In Vitro Evaluation of Antimicrobial Efficacy of the Different Concentrations of Chitosan against *Staphylococcus epidermidis* in Disinfection of the Root Canal System

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Abstract

During endodontic procedures, along with mechanical instrumentation, irrigants are imperative for pathogen eradication. As the strains of pathogens are becoming antibiotic resistant at a constant ascending pace and also the potentially harmful effect is associated with synthetic drugs, so studies on natural alternatives for irrigation are being done. Chitosan, a natural alternative, has been evaluated as an antimicrobial medicine. Recently, in cases presenting with persistent infections after endodontic treatment, it was noted that of all the microbial species identified, the prevalence of *s.epidermidis* was the most. In this current study, an analysis of the antimicrobial effectiveness of chitosan as a potent root canal irrigant, for disinfecting the root canal was conducted. **Methodology:** By the serial dilution method, the minimum inhibitory concentration was determined. Dilution of the test solution was done using 10% DMSO solution. After which, incubation in a rotary incubator, at 180 rotations per minute, the temperature of 37℃, for 24 hours was done. For obtaining the zones of inhibition of different concentration (0.2%, 0.5%, 0.75, 1%) of chitosan irrigant, the agar diffusion method was applied and the resultant clear zone was measured with a ruler. **Result:** Chitosan (liquid) had promising antibacterial activity against *Staphylococcus epidermidis*, with a 16 mm zone of inhibition at 100 µg/ml. **Conclusion:** Within the limitations of the present study, it was observed that, even at lower concentrations, chitosan (liquid form) effectively hindered the further growth of *S.epidermidis*.

Keywords*: Chitosan, Antimicrobial efficacy, S. epidermidis, root canal system*

Introduction

It has been established that the preeminent cause behind pulp infection and periapical disease is microbes.[1,2] Polymicrobial character is specific to endodontic infection, which is of intraarticular or extraarticular origin. In primary intraarticular infection, apart from fungi(*candida spp*), viruses, the commonly found pathogens are gram-negative anaerobic rods, *Fusobacterium, dialister*, spirochetes, *Tannerella forsythia,* gram-positive anaerobic rods, gram-positive cocci. Endodontic pathogens associated with extraarticular infection are usually anaerobic bacteria, *Actinomyces spp, Prevotella spp., Fusobacterium nucleatum* to name a few.

Enterococcus faecalis (E. faecalis) and *Candida albicans (C. Albicans*) are commonly detected in root canals that are already endodontically treated but still showing signs of infection.[3-5] But in some recent studies, the presence of microbes other than commonly found ones are mentioned. *Murad et al* stated that, when investigated by checkerboard DNA-DNA hybridization, *S.epidermidis* along with *E.faecium* were the most prevalent microbes in cases of persistent

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endodontic infection.[6] With the total occurrence rate of staphylococcal species in the oral cavity (saliva) being 83.9 %, s.epidermidis is the second most frequently found species in the oral cavity (41.4%). Bacterium *s.epidermidis* is a facultative anaerobe, i.e., it can survive in both the aerobic and anaerobic situation.[6]

During endodontic procedures, in conjugation with mechanical instrumentation, irrigants are imperative for thorough pathogen eradication from the root canals. The antimicrobial efficacy as shown by irrigants is of extensive levels. The features supposed to be present in quintessential endodontic irrigants are superior antimicrobial efficacy, non-cytotoxic, prudent towards the tissues of the periapical region, endotoxin inactivation, lubricating nature and breakdown of tissue.[7-9]

Of all the irrigants used in endodontics, Sodium Hypochlorite(NaOCl) is considered as the gold standard.[7] The major disadvantage of NaOCl is that when near vital tissues, it irritates leading to severe inflammatory reactions.[10-12]

As the strains of pathogens are becoming antibiotic resistant at a constant ascending pace and also the potentially harmful effect is associated with synthetic drugs, so studies on natural alternatives for irrigation are being done.

Chitosan, a natural alternative, has been evaluated as an antimicrobial medicine. It is a Polysaccharide and cationic. The chemical structure of chitosan (Figure1) comprises of "copolymers of glucosamine and N-acetylglucosamine". The main characteristic feature of chitosan being biocompatible, biodegradable, bioadhesive, non-cytotoxic along with its ability to chelate for varying metal ions, even in acidic condition, makes chitosan an enticing potent irrigant in endodontics.[7]

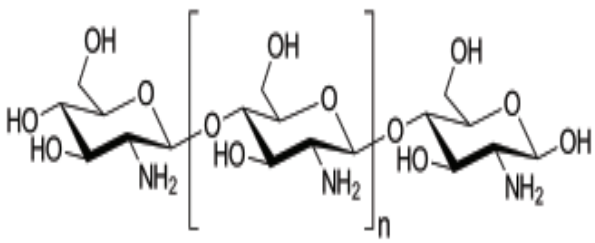

Fig 01: chemical structure of chitosan

So, the current study mainly aims at the analysis of the effectiveness of chitosan, in two forms, the powder and the liquid form, against *S.epidermidis* as a potent irritant in the root canal system.

