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Antimicrobial Evaluation for Novel Solution of Iron Oxide Nanoparticles Functionalized with Glycine and Coated by Chitosan as Root Canal Final Irrigation

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ABSTRACT

Background: This study aims to evaluate the antibacterial activity of chitosan-coated iron oxide nanoparticles (Chi-IONP) by agar direct diffusion method and determine the minimum inhibitory concentration and minimum bactericidal concentration (MIC/MBC) of this nano-solution against *E.faecalis, S.mutans and Candida albicans* the three oral microorganisms commonly isolated from endodontic infections.

Methods: The nanoparticles solution was prepared by functionalizing the 8nm iron oxide nanoparticles with glycine and coating them by chitosan dissolved in 0.25% v/v acetic acid. *E.faecalis, S.mutans and candida albicans* were isolated and identified with Vitek 2 system. Agar diffusion method was used to test three groups of materials, NaOCl 5.25% serve as positive control, 0.25% Acetic acid as negative control and Chi-IONP as experimental material. Broth microdilution method used to determine the MIC/MBC of the experimental material.

Statistical analysis used: Statistical analysis was conducted by independent variables t-test performed by using IBM-SPSS program version 22.

Result: The used microbial isolates were confirmed with Vitek 2 system with excellent confidence level of diagnosis. Chi-IONP showed antifungal activity higher than NaOCl 5.25%, and comparable effect to NaOCl 5.25% against *E.faecalis* and *S.mutans*. The negative group showed no effect on the tested microorganisms. The MIC/MBC were determined as following 125/250 µg/ml for ***E.faecalis,*** 250/ 500 µg/ml for ***S.mutans*** and the MIC/MFC was 62.5/ 125 µg/ml for ***C.albicans.***

Conclusion: Chi-IONP was a powerful antimicrobial agent against the tested microorganisms, and this test results shows antifungal activity of Chi-IONP higher than NaOCl 5.25% and comparable antibacterial effect to NaOCl 5.25%.

Keywords: Nanoparticles; antibacterial; Iron oxide; root canal; drugs

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INTRODUCTION

Endodontic infection is considered the major etiological factor of apical periodontitis and can be defined basically as the dental root canal system infection[1]. Root canal system infection occurs when the enamel is breached, dentine invaded and the pulp immune response defeated by microorganisms when it tries to enter and settle in the remaining necrotic tissue [2].

Pulpal inflammation (pulpitis) is caused mainly by bacteria when it enters the pulp through a deep caries lesion or improper coronal seal. Bacterial products can also cause pulpitis. Loss of the mineralized tooth' structures open a track for entering of bacteria into the root canal system, from the oral cavity [3].

E.faecalis, S.mutans and candida albicans are the three common microorganisms isolated from root canal infection with apical periodontitis [4-5-6]

Chitosan is biopolymer with interesting properties such as biocompatibility, biodegradability and antimicrobial activity. Chitosan is derived from chitin one of the most plentiful natural polysaccharides [7].

Sodium hypochlorite (NaOCl 5.25%) is a potent antimicrobial agent used widely in endodontics. The antibacterial activity of NaOCl increases with increase of its concentration and toxicity [8].

The aim of the present study was to evaluate the antibacterial activity of ultrafine chitosan coated iron oxide nanoparticles by agar direct diffusion method and determine the minimum inhibitory concentration and minimum bactericidal /fungicidal concentration of this nano-solution against *E.faecalis, S.mutans and Candida albicans* the three oral microorganisms commonly isolated from endodontic infections.

METHODS

Chitosan coated iron oxide nanoparticles:

The coating method was done in two steps procedure:

First: Functionalizing of iron oxide nanoparticles with glycine, as following;

Mechanical mixing of 100 mg of the iron oxide nanoparticles with average size 8nm (US Research Nanomaterials, USA) dried powder, and 200 mg of Glycine HCL powder (Sigma-Aldrich, Germany). The two powders were mixed and grounded very well for 20 minutes with the pestle and mortal. Then the mixture was dissolved in 5ml of deionized water and filtered using syringe filter (0.1 µm)[9].

Second: Preparation of chitosan solution by dissolving 250 mg of low molecular weight chitosan powder (Sigma-Aldrich, Germany) in 100ml of 0.25% v/v acetic acid solution (Sigm- Aldrich, Germany) then mixed well with vortex mixer (Microyn Technologies Inc., USA).

