ANTIMICROBIAL ACTIVITY AND ANTI BIOFILM FORMATION OF NATURAL SPICES AGAINST STREPTOCOCCUS MUTANS FROM DENTAL PLAQUE OF SUBJECTS UNDERGOING ORTHODONTIC TREATMENT FOR PREVENTION OF WHITE SPOT LESIONS. - AN IN VITRO STUDY

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Abstract

Orthodontic treatment poses an increased risk of tooth decalcification due to plaque accumulation and biofilm formation around orthodontic appliances. Disruption of biofilm formation activity leads to inhibition of microbial virulence through the utilisation of QS inhibitors. This study was aimed to test three commonly used spices such as Cuminum cyminum (cumin seeds), Trachyspermum ammi (carom seeds) and Trigonella foenum-graecum (fenugreek seeds) for their anti-quorum sensing and antibiofilm activity against S. mutans which is the most common pathogen responsible for white spot lesions in orthodontic subjects. Plaque samples were collected from patients undergoing fixed appliance therapy and biochemical characterization was done. Solvent extraction of the three spices were obtained. In vitro analysis of antimicrobial activity and antibiofilm activity were performed by microdilution and crystal violet staining assay. The antimicrobial and antibiofilm activities of methanol extracts of the spices were tested against S. mutans. At a low concentration level (10mg/ml), the methanol extract of fenugreek seeds and 2.5mg for cumin and carom seeds, potentially inhibited the bacterial growth. At the lowest concentration level of 0.781, 0.390 and 0.195mg/ml of cumin extract significantly reduced the production of biofilm formation in S. mutans to the level of 62%, 60% and 58% respectively. Methanol extract of Cumin seeds had a potential inhibitory effect on the biofilm formation in S. mutans and all three spices showed antimicrobial activity.

Keywords: Antibiofilm, Anti-quorum sensing, quorum sensing inhibitors, spices, cumin, fenugreek, carom

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1. Introduction

The most common oral diseases such as dental caries and periodontal diseases depend on the ability of oral microorganisms to form complex biofilms on the hard tissues of the oral cavity. Orthodontic treatment poses an increased risk of tooth decalcification due to plaque accumulation around the elastomeric modules surrounding brackets, leading to the formation of white spot lesions.[1] Conventional ligation using elastomeric modules harbor more bacteria when compared to self ligating systems.[2]

Zachrisson and Zachrisson stated that the etiology and pathogenesis of periodontal diseases are multifactorial but dental plaque certainly is an essential precursor.[3] These microbial communities form microcolonies that interact with each other using very sophisticated communication system called autoinducers. [4] The use of antimicrobial chemotherapy to control microbial growth is one of the approaches. The effectiveness of antimicrobial agents as concluded in vitro tests is not evident in-vivo. This may be due to decreased sensitivity of the mature and intact biofilms to chemotherapeutic agents.[5] Indiscriminate use of antimicrobials has led to the rise of resistant bacteria called superbugs with high levels of antibiotic resistance.

Analysing the mechanisms of biofilm formation help in knowing how bacteria communicate in the their environments. This knowledge will help to form strategies for controlling and preventing dental diseases and also for the prevention of diseases and conditions in which bacterial accumulation play a prominent role. Numerous bacteria–host and bacteria–bacteria interactions occur in plaque biofilms but the important ones are the interactions that allow bacteria to adhere to the tooth surface and stabilize the three-dimensional plaque matrix.[4]

Recent discoveries have led to several approaches to control of oral biofilms such as preventing biofilm formation and disrupting existing biofilms. Moreover, the control of dental biofilm requires not only bacteriostatic but also anti-adhesive actions in order to inhibit the initial bacterial adherence. [6] Disrupting the communication system or bacterial quorum sensing (QS) activity leads to attenuation of microbial virulence. Inactivation of QS signal molecules is known as QS inhibition or quorum quenching (QQ) which can be done by ways like development of antibodies, enzymatic demolition and using agents which block QS leading to interference in cell to cell communication.[7][8]