Material and Methodology

The Agar-well diffusion method was used for antibacterial assays of chitosan.

Bacterial strain: Biofilm-positive strain of *s. epidermidis* was incorporated in this study. [Strain: RP62A / ATCC 35984]

Bacterial Culture*:* Agar was taken as the culture media for the culture of the *s.epidermidis*. For testing the efficacy, three Petri dish were taken, one for the reference control group ampicillin, and remaining two for the two forms of chitosan viz., the powdered form and the liquid form. Into all three of the pre-sterilised Petri dish of 6 mm depth, the culture media was dispensed and the bacterial lawn was prepared that was fully punched. After 30 minutes of the preparation of the lawn, 5 wells were prepared on the lawn. 50 µl of molten nutrient agar was then put as a base in all the 5 wells. Following which, 100 µl of the test compound, chitosan was poured in the well, containing the molten nutrient agar. Each of the 5 wells in the Petri dishes, had a different concentration of chitosan(0.25%,0.5%,0.75%,1%). Incubation of the plates was done at a temperature of 37°C, duration of 18-24 hours and was then refrigerated until further use.

The powder form of chitosan was from Bangalore Fine Chemicals (BFC), India. (Item model number: BFC260274). To prepare the stock solution of powder form of chitosan, the addition of 1gram of chitosan powder to 100 ml of 0.1M acetic acid under mild heat (40-50 °C) with continuous agitation for almost 5 hours was done. The insoluble chitosan of higher molecular mass was removed by filtration.

The chitosan in the liquid form came in a glass bottle of 2% concentration. The different concentrations of both the forms of chitosan were achieved by diluting the stock solution with 10% DMSO solution(dimethyl sulfoxide)

The Minimum Inhibitory Concentration (MIC): The lowest concentration at which no visibly appreciable turbidity can note is known as Minimum Inhibitory

Concentration. The preparation of the bacterial culture was done as such so that the McFarland standard turbidity of 0.5 could be obtained. The determination of MIC was gained by serial dilution of 0.25%, 0.5%,0.75% and 1% of chitosan. At 180 rotations per minute, the incubation of the well plates was done for 24 hours, at a temperature of 37°C

Measuring The Zone Of Inhibition: For determining the zone of inhibition, agar well diffusion method was chosen for this study. Onto a trypticase soy agar plate, *S. epidermidis* was sub-cultured, followed by incubation for a period of 24hours, at a temperature of 37°C. As a reference for adjusting the turbidity, 0.5 McFarland's standard was considered. On completion of 24hours, suspension of bacterial colonies in the culture medium was done. The turbidity of the bacterial suspension was adjusted according to the standard. (Figure 2).

On the superficial layer of the trypticase soy agar plate, an even layer of bacterial suspension was laid out. It was allowed to dry for some time of 5minutes. Already sterilized Whatman disc, having a radius of 3mm was saturated in 20 µL of chitosan, in both powdered and liquid form, at different concentrations. While maintaining an adequate distance between the discs, the placement of the trypticase soy agar plate was carefully executed. Following which, incubation for 24 hours and at a temperature of 37°C was done. From the zone of inhibition, the evaluation of the antibacterial activities was done by measurement of the diameter of the zone, using an electronic digital calliper (6 inches).

Reference Control: As a reference control, ampicillin having a concentration of 30µg/ml was taken.100µl aliquot was analysed. The zone of inhibition of ampicillin had a diameter of 21mm (average).10% DMSO solution having null cytotoxic and antibacterial activity was taken.

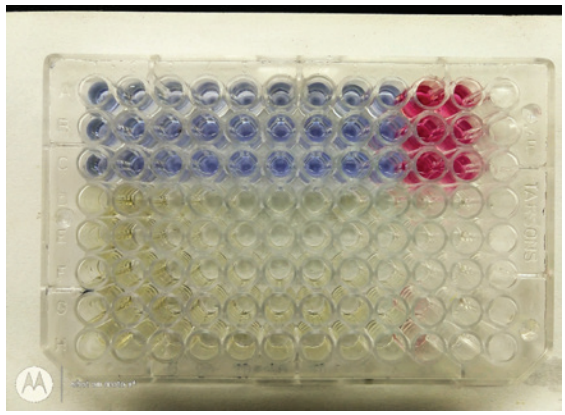

Figure 02:Minimum Inhibitory Concentration of chitosan against Staphylococcus epidermidis