The final solution was done by mixing of 1:4 volumes of functionalized iron oxide nanoparticles solution with chitosan solution.

Preparation of culture media

Six types of culture media were used in this study as following: blood agar, Mueller Hinton broth (MHB), Mueller Hinton agar (MHA), Pfizer selective enterococcus agar, Mitis Salivarius agar (MSA) selective media for *S.mutans,* and Sabouraud's Dextrose agar (SDA) selective media for *Candida albicans.* All the types of culture media

were prepared according to manufacturer instructions (Himedia, India).

Sampling procedure

The sampling procedure was done in specialized dental center in Karbala city, the patients who were included in the study were informed about the procedure details of this study and they signed the informed consents. Ten patients aged 25-45 with pulpal necrosis, periapical changes of single rooted teeth were selected for this study. The teeth were asymptomatic and the diagnosis was confirmed by clinical and radiographic examination indicated for endodontic treatment.

The exclusion criteria included the patients under any antibiotic treatment with in the last three months and the teeth with severely destructive crown that interfere with placement of rubber dam.

All the teeth were cleaned by pumice, then rubber dam was used to isolate the field, the disinfection was done with 35% hydrogen peroxide (H2O2) and 5.25% sodium hypochlorite (NaOCl) respectively.

Two burs were used to do access cavity, the first one was large carbide bur mounted on low speed hand piece, used to remove the caries and not gone deep to the pulp chamber and the second new carbide bure was used to remove the roof of the pulp chamber and clean it.

All the burs must not enter deeply into pulp chamber in order to not disturb the pulp micro flora [10].

The sampling procedure was done after minimal instrumentation by a file size 20# without use of any irrigation. The canal was flooded with sterile saline solution and agitated by file to form a bacterial suspension inside the canal [11-12]. Two paper points were used for sampling procedure by inserting them inside the canal to full working length for 60 seconds then each paper point was transferred to a tube of sterile transport media (AMIES) [13]. Half of the samples were transported to the laboratory by anaerobic gar and the other half transported aerobically.

Isolation and Identification of micro-organisms

Suspensions were prepared from the transported samples mixed well by vortex then cultured and spread on selective media, Pfizer for *E.faecalis,* MSB for *S.mutans* and SDA for *candida albicans*, and blood agar media to refresh the microorganisms for backup in case if not grown in selective media.

The plates were incubated in anaerobic and aerobic conditions at 37 C° for 48 hours. The growth of microorganisms can be identified based on their morphological appearance of colony by size, shape and color in public health laboratories\ Kabala.

Each type of pure colony was sub-cultured on blood agar for 18 hours to prepare a pure culture of each microorganism to confirm the identification by using vitek 2 system (Figure 1) (biomerieux, USA).

Confirmation by Vitek 2 compact system

The procedure was done according to the instruction of manufacturer as following:

Preparation of an inoculum from pure culture by transferring sufficient number of colonies that have the same morphology to clear polystyrene tub contain 3.0 ml sterile saline (0.45% to 0.5% NaCl, pH 4.5 to 7.0).

The final density of microorganism's suspension was adjusted by VITEK 2 DensiCHEK Plus devise to be 0.5 McFarland for selected bacteria and 2.0 McFarland for *C.albicans*. Fill the Vitec 2 card GP (for tested the gram positive bacteria) and YST card (for *C.albicans*). After the results were completed, the identification level of excellent, very good and good depended in the study [14-15].

Figure 1: Vitek 2 system that was used for bacterial identification.

Test of the sensitivity of the selected microorganisms to Chi-IONP

Thirty petri dishes of MHA were prepared and inoculated with 100 µl of freshly prepared (0.5 McFarland turbidity standard) suspension of *E.faecalis, S.mutans and C.albicans*. The inoculum was spread on a petri dish by mean of sterilized cotton swap the spreading must be with gentle touch and in all direction of the petri dish.

Three wells of 6mm diameter and 4mm depth were punched in each petri dish, the wells were filled with Chi-IONP, 0.25% acetic acid (A.A) as negative control and sodium hypochlorite (NaOCl) 5.25 % as positive control. All the petri dishes were incubated for 24 hours at 37C° in ambient air condition for *E.faecalis* and *C.albicans*, but regarding *S.mutans* incubation was performed in anaerobic condition using anaerobic jar with gas pack [16-10].