Spices are common used ingredients in diets of many ethnicities. Spices like cinnamon, clove, rosemary and oregano are reported to have significant anti-oxidant, antimicrobial, and/or anticarcinogenic activities. Many a spices and herbs possess anti-adhesive properties as they have contain compounds which don't allow adhesion of microbes on to the host tissue thereby avoiding primary infection.[9] Quorum sensing controls pathogenicity and virulence in many a relevant bacteria. Anti-quorum sensing (AQS) compounds that interfere with the QS system attenuates bacterial pathogenicity. Bacteria are not killed by them but their growth is inhibited and they are less likely to develop resistance as compared to the existing antibiotics.[8]

The present study was aimed to assess the anti microbial and anti biofilm activity of the commonly used culinary spices. Three commonly used spices such as Cuminum cyminum (cumin seeds), Trachyspermum ammi (carom seeds) and Trigonella foenum-graecum (fenugreek seeds) were tested for their anti-quorum sensing virulence traits and antibiofilm formation activity against S. mutans which is the most common pathogen responsible for initial enamel decalcification in orthodontic subjects.

2. Materials and Methods

Sample collection

Oral plaque samples were collected from patients undergoing fixed appliance with pre-adjusted edgewise metal brackets and conventional ligation with elastomeric modules not under any topical antiseptics and systemic antibiotics. A sterile explorer was used to collect plaque from buccal surfaces of upper premolars and labial surfaces of lower incisors. The plaque samples were transferred to Eppendorf tubes containing 1ml Brain Heart Infusion (BHI) broth.

Biochemical characterization

A total of 10 samples each were collected from 5 patients and were incubated at 37 °C for 24 h in the shaker incubator. After 24 h of incubation, 0.1ml of cultures were smeared on the surface of BHI agar plate. The plates were incubated at 37 °C for 24 h. Then a single colony of bacteria was sub cultured in the BHI broth and the tube was incubated at 37 °C for 24 h. The preliminary screening of bacteria was identified based on the colony morphology, texture and other parameters such as indole, Methyl

Red, Voges–Proskauer, Gram staining were compared with Bergey's manual of determinative bacteriology. [10]

Collection of Spices

The natural spices such as Cuminum cyminum (cumin seeds), Trachyspermum ammi (carom seeds) and Trigonella foenum-graecum (fenugreek seeds) were obtained locally procured in Chennai, India and were used in the study.

Solvent Extraction

The collected dry spices were separately pulverised into a coarse powder form using a mechanical grinder. Each of the samples (10g) were transferred into a conical flask and 100 ml of methanol was added. The flasks were incubated at room temperature in a shaker incubator (RPM 150) for 3 days. The solvent was filtered using Whatman no 1 filter paper (Himedia, Mumbai). The collected extracts were dried using a rotary flash evaporator in a hot condition (50^0C) and the dried extracts were stored in a refrigerator for further analysis.

Determination of minimum inhibitory concentration and sub MIC

For evaluation of MICs of methanol extracts of Cuminum cyminum (cumin seeds), Trachyspermum ammi (carom seeds) and Trigonella foenum-graecum (fenugreek seeds) the method used was broth microdilution (two fold). MICs of the methanol extracts of the spices against S. mutans used in the assay were assessed concentrations differing in range from 10 to 0.019 mg/ml. Control and sterility control were also maintained. The bacterial growth was visualised by addition of 2,3,5-triphenyl tetrazolium chloride (TTC) salt which acted as an indicator. The lowest concentration of the extract without any visible growth was noted as MIC.

