alcohol, polyvinylpyrrolidone iodine, quaternary ammonium compounds. Recently, the use of newer methods like electron beam sterilization has also been tried. However, none of these methods have been proved as fully as 100% effective. Currently, the recommended method for decontamination of GP points consists of treating the cones using a 1% Sodium hypochlorite for 1 minute (Milton's solution), or 0.5% Sodium hypochlorite for 5 minutes (Dakin's solution)[9]. In this case, the risk of sodium hypochlorite causing crystal deposition within the canals which can impede the obturation cannot be ignored, which will cause failure of root canal treatment in future. Neem is known for its anti-inflammatory, anti-malarial, anti-fertility, antimicrobial, anti-acne, acaricidal, and nematicidal activities. The main active component found in neem is AZA, which is commonly used as a biological marker for this plant. There are several studies on the antimicrobial activity of neem. Some of them have demonstrated activities of extracts from seeds and leaves against *Staphylococcus aureus, Escherichia coli*, as well as, negative results against *Bacillus subtilis, S. paratyphi*, *S. dysenteriae* and *Candida albicans*. Neem leaves are efficient against pathogenic fungi, such as *Trichophyton, Epidermophyton, Microsporum, Trichosporon* and *Geotricum*. The activity in inhibiting the protease of *Trichophyton*, the production of aflatoxin of *A. parasiticus*, antifeedant activity and the antifungal activity against *Penicillium expansum* have been confirmed. Black pepper is traditional spice which is used in human diet. It is included in the prescriptions of Ayurveda and other traditional medicinal systems. Pepper is also used in folk medicine as aphrodisiac, carminative, stomachic, antiseptic diuretic and for the treatment of cough, rheumatoid arthritis, peripheral neuropathy, melanoderma and leprosy due to the presence of volatile compounds, tannins, phenols and other unknown substances.

In this research work, neem and black pepper was studied for their antimicrobial activity against the oral pathogens. The MIC of the extracts was performed. Also the decontamination of GP cones was carried out with the extracts.

Materials and methods

Test Organisms: The common GP contaminants were included in the study are *Streptococcus mutans, Enterococcus faecalis, Escherichia coli and Staphylococcus aureus.* The culture of *S.mutans* was ordered from Microbial Type Culture Collection and Gene bank (MTCC). Culture of *E.faecalis* was ordered from National Collection of Industrial Micro-organisms (NCIM). The cultures of *E.coli* and *S.aureus* were obtained from the Patkar College, Microbiology laboratory, Goregaon, Mumbai. After the cultures were received they were checked for viability.

Current method used to decontaminate GP cones: Sodium hypochlorite solution was kept both in sunlight and incubator at 37°C for 24-48 hours. Further it was checked for its crystal formation under microscope at 10x, 40x and 100x (oil immersion). The sodium hypochlorite solution was mixed with neem extract in 1:1 ratio. The solution was poured in the petriplates and were kept in sunlight and incubator. This was done to check if crystallization of sodium hypochlorite yet exists.

Preparation of the extracts: Neem leaves, neem bark and kernels and black peppercorns were used in the process to obtain various extracts. All the raw materials were washed with distilled water and sun dried. The neem leaves were dried at 80°C in the hot air oven for 48 hours. Similarly, neem bark and kernels were dried. The black peppercorns were already in dried form, which were obtained from a grocery store. All the dried materials were grinded to a fine powder using grinder and stored in air-tight container.

Ethanolic extracts were prepared of the individual dried powders and a combination of all three powders together. Four different concentrations were prepared: 5%, 10%, 20% and 30%. For 5% concentration of the extract, 10gms of each powder was mixed in 200ml of ethanol. Similarly, other concentrations of extracts were prepared. The mixtures were mixed properly by using a stirrer, plugged with cotton and were kept on the shaker for one week. The solutions were then filtered using muslin cloth and extracts were stored at room temperature.

Antimicrobial activity of the extracts: The agar well diffusion method prescribed by NCCLS (2000) was employed in the study. Each sterile Blood Agar plate was uniformly seeded with 1 ml of the broth culture and the plates were left on the bench for solidification. Four wells of 5mm in diameter, about 2cm apart were punched in the BA plate with a sterile cork-borer. 10 µl of the extracts of different concentrations viz. 5%, 10%, 20% and 30% were dropped into each well. The plates were incubated at 37°C for 24 hrs. After incubation, the zone of inhibition was measured and recorded in millimeters.

Minimum Inhibitory Concentration: The minimum inhibitory concentration of neem leaves, neem barks and kernels, black pepper and mixture of cumulative extracts were studied. Suspensions of individual bacteria were made in sterile normal saline and adjusted to the 0.5 McFarland's standard. The extracts were incorporated into sterile Nutrient broth at concentration ranging from 0.1 – 1.0 mg/ml along with 0.1 ml of bacterial suspension in each tube. A negative control contained the media and the bacteria and was refrigerated. A positive control contained the growth media and bacteria. A media control contained only media. All the tubes, except negative control, were incubated at 37°C for 24 hrs. No turbidity in the first tube from the set was determined as its MIC since there would be no growth.

Decontamination of GP cones: Broth culture of *S.mutans* was made and adjusted to 0.5 McFarland's standard. The GP cones were dipped in extracts of various concentrations viz. 5%, 10%, 20% and 30% for about 20mins at room temperature. Further 20ml of freshly prepared Thioglycolate broth was used as

growth media to which 1ml of the broth stock culture was added. The GP cones were added to the tubes. The tubes were incubated at 37°C for 24 hours. Turbidity is interpreted as growth of bacteria, while no turbidity indicated that the given concentration of the extract decontaminated the GP cones.