Determination of MIC (minimum inhibitory concentration) and MBC /MFC (minimum bactericidal / Fungicidal concentration) of Chi-IONP

Determination of MIC (minimum inhibitory concentration)

Broth microdilution method was done to determine the MIC\MBC by using 96 well plate and MHB.

Two hundred µl of Chi-IONP (2mg/ml) was added to the first well of the first line in 96well plate then in the next seven wells 100 µl MHB were added.

After that 100µl from the first well (Chi-IONP) was transferred to the next well added to previous 100µl of MHB mixed well then 100µl of the diluted Chi-IONP was transferred to the next well. The procedure was repeated until the last well. In the last well, the final volume was 200µl so 100µl was discarded to obtain eight wells with 100µl in each.

Bacterial inoculation was prepared by direct colony suspension method by suspending fresh-cultured microorganisms in MHB to make a suspension of 0.5 McFarland turbidity.100µl of this inoculation was added to each well. So the range of concentration in eight wells was 1mg/ml - 7.8 µg/ml.

Two hundred µl of non-inoculated media and inoculated media were added into two separated wells to serve as negative and positive control respectively. The plate was sealed with parafilm and incubated to 16-20 hours and after incubation period the wells were examined for bacterial growth (turbidity) [16-10].

Determination of minimum bactericidal/ fungicidal concentration (MBC)

The MBC determination procedure was done by culture of 10 µl from each well after well mixing on agar plate to identify the higher concentration with no growth [17-11].

RESULT

Chitosan coated iron oxide nanoparticles

The coating procedure was done successfully and the coating of chitosan to iron oxide nanoparticles was proved by atomic force and transmission electron microscopies. The average size of the coated particles was 30nm (the detailed data are mentioned in a separate study).

Identification and confirmation of the isolated microorganisms

Growth and colony morphology on selective media

All the isolated microorganisms were tested under microscope and the basic characteristics were confirmed by public health laboratories\ Karbala.

E.faecalis identification

E.faecalis colonies appeared as circular smooth elevated entire edged with wet appearance and the size of colony about 1-1.5 mm, and grow well on Pfizer agar with blackening around the colony. Figure (2).

S.mutans identification

S. mutans colonies appeared swell –shaped "udule-shaped" stacked colonies about 1mm with a granular glass appearance with frosted surface. Figure (2).

C.albicans identification

C.albicans Colonies are white creamy colored, raised, entire, smooth and yeast-like in appearance. Figure (2).

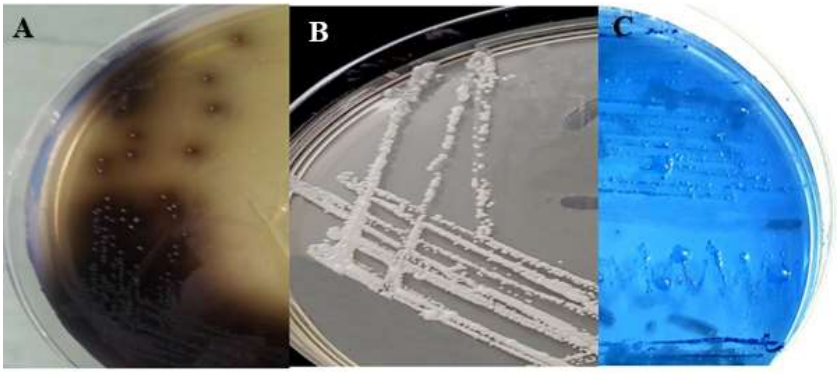

Figure 2: A- *E.faecalis* on Pfizer agar, B- *C.albicans* on SDA, C- *S.mutans* on MSB agar.

Confirmation by vitek 2 system

The clinical isolates were identified and confirmed at the species level by Vitek 2 compact system. The identification report of Vitek 2 system shows six confidence probability levels:

Excellent 96-99% Probability, Very Good 93-95% Probability, Good 89-92% Probability, Acceptable 85-88% Probability, Low Discrimination, and unidentified. The isolates with excellent probability were used in this study. Figure (3).