Microtitre Biofilm Inhibitory Assay

To evaluate the inhibition of biofilm formation static microtitre plate assay was used. It was assessed for Cuminum cyminum (cumin seeds), Trachyspermum ammi (carom seeds) and Trigonella foenum-graecum (fenugreek seeds) against the clinical isolate of S. mutans. Concentrations ranging from 12.5 to 0.02 mg/mL for methanol extract of fenugreek and from 3.125 to 0.005 mg/mL for cumin and carom seeds extract was loaded to each well containing 80**µ**l of the BHI broth and was well mixed. Following this the culture (20 µl) containing the test pathogens was loaded followed by incubation of the microtiter plate for 24 hours at 37 °C. After 24 hours of incubation, the planktonic cells were removed and read at 600 nm. This plate was washed with sterile distilled water for removing the excess planktonic cells. Crystal violet (10 µl, 0.1% (w/v) in water) was added to each of the wells and the plate was incubated for 15 minutes at room temperature. [11] The crystal violet was removed from the wells after 15 minutes and washed gently with sterile distilled water for removing the unbound crystal violet stain. (Figure 1) The adhered biofilm was eluted in ethanol (95%) then the absorbance was measured at 525 nm (Robonik Elisa Plate Reader). The presence of growth (planktonic cells) of the treated strains was compared with the untreated control strains by measuring the OD at 600 nm.

Figure 1 : Microtitre biofilm inhibitory assay

3. Results

Determination of MIC

The minimum inhibitory concentrations were performed by microdilution method. The methanol extracts of Cuminum cyminum and Trachyspermum ammi potentially prevented the bacterial growth at the concentration level of 2.5mg/ml. (Figure 2-3) Similarly, the methanol extract of Trigonella foenum-graecum inhibited the bacterial growth (S. mutans) at the concentration level of 10mg/ml. (Figure 4)

S No.	Seed Extract	1	2	3	4	5	6	7	8	9	10
		10mg	5mg	2.5mg	1.25mg	0.625mg	0.31mg	0.15mg	0.078mg	0.039mg	0.019mg
1	Cumin	MIC	MIC	MIC	G	G	G	G	G	G	G
2	Carom	MIC	MIC	MIC	G	G	G	G	G	G	G
3	Fenugreek	MIC	G	G	G	G	G	G	G	G	G

Table 1: Determination of MIC (MIC - Minimum inhibitory concentration, G - Microbial growth seen)

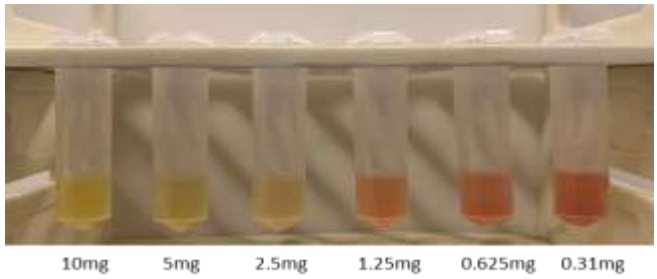


Figure 2 : Determination of MIC - the methanol extract of cumin seeds showed antimicrobial activity at concentration of 2.5mg/ml

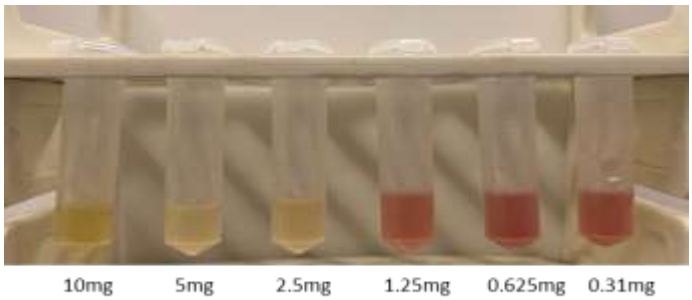
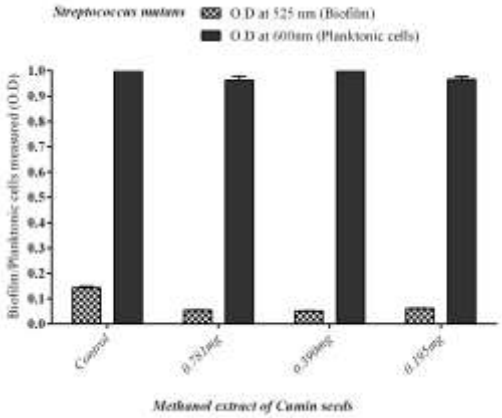


Figure 3 : Determination of MIC - the methanol extract of carom seeds showed antimicrobial effect at concentration of 2.5mg/ml

Figure 4 : Determination of MIC - the methanol extract of fenugreek seeds showed antimicrobial effect at the lowest concentration of 10mg/ml

Antibiofilm activity (Crystal violet staining assay)

At the lowest concentration levels of 0.781, 0.390 and 0.195mg/ml cumin extract had significantly reduced the production of biofilm formation in S. mutans by 62%, 60% and 58% respectively. (Graph 1) However, fenugreek and carom seeds did not inhibit the biofilm formation in S. mutans.