Results and discussion

Current method used to decontaminate GP cones: In endodontics, GP decontamination is important to prevent any microbial contamination of the root canal during the procedure. Thus, it is necessary to employ a quick, reliable, inexpensive yet effective decontaminant which can be used instantly in the dental clinics itself. An effective example of chemo-sterilizer is glutaraldehyde. 2% glutaraldehyde have a broad spectrum of action when used in aqueous solution and has been used efficiently to decontaminate endodontic files prior to sterilization in a glass bead sterilizer[10]. Though, *Bacillus subtilis* spores are resistant to treatment with glutaraldehyde. 70% concentrated ethanol is widely used in most laboratories and dental clinics also. But studies show that it gives only intermediate level of disinfection. Also the object needs to be dipped in the solution for minimum 10 minutes for its surface decontamination[11]. 2% Chlorhexidine disrupts the cell membranes and induces precipitation of the cytoplasm which ultimately kills the pathogens. It has however been reported by Sequeira that surface exposure after 10 minutes is ineffective and requires much longer duration of contact with the disinfectant[12-13]. Thus other irrigants need to replace the ineffective chemicals.

Sodium hypochlorite is the one of the common irrigants used to decontaminate GP cones before root canal treatment. Generally, 1% for 1 minute or 0.5% for 5 minutes is used. The common cause of root canal treatment failure which is precipitation in the form of crystallization of the sodium hypochlorite around the GP cones in the cavity of the tooth. When its solution is kept in sunlight as well as in incubator at 37°C which mimics human body temperature in-vitro, the solution crystallizes. Precipitation was also observed when the solution is mixed with the neem extract solution in 1:1 ratio. But when the crude neem extract is kept in sunlight, it however, does not precipitate and doesn't cause any crystals. Thus, if sodium hypochlorite solution mixed with neem extract would yet cause crystallization, if used, post root canal treatment and lead to its failure (Figure-1, 2 and 3).

Antimicrobial activity of the extracts: The agar well method was studied with different concentrations of the extracts and against the organisms grown on Blood Agar plate. *S.aureus* gave clear beta-hemolysis on Blood Agar. *E.faecalis* also showed lysis of the red blood cells and gave beta hemolysis. *S.mutans* and *E.coli* did not give any hemolysis. The zone of inhibition varies from organism to organism as per the concentration of the extracts. Our experiment proved that black pepper extract has good anti-microbial activity against the four organisms used in our study. Also the cumulative antimicrobial activity of mixture extract was better as compared to the individual extracts.

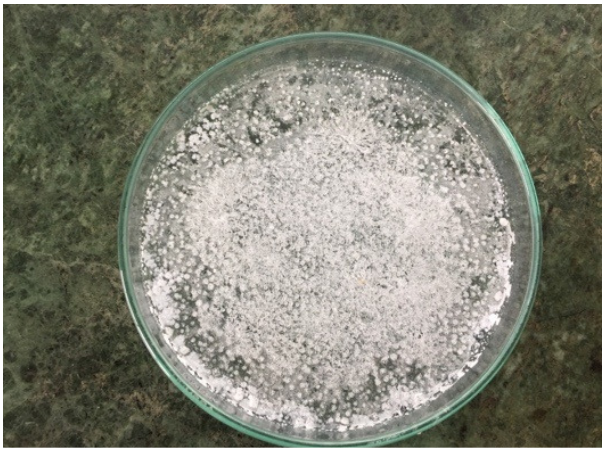

Figure-1: Crystals of sodium hypochlorite.

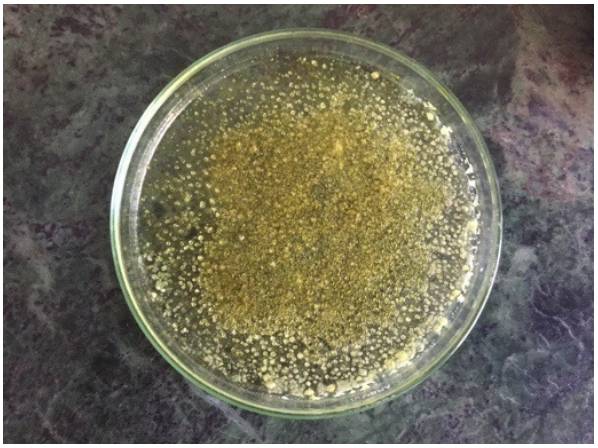

Figure-2: Crystals of sodium hypochlorite and neem extract mixture.

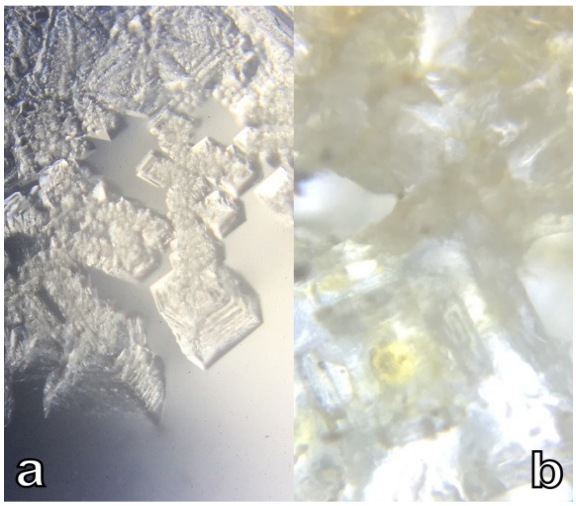

Figure-3: (a) Crystallization of sodium hypochlorite under the microscope. (b) Crystallization when sodium hypochlorite is mixed with neem extract under microscope.