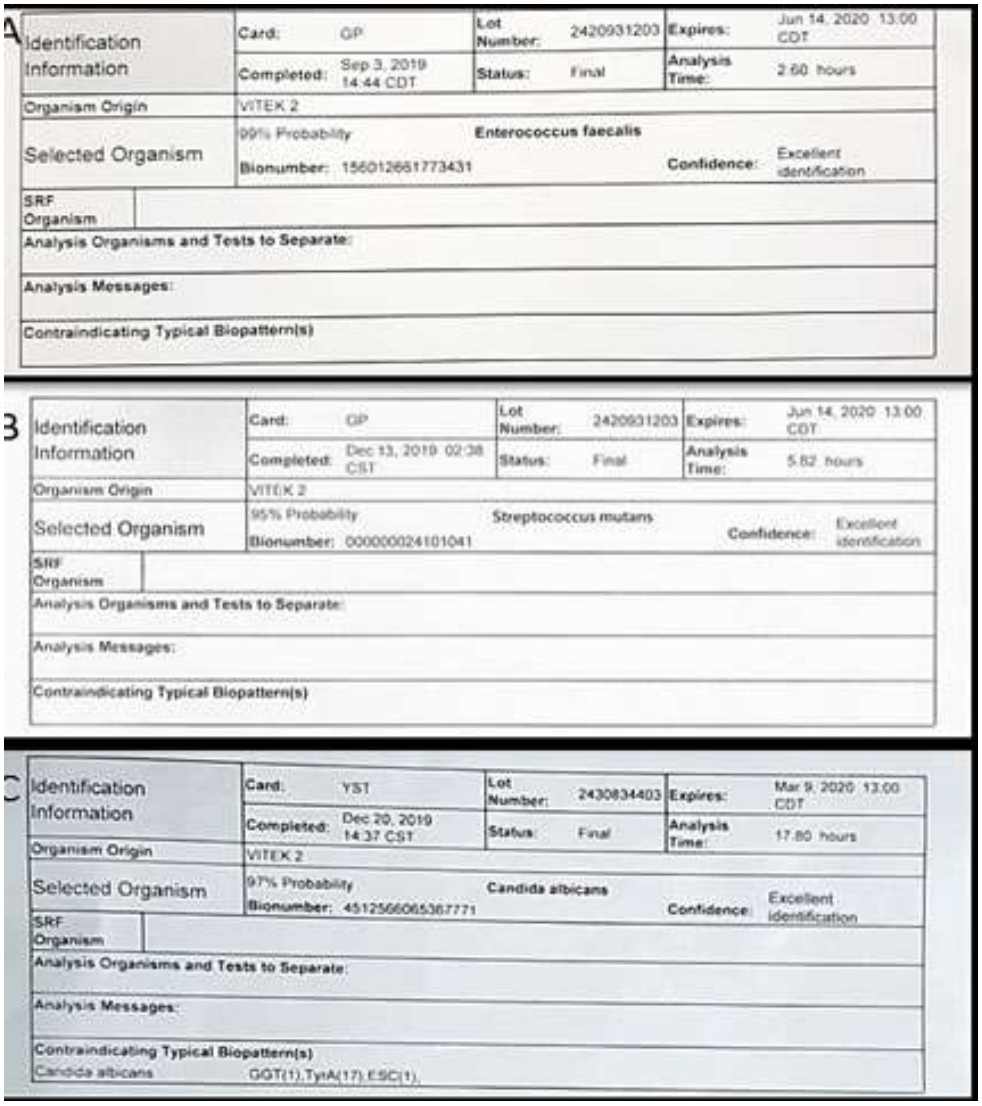

Figure 3: Vitek 2 identification reports, A. *E.faecalis*, B. *S.mutans*, C. *C.Albicans*

The sensitivity of the selected microorganisms to Chi-IONP

E.faecalis

Mean, maximum, minimum and standard deviation values of inhibition zones around each tested group were shown in table (1) and figure (4,5).

Table 1: Mean, maximum, minimum, SD values of inhibition zone *(E.faecalis).*

Groups	N	Minimum	maximum	Mean	Std. Deviation
NaOCl	10	23	28	26.4000	1.57762
Chi-IONP	10	24	28	26.1000	1.52388
A.A	10	0	0	0	0

NaOCl group shows the highest inhibition zone with slight differences from the Chi-IONP. In addition, 0.25% acetic acid (A. A) shows no effect on *E.faecalis* at all. In order to compare the results of NaOCl and Chi-IONP Independent sample t-test was done, and the result shows no significant differences between the groups (Table 2).