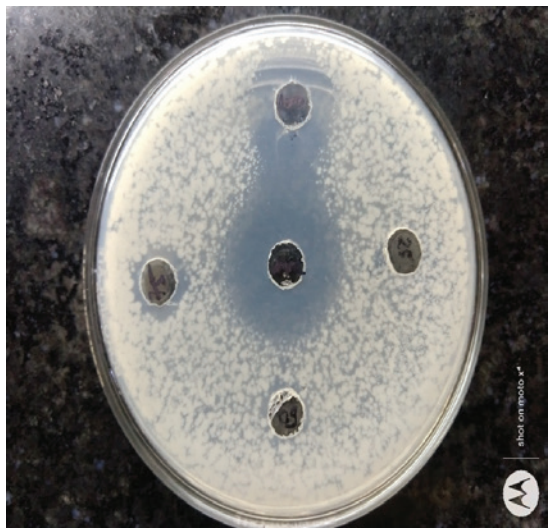

Figure 03: Antibacterial effectivity of chitosan (powdered form) against *Staphylococcus epidermidis*

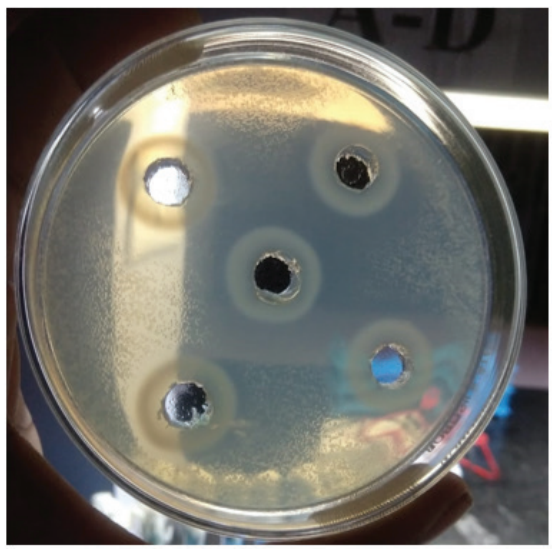

Figure 04: Antibacterial effectivity of chitosan (Liquid form) against *Staphylococcus epidermidis*

Results

The powdered form of chitosan had negligible antibacterial activity against *Staphylococcus epidermidis* (fig.03). Only 6 mm zone of inhibition was observed, which was very low when compared to the positive control group of standard antibiotic Ampicillin (30 µg /ml). (table:01). On the other hand, Chitosan in the liquid form had promising antibacterial activity against *Staphylococcus epidermidis* (fig.04). A 16 mm zone of inhibition at 100 µg/ml was observed, which was comparatively slightly higher than the positive control group. Even at lower concentrations of 0.25, 0.5 & 0.75 chitosan in the liquid form exhibited moderate antibacterial activity. (table:01) The Minimum inhibitory concentration (MIC) of chitosan (liquid) was recorded at 30 µg/ml.

Table 1: Zone of inhibition and minimum inhibitory concentration of the different concentrations of chitosan in both the powder and the liquid form

Sample	Concentration	Zone of Inhibition	MIC
Chitosan (powdered form)	25µg/ml	-	- - - -
	50µg/ml	-	
	75µg/ml	-	
	100µg/ml	6 mm	
	Ampicillin 30 µg/ml Positive Control	15 mm	
Chitosan (Liquid form)	25µg/ml	10 mm	30 µg/ml
	50µg/ml	12 mm	
	75µg/ml	15 mm	
	100µg/ml	16 mm	
	Ampicillin 30 µg/ml Positive Control	15 mm	

Discussion

Bacterium *S. epidermidis* is a facultative anaerobe, i.e., it can survive in both the aerobic and anaerobic situations. Character specific to S.epidermidis is biofilm mode of growth and its attachment to the uncoated abiotic surfaces. As endodontic infection is primarily biofilm-oriented, so for successful treatment and prognosis, disruption of the pathogens through thorough irrigation and ultimately leading to its eradication is of prime concern. [11]

For a good prognosis and for the outcome of endodontic treatment to be successful, along with proper instrumentation of the root canal, irrigation is also of prime importance. Chitosan, a potent irrigant, is isolated from the crustaceans (Phylum: Arthropoda). The shells of the organisms are then treated with a substance, alkaline in nature, preferably sodium hydroxide. When the chitin shells are deacetylated partially, chitosan as an end-product is acquired. It demonstrates antimicrobial activity against a wide range of microbes, including bacterial species, fungal species and also viruses as it interferes with the cellular walls and membranes of microbes, leading to lysis of the cell. Chitosan is biocompatible, biodegradable, bioadhesive and is non-cytotoxic by nature. [12]