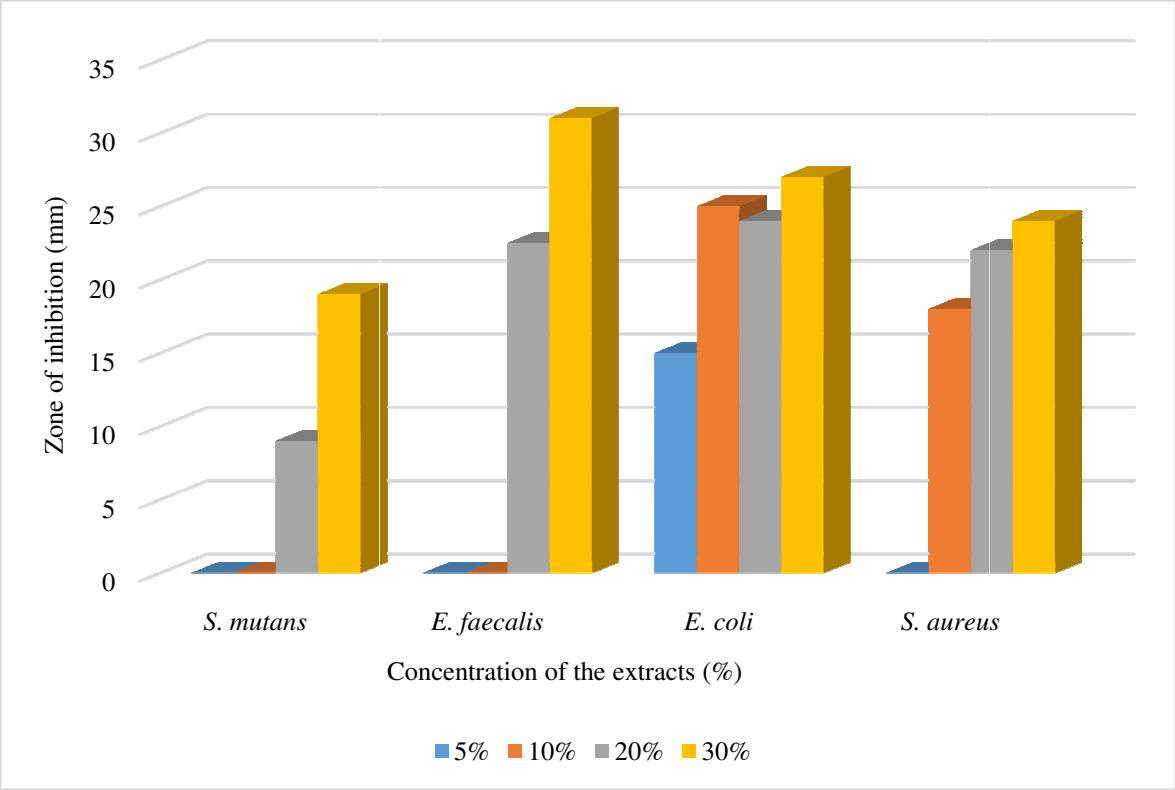

Figure-4: Zone of Inhibition for neem leaves extract.

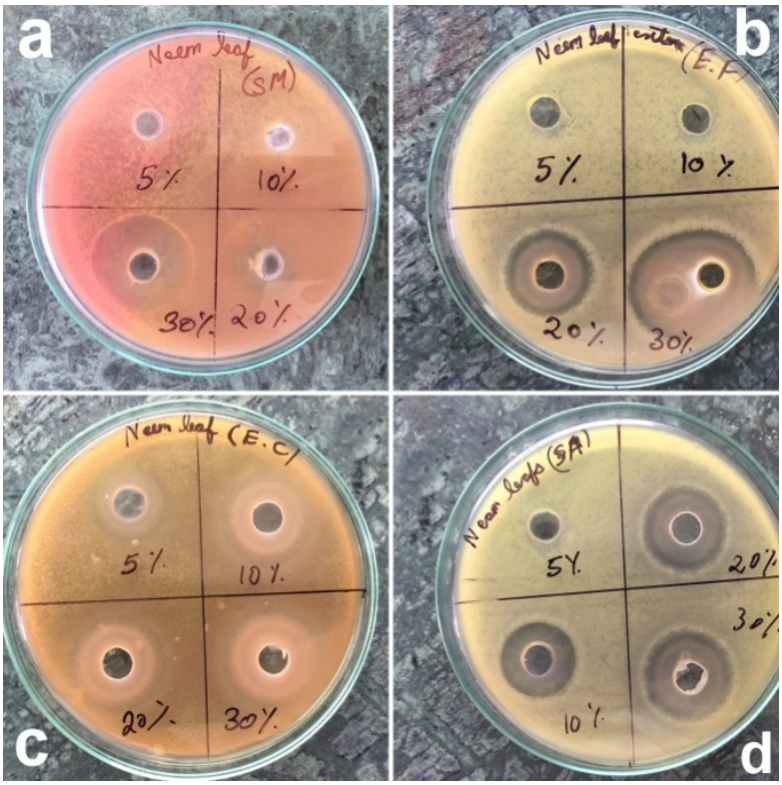

Figure-5: Antimicrobial activity of neem leaves extract using agar cup method: (a) Antimicrobial activity against *Streptococcus mutans.* (b) Antimicrobial activity against *Enterococcus faecalis.* (c) Antimicrobial activity against *Escherichia coli.* (d) Antimicrobial activity against *Staphylococcus aureus.*