Table 2: Independent sample t-test to compare NaOCl and Chi-IONP groups *(E.faecalis)*.

Groups	Mean Difference	S.E Difference	P-value	Description
NaOCl and Chi-IONP	.30000	.69362	.671	NS

S.mutans

The descriptive statistics of inhibition zone around each tested group were listed in table (3) the NaOCl group showed the highest inhibition zone and the A.A group showed no zone at all. Figure (4, 5).

Table 3: Mean, maximum, minimum, SD values of inhibition zone *(S.mutans)*.

Groups	N	Minimum	Maximum	Mean	Std. Deviation
NaOCl	10	25	31	27.8000	1.81353
Chi-IONP	10	23	29	25.2000	1.98886
A.A	10	0	0	0	0

Independent sample t-test was done to compare means of NaOCl and Chi-IONP groups and the result showed high significant differences between the groups (Table (4).

Table 4: Independent samples t-test to compare control and Experimental groups *(S.mutans)*.

Groups	Mean Difference	S.E Difference	P-value	Description
NaOCl and EXP.	2.60000	.85114	.007	HS

C.albicans

The descriptive statistics of inhibition zone around each tested group were listed in table (5) the Chi-IONP group showed the highest inhibition zone and the A.A group shows no zone at all. Figure (4, 5).

Table 5: Mean, maximum, minimum, SD values of inhibition zone *(C.albicans*).

Groups	N	Minimum	maximum	Mean	Std. Deviation
NaOCl	10	20	28	25.5000	2.54951
Chi-IONP	10	27	33	29.3000	1.88856
A.A	10	0	0	0	0

Independent sample t-test was done to compare means of NaOCl and Chi-IONP groups and the result showed high significant differences between the groups (Table 6).

Table 6: Independent samples t-test to compare control and Experimental groups (C.albicans).

Groups	Mean Difference	S.E Difference	P-value	Description
NaOCl and Chi-IONP	3.80000	1.00333	.001	HS

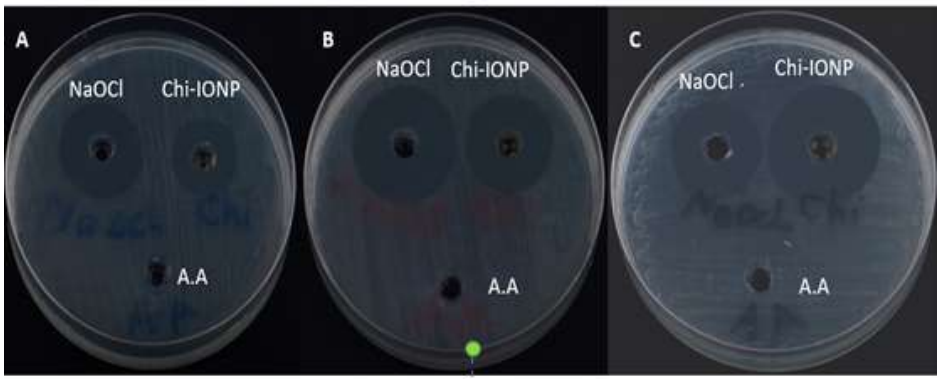

Figure 4: Inhibition zones of A, *E.faecalis*, B. *S.mutans*, and C. *Candida albicans*.

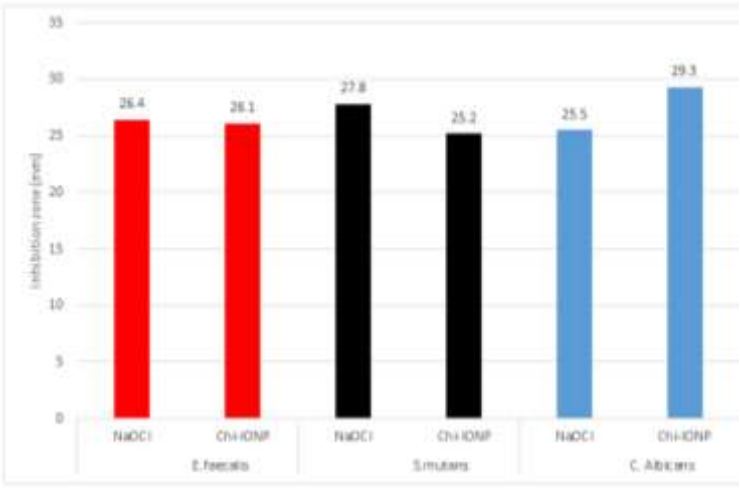

Figure 5: Means of Inhibition zones of *E.faecalis, S.mutans*, and Candida albicans.