The production cost being comparatively low, the serviceableness of chitosan has increased manifold in the field of medicine and pharmaceuticals. Not only in the medical field, but also in dentistry, in the branch of periodontology, the successful usage as a "barrier membrane" is evidenced. Another commendable property of chitosan is its ability to chelate for varying metal ions, even in acidic condition. The antimicrobial efficacy of different forms and concentration of chitosan was evaluated in this study. The current study revealed that 1% of chitosan had an antimicrobial effect compared to the control group,i.e., ampicillin 3%. The agar diffusion method and MIC was used to determine the concentration of chitosan against *S.epidermidis.*[13-15]

Previous studies advocated that despite the root canal system is complex, the antimicrobial property of chitosan was adequate. The outcome of this current study also complies with the already published literature. The cation active structure of chitosan ascribed to the superlative antimicrobial property of chitosan.[15] As the bacterial cell wall is negatively charged, so when the oppositely charged chitosan binds easily to it, the imperative functions of the bacterial cell wall gets disrupted, ultimately leading to apoptosis.

The effectiveness of the antibacterial property of a test material/compound is directly related to the size of the zone of inhibition. The larger the clear area on the disc, the more is the antibacterial efficacy of the compound against the microbe. Standard antibiotic, Ampicillin, which is a broad-spectrum antibiotic was taken as the reference control group. This group exhibited a zone of inhibition of 15mm.

Chitosan-liquid showed a zone of inhibition of 16mm at a concentration of 1%, which was slightly higher, by 1mm, than the reference control group, ampicillin. At a concentration of 0.25%, 0.5% and 0.75%, the zone of inhibition was found to be 10mm,12mm and 15mm respectively. So even at a lower concentration, Chitosan-liquid resulted in moderate antibacterial activity. At a concentration of 1%, Chitosan-powder showed a zone of inhibition of just 6mm, which is very low, as compared to the reference control group. At lower concentration of 0.25%, 0.5%, 0.75%, no zone of inhibition could be appreciated. When compared between the different forms of chitosan, i.e, the powder and the liquid form, it was seen that even when the concentration was kept the same, the two forms of chitosan showed a stark difference in the result. At a concentration of 1%, while chitosan-liquid showed result superior to that of the control group, chitosan-powder showed the almost negligible result.[16]

Also at lower concentrations (0.25%,0.5%,0.75%), the moderate antibacterial activity could be seen in case of the liquid form of chitosan. But no activity could be seen in the powdered form. So, on comparing the antibacterial activity between the two forms of chitosan, the liquid form showed a promising result. The poor result of the chitosan in the powdered form may be related to the particle size and particle solubility. The size of the particle influences the bacterial cell wall penetration. Also higher the degree of deacetylation, more is the solubility of chitosan in 0.1M acetic acid. It has been reported that lowering the particle size has a positive effect on its antibacterial property. Of all the methods of investigating the antibacterial properties, the

agar diffusion test is one of the entrenched ones. The major benefit of the agar diffusion method lies in the fact that the chemical properties of the test medicament don't undergo any change in the whole process. Just by asserting the specific microbial isolates present in the antimicrobial disc, the detection of the antimicrobial efficacy of the medicaments can be accomplished. In the current study, the largest zone of inhibition was exhibited by the liquid form of 1% chitosan against *S. epidermidis*. It showed a zone of inhibition which was more than the control group. After taking into account the disparity in the data of minimum inhibitory concentration and the zones of inhibition evaluated in the current study, the speculation that can be contrived is, even at a low concentration, liquid form of chitosan can be an irrigant of choice, a potent irrigant against *S. epidermidis*.[17]

Further researches including the antimicrobial effect of chitosan in the presence of other microbes, either a single species or multiple species, that are found in the biofilm is obligatory.

Conclusion

The antimicrobial efficacy of chitosan, a natural alternative to the conventional irrigant was evaluated in this study. Within the limitations of the present study, it was observed that, at a concentration of 1%, chitosan-liquid constrained the microbial growth of *S. epidermidis*, showing result superior to that of the control group(ampicillin), while chitosan-powder showed almost negligible result. At a concentration of 0.3 %, chitosan in the liquid form showed promising antimicrobial efficacy against *s. epidermidis.* So considering the antimicrobial properties that were evaluated in the study, chitosan can be possibly used as an endodontic irrigant.

Ethical Permission: Not Required

Conflict of Interests: None

Funding: None

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