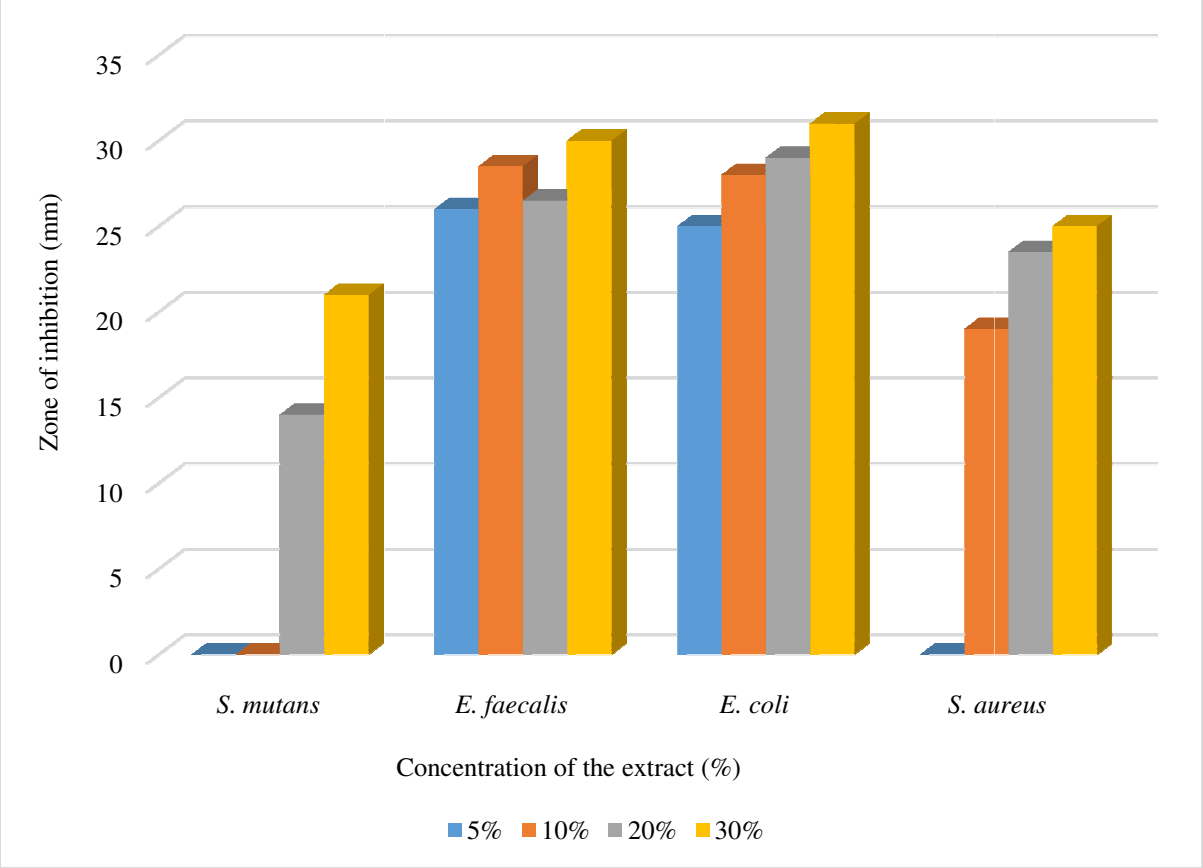

Figure-6: Zone of Inhibition for neem bark and kernels extract.

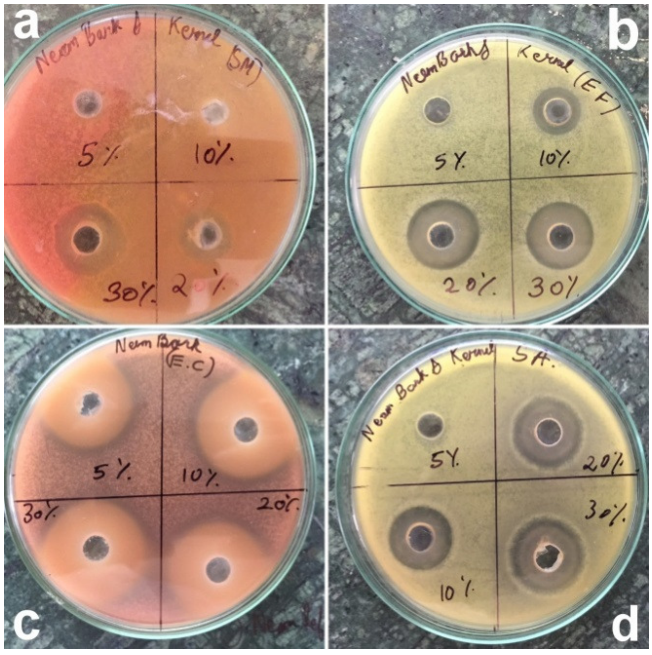

Figure-7: Antimicrobial activity of neem bark and kernels extract using agar cup method. (a) Antimicrobial activity against *Streptococcus mutans.* (b) Antimicrobial activity against *Enterococcus faecalis.* (c) Antimicrobial activity against *Escherichia coli.* (d) Antimicrobial activity against *Staphylococcus aureus.*