Determination of MIC (minimum inhibitory concentration) and MBC /MFC (minimum bactericidal / Fungicidal concentration) of Chi-IONP

The MIC of Chi-IONP was 125 µg/ml, 250 µg/ml and 62.5 µg/ml for *E.faecalis, S.mutans, C.albicans* respectively.

The MBC/MFC of Chi-IONP was 250µg/ml, 500µg/ml and 125µg/ml for *E.faecalis, S.mutans, C.albicans* respectively.

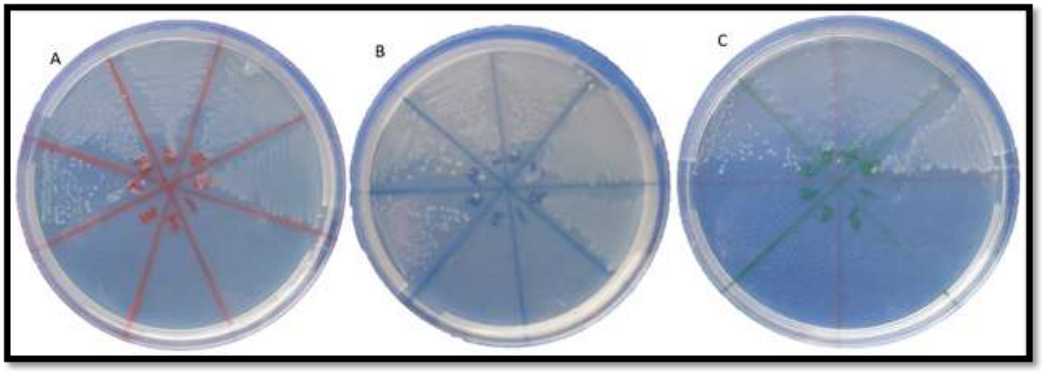

Figure 6: Subculture of microorganisms to determine MBC\MFC. *A. E.faecalis, B. S.mutans, C. C.albicans.*

DISCUSSION

The endodontic accounts to 40-50% of all oral diseases and there is a high but not complete success rate of treatment. [18].

The removal of all microorganisms from the infected root canal system is a very difficult procedure and the incomplete removal of these microorganisms is considered the main cause of failure of endodontic treatments.

The prime target of endodontic treatment is eradication of all microorganisms and their byproduct from the root canal system, by using antimicrobial agents in different ways to produce sterile root canal space [19-20].

The use of nanoparticles in root canal disinfection is increasing in recent years with very high efficiency than conventional disinfectants. This is mainly due to nanoscale size enabling the particles to penetrate deeply in dentinal tubules and for their broad-spectrum antibacterial activity [21-22].

For the antibacterial assessment of the tested material agar diffusion method was used which is a common method to evaluate the antibacterial activity of materials with accurate result and provide opportunity for direct comparison of the tested materials [21-23].

The presence of *E. faecalis, S.mutans and candida albicans* in infected root canal with apical periodontitis was confirmed in several previous studies [4-5-6].

Vitek 2 system was used widely to identify oral isolated microorganisms with considerable period of time for identification and high accurate results. [24-25-26].

Evaluation of the antimicrobial activity of Chi-IONP against *E. faecalis*

E.faecalis is the microorganism that can be isolated from endodontic infections and mostly isolated from failure endodontic cases. It can survive in deep area inside dentinal tubules under difficult conditions as high PH, resist acidity and conditions of scant available nutrients [27-28].

This bacterium is highly resistant to various antimicrobial agents, irrigants and medicaments that were used for endodontic treatment, especially when they penetrate deeply inside the dentinal tubules [29-30].

In the present study Chi-IONP group showed inhibition zone against *E.faecalis* comparable to 5.25% NaOCl irrigant with no statistical significant difference.

Several studies showed that 5.25% NaOCl is the most potent antibacterial agent against *E.faecalis* when compared with another endodontic irrigants such as chlorhexidine, Ethylene diamine tetra-acetic acid, nano- silver irrigants and herbal extracts [31 -32- 10- 30].