Graph 1 : Antibiofilm activity of cumin seeds

4. Discussion

Targeting of the QS system is gaining much attention as an infection control strategy, especially in the wake of widespread drug resistance among bacterial pathogens. In this study, three commonly used spices Cuminum cyminum (cumin seeds), Trachyspermum ammi (carom seeds) and Trigonella foenum-graecum (fenugreek seeds) were investigated for antibacterial and anti-quorum sensing activities. Cuminum cyminum (Cumin seeds) showed anti-quorum sensing activity. At the lowest concentration of 0.781mg/mL, Cumin seeds exhibited significant reduction of biofilm formation in S. mutans. All three spices demonstrated antimicrobial activity. At the lowest concentration of 10mg for fenugreek seeds and 2.5mg for cumin and carom seeds, microbial growth was inhibited. Very few reports are available related to the antimicrobial activity of Indian spices.[12] In a study by Minakshi De et al, chili, clove, horseradish, cinnamon, bishop's weed, cumin, tamarind, pomegranate seeds cumin, cambodge, nutmeg, garlic, onion, tejpat, celery, have good antimicrobial activities against the test organisms Escherichia coli (ATCC 10536) Bacillus subtilis (ATCC 6633), and Saccharomyces cerevisiae (ATCC 9763). [13] Bactericidal effect of garlic was apparent within one hour of incubation and demonstrated antifungal activity along with clove. [14] The antimicrobial compounds of herbs are mostly in the essential oil fraction. Ceylan E at al, stated that the major antimicrobial components in spices are the hydrocarbons, alcohols, ketones, aldehydes and ethers. [15] The degree of observed microbial inhibition depends on the method employed. [16] Thus spices and herbs could be potentially developed as antimicrobial agents and as preservatives in food. [17] Venkadesaperumal et al showed anti-quorum sensing activity of nanoemulsions of spice oils of cumin (Cuminum cyminum), pepper (Piper nigrum), and fennel (Foeniculum vulgare) oil against food borne pathogens. [18] Mutungwa et al noted that S. aromaticum effectively inhibited biofilm production by quorum sensing and virulence factors against clinically isolated strains of P. aeruginosa. [19] Carum copticum [20], Cinnamomum zeylanicum, Ocimum basilicum [21], Citrus maxima [22] and several other dietary products have been proved to have effective quorum sensing inhibitory effects. [23] Evalation of bioactivity of medicinal plants is needed as traditional medicine is used widely throughout the world.[24,25] The QS system plays an important role in initiation, attachment and maturation of biofilms and also in drug resistance and disease pathogenesis. Therefore interfering with the QS system may prevent biofilm matrix assembly and further infection in host cells. This approach may help in the discovery of a newer and safer antimicrobial and antibiofilm drugs from dietary sources with reduced toxicity and risk of antibiotic resistance. The antibiofilm and QS inhibition activity of cumin seeds can be useful alone or in combination with other dietary quorum sensing

inhibitors for oral benefits. Further studies on the pharmacokinetic properties of Cumin seeds are required to make full use of their clinical properties. Future studies should be conducted to identify more natural products which are rich sources of antimicrobial and antipathogenic agents.

5. Conclusion

Methanol extract of Cumin seeds had potential biofilm inhibition effect by S. mutans and all three spices showed antimicrobial activity. These observations from the present study results suggest that phytochemicals obtained from natural spices have inhibited biofilm formation and also inhibited QS related virulence processes in pathogenic bacteria thus opening a strategy for antimicrobial chemotherapy.

Conflict of Interest

The authors declare that they have no competing interests.

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