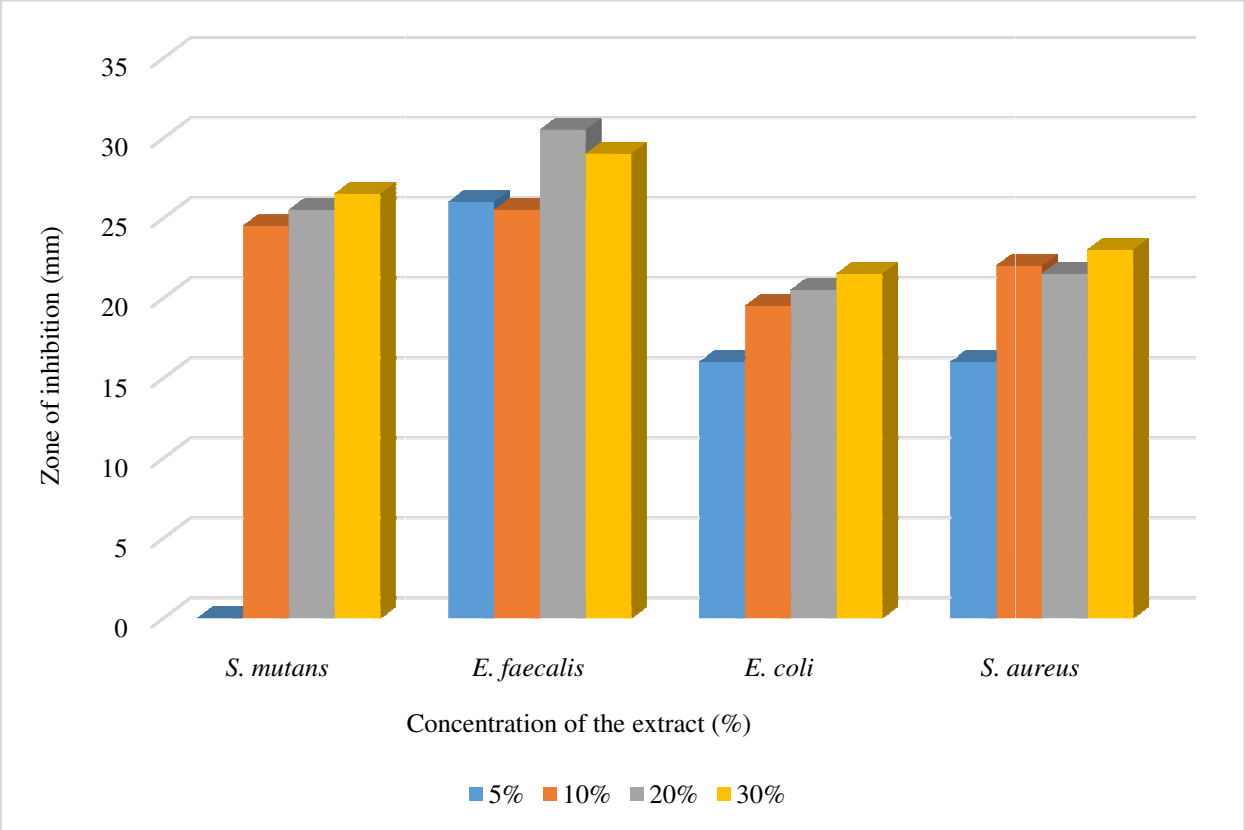

Figure-8: Zone of Inhibition for black pepper extract.

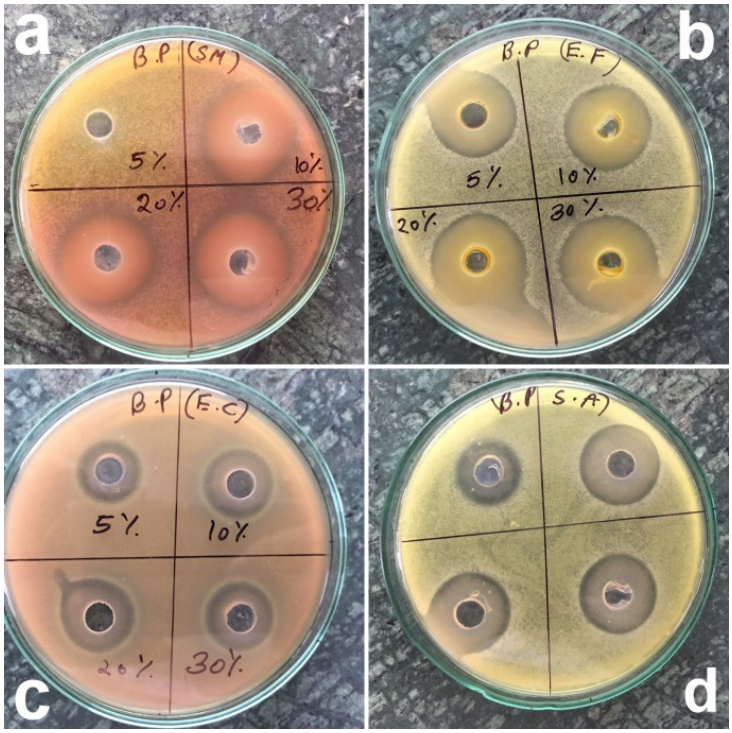

Figure-9: Antimicrobial activity of black pepper extract using agar cup method. (a) Antimicrobial activity against *Streptococcus mutans.* (b) Antimicrobial activity against *Enterococcus faecalis.* (c) Antimicrobial activity against *Escherichia coli.* (d) Antimicrobial activity against *Staphylococcus aureus.*

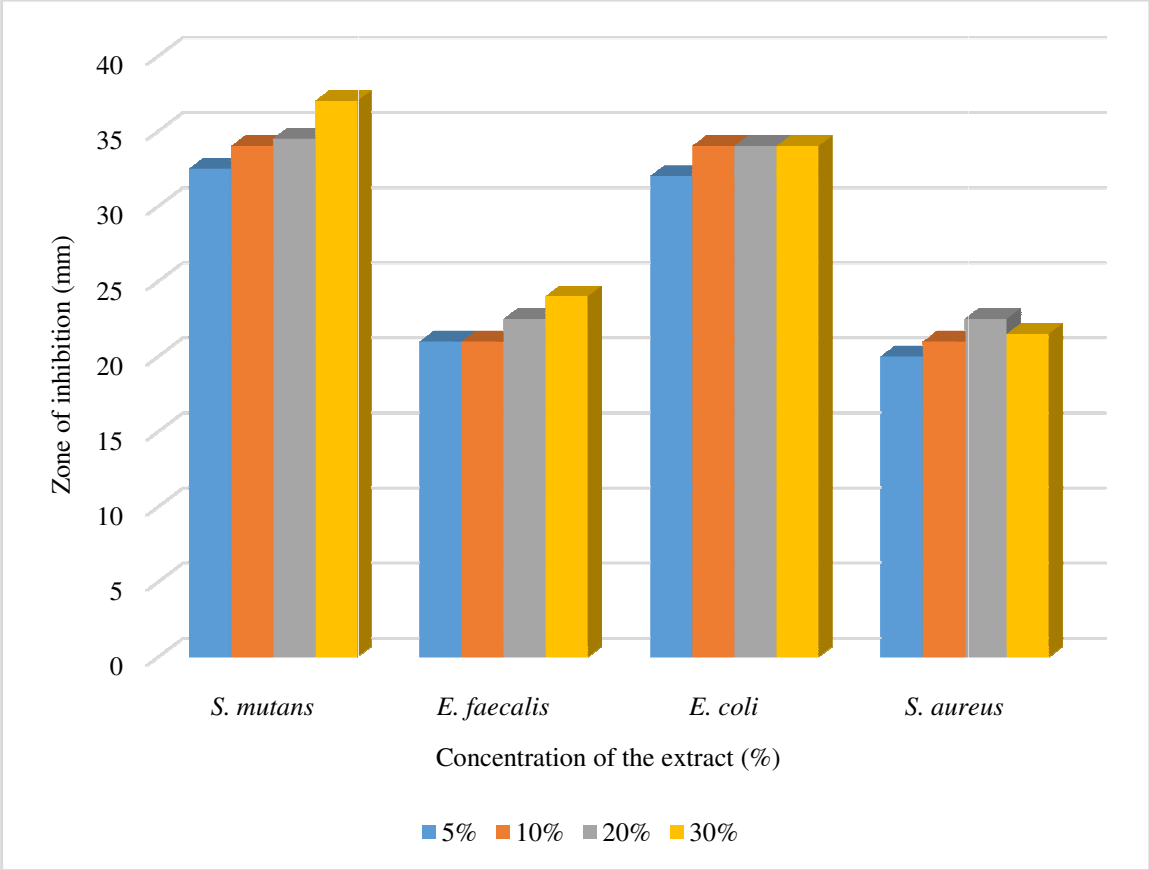

Figure-10: Zone of Inhibition for mixture extract.

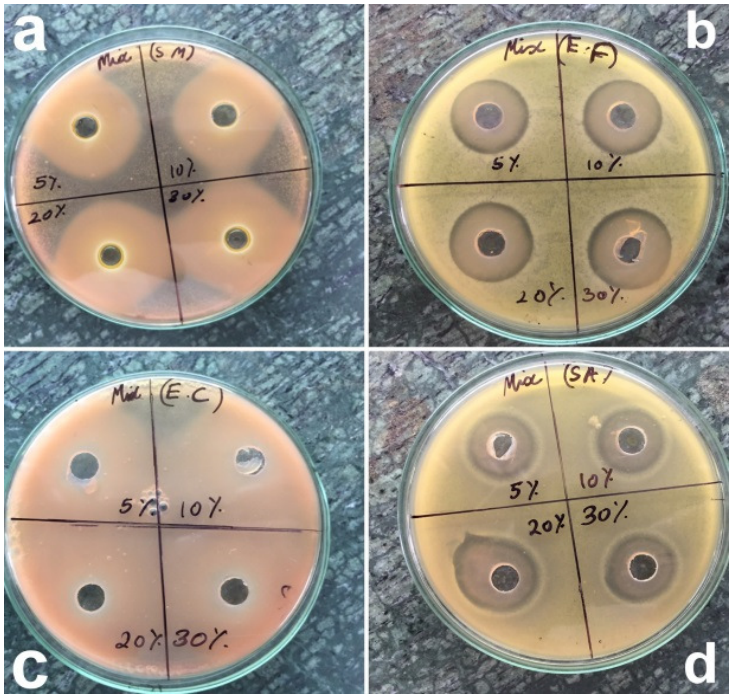

Figure-11: Antimicrobial activity of mixture extract using agar cup method. (a) Antimicrobial activity against *Streptococcus mutans.* (b) Antimicrobial activity against *Enterococcus faecalis.* (c) Antimicrobial activity against *Escherichia coli.* (d) Antimicrobial activity against *Staphylococcus aureus.*

Minimum Inhibitory Concentration: The minimum inhibition concentration was studied for each extract against each organism. For neem leaf extract, the MIC for E.coli was 0.6 mg/ml and 0.8 mg/ml for other three bacteria. Neem bark and kernels extract showed MIC of 0.6 mg/ml for *E.coli. S.mutans* and *S.aureus* showed 0.8 mg/ml and *E.faecalis* showed 1.0 mg/ml. The MIC of black pepper was as low as 0.4 mg/ml for all organisms except *S.mutans*; which showed MIC of 0.6 mg/ml. The mixture extract showed the MIC of 0.4 mg/ml for *E.coli* and *S.aureus* and 0.6 mg/ml for *S.mutans* and *E.faecalis.*

Table Key: +: Turbidity, -: No turbidity, PC: Positive Control, NC: Negative Control, MC: Media Control.

Table-1: Minimum inhibitory concentration for neem leaves extract.

Conc. of neem leaves extract (mg/ml)	Observation			
	S. mutans	*E. faecalis*	*E. coli*	*S. aureus*
0.2	+	+	+	+
0.4	+	+	+	+
0.6	+	+	_	+
0.8	_	_	_	_
1.0	_	_	_	_
PC	+	+	+	+
NC	_	_	_	_
MC	_	_	_	_

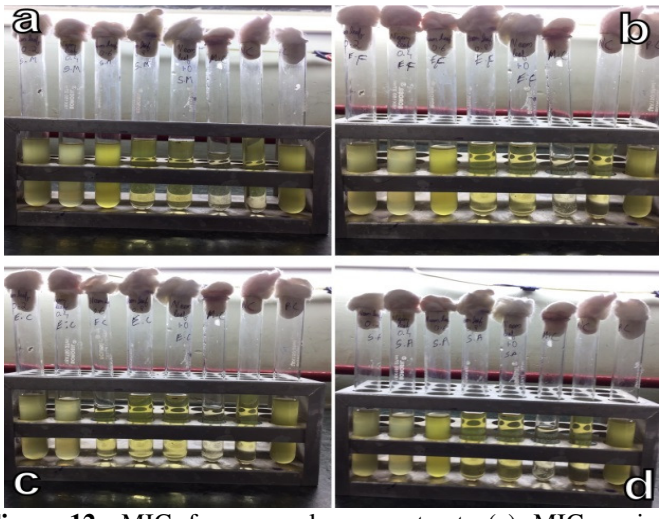

Figure-12: MIC for neem leaves extract. (a) MIC against *Streptococcus mutans.* (b) MIC against *Enterococcus faecalis.* (c) MIC against *Escherichia coli.* (d) MIC against *Staphylococcus aureus.*

Table-2: Minimum inhibitory concentration for neem bark and kernels extract.

Conc. of neem bark and kernels(mg/ml)	Observation			
	S. mutans	*E. faecalis*	*E. coli*	*S. aureus*
0.2	+	+	+	+
0.4	+	+	+	+
0.6	+	+	_	+
0.8	_	+	_	_
1.0	_	_	_	_
PC	+	+	+	+
NC	_	_	_	_
MC	_	_	_	_

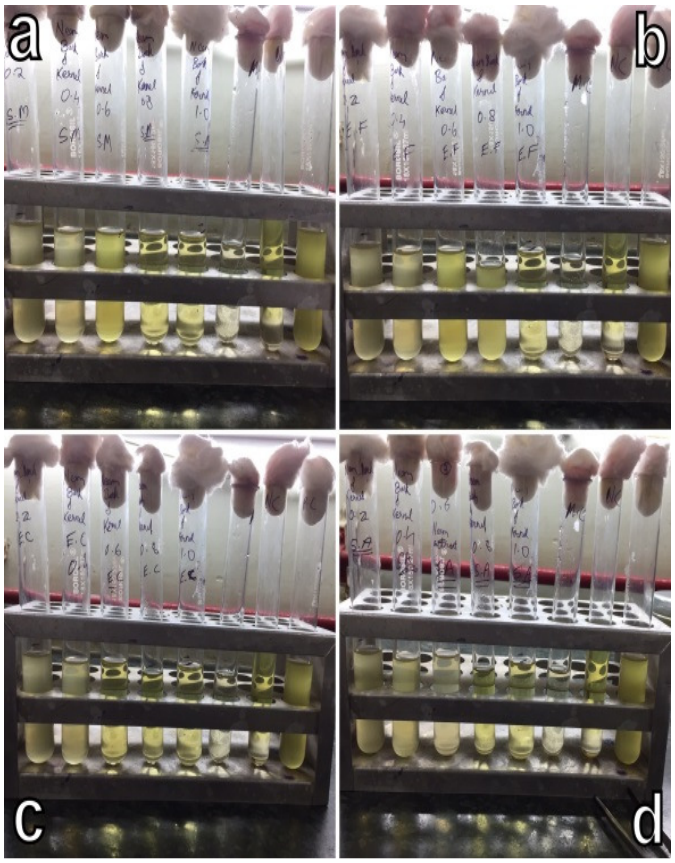

Figure-13: MIC for neem bark and kernels extract. (a) MIC against *Streptococcus mutans.* (b) MIC against *Enterococcus faecalis.* (c) MIC against *Escherichia coli.* (d) MIC against *Staphylococcus aureus.*

Table-3: Minimum inhibitory concentration for black pepper extract.

Conc. of black pepper(mg/ml)	Observation			
	S. mutans	*E. faecalis*	*E. coli*	*S. aureus*
0.2	+	+	+	+
0.4	+	_	_	_
0.6	_	_	_	_
0.8	_	_	_	_
1.0	_	_	_	_
PC	+	+	+	+
NC	_	_	_	_
MC	_	_	_	_

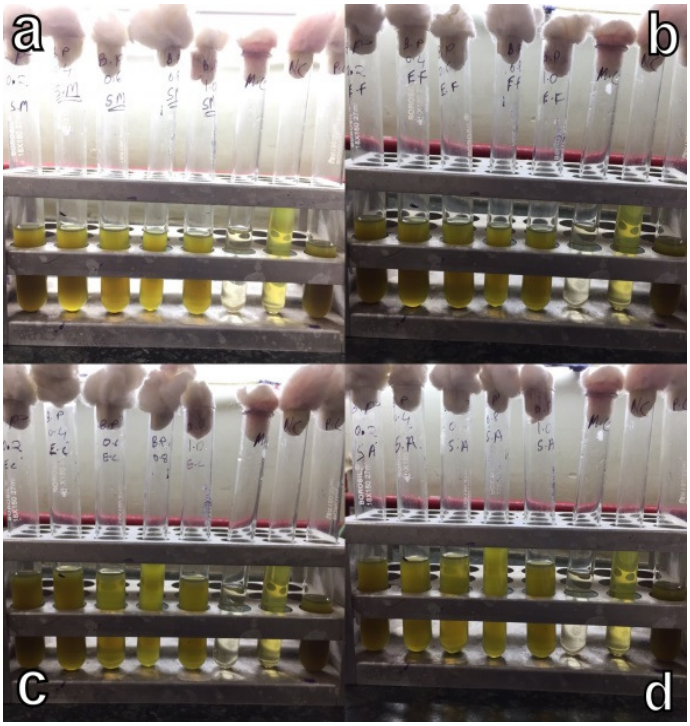

Figure-14: MIC for black pepper extract. (a) MIC against *Streptococcus mutans.* (b) MIC against *Enterococcus faecalis.* (c) MIC against *Escherichia coli.* (d) d. MIC against *Staphylococcus aureus.*

Decontamination of GP cones: The GP cones were decontaminated using Thiogylcolate broth against the *S. mutans*. The GP cones were dipped in four individual extracts for 15 minutes to 20 minutes and then put in the broth which had *S.mutans* inoculated in it. Different concentrations of the extracts were used viz 5%, 10%, 20% and 30%. 15 mins were chosen so as to be rapid and fast enough to disinfect the cones.

Table-4: Minimum inhibitory concentration for mixture extract.

Conc. of mixture (mg/ml)	Observation			
	S. mutans	*E. faecalis*	*E. coli*	*S. aureus*
0.2	+	+	+	+
0.4	+	+	_	_
0.6	_	_	_	_
0.8	_	_	_	_
1.0	_	_	_	_
PC	+	+	+	+
NC	_	_	_	_
MC	_	_	_	_

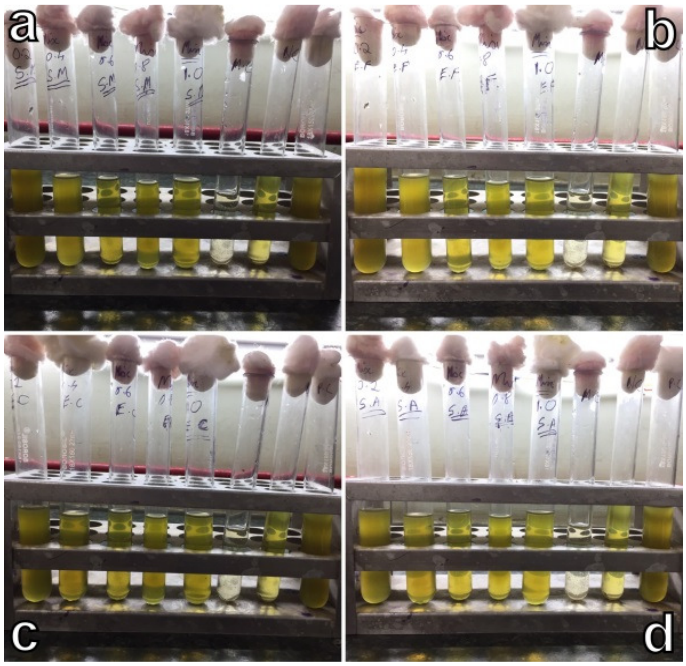

Figure-15: MIC for mixture extract. (a) MIC against *Streptococcus mutans.* (b) MIC against *Enterococcus faecalis.* (c) MIC against *Escherichia coli.* (d) MIC against *Staphylococcus aureus.*

The neem leaf and neem bark and kernels extract decontaminated GP cones at 20% and 30% showing no turbidity in the broth. Black pepper decontaminated the GP cones at 10% concentration of extract and the mixture showed clear solutions in all tubes.

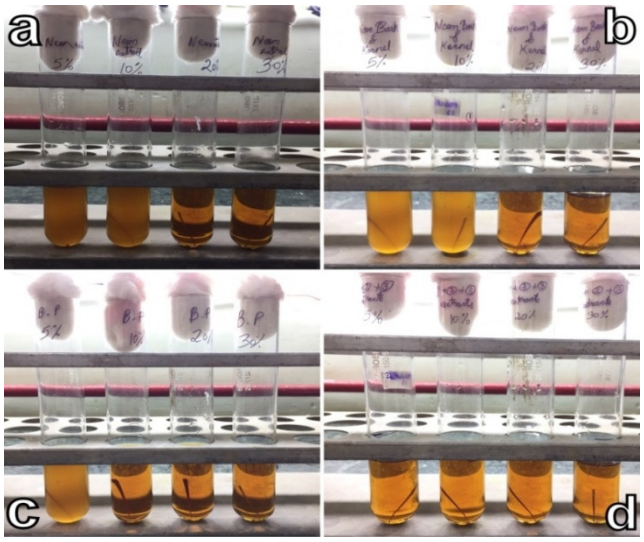

Figure-16: Decontamination of GP cones against *S.mutans* with different extracts. (a) With neem extract. (b) With neem bark and kernels extract. (c) With black pepper extract. (d) With mixture extract.

Conclusion

There are various chemicals used as irrigants in endodontic treatment, but every chemical has certain disadvantages. Sodium hypochlorite crystallizes even when mixed with a herbal neem extract. Thus it would be beneficial if an irrigant is used which is not a chemical, but yet would provide rapid decontamination of GP cones. Various substances are used like aloe vera gel, turmeric, clove, cinnamon, ginger, etc. In our study, we have used parts of neem plant and black pepper. Neem, as it is known for its anti-inflammatory, anti-malarial, anti-fertility, anti-microbial, anti-acne, acaricidal, nematicidal properties, is an excellent irrigant against the GP cones contaminants. Similarly, the black pepper extract also shows anti-microbial activity against various oral organisms. The cumulative extracts of neem and black pepper would make a root canal treatment successful as it inhibits the organisms used in our study, especially *S.mutans* which is the main causative agent for dental caries. Thus the cumulative extract can further replace the sodium hypochloride which is the recommended method to decontaminate the GP cones. These extracts should be tested against various other oral pathogens. The concentrations at which extracts inhibits organism should be further experimented on model of teeth with biofilms, or with people suggested to undergo the root canal treatment under trial basis. The extracts can also be incorporated in various oral products so as to maintain oral hygiene and thus to avoid occurrence of dental caries and formation of biofilms.

Acknowledgements

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