Chitosan is a biocompatible biopolymer with antibacterial activity that is widely known in medical and dental field. The antibacterial activity of nano-chitosan against *E.faecalis* was discussed in many studies all agreed on the nano chitosan is powerful antibacterial agent against *E.faecalis* [33-34-35].

Moukarab in 2020[36], made a comparison between nano-chitosan and NaOCl 5.25% as root canal irriginat and the author found that there is no significant difference in antibacterial action between the groups.

Evaluation of the antimicrobial activity of Chi-IONP against *S.mutans*

The result of the present study showed that *S.mutans* was more sensitive to NaOCl 5.25% with average inhibition zone 27.8 mm than Chi-IONP with average inhibition zone 25.2 mm.

The result of sensitivity of *S.mutans* to 5.25% NaOCl more than *E.faecalis* came in agreement with Mahdi et al., in 2018[10] and Darrag in 2013[37], who found that the activity of 5.25% NaOCl on *S.mutans* more than *E.faecalis.*

The result of antibacterial activity of Chi-IONP against S.mutans came in coincidence with several studies who tested the antibacterial activity of chitosan and nano-chitosan materials [38-39-40].

Moreover, the antibacterial activity of chitosan coated iron oxide nanoparticles against different bacterium and fungi other than *E.faecalis* and *S.mutans* was assessed in different studies [41-42].

Evaluation of the antimicrobial activity of Chi-IONP against *C.albicans*

The results of this study showed that *C.albicans* presented high sensitivity to Chi-IONP with average inhibition zone 29.3 mm more than the inhibition zone of 5.25% NaOCl that is 25.5 mm.

Several studies discussed the efficacy of 5.25% NaOCl against C.albicans and the results showed high antifungal activity of NaOCl [43-44-45].

Chitosan and nano-chitosan anti-fungal activity were discussed in several studies separately, and they concluded that the chitosan showed antifungal activity in different concentrations and different chemical forms [46-47].

Abdeltwab et al. [48], in 2019 studied the differences in antifungal activity of chitosan and nano-chitosan and they concluded that nano-chitosan was more effective to eradicate *C.albicans* than chitosan.

Interestingly, there is one study that assessed the anti-fungal effect of chitosan coated iron oxide nanoparticles against C.albicans which was by Nehra et al., in 2018 and the resulted inhibition zone was smaller than the present study. This difference may be due to differences in coating procedure, nanoparticle size and chitosan concentration.

The antibacterial mechanism of NaOCl could be due to chlorine and high PH. Chlorine which is a strong oxidant that inhibit bacterial enzyme such as irreversible oxidation of sulfhydryl group of essential bacterial enzyme. The high pH of NaOCl may affect the cytoplasmic membrane integrity of microorganisms [49].

Moreover, NaOCl acts as a solvent for organic tissue of the root canal system by neutralizing the amino acids [50-49].

Several studies reported that NaOCl causes severe tissue irritation accompanied by pain, swelling and paresthesia as a result of accident interaction with periradicular tissue [51-52].

Unfortunately, the antibacterial efficacy and ability to dissolve tissues of NaOCl is a prortionally related with its concentration which increases its toxicity [50-53].

Chitosan is biocompatible biopolymer derived from chitin. The antimicrobial mechanisms of chitosan were ambiguous. The most acceptable mechanism is that the positive charge of amine group in chitosan interact with negatively charged of bacterial cell wall causing rupture and leakage of cell components [54]. Another mechanism that is widely accepted is the penetration of bacterial cell wall by chitosan (especially low molecular weight chitosan) and binding to DNA and inhibit cell replication [55].

Kravanja et al.[56], in 2019 mentioned that the chelating effect of chitosan is another antibacterial mechanism of chitosan by interaction of chitosan with metal ions on the bacterial cell wall and inhibit the microbial growth.

The antimicrobial activity of chitosan increased with increasing degree of deacetylation, lowering the molecular weight, decreasing the size of particles and presence in acidic medium [56- 57- 58]. The nano size chitosan showed higher antifungal and antibacterial activity than macro sized chitosan [48-59].

Determination of MIC (minimum inhibitory concentration) and MBC /MFC (minimum bactericidal / Fungicidal concentration) of Chi-IONP

In the present study the results of MIC of Chi-IONP were 125 µg/ml, 250 µg/ml and 62.5 µg/ml for *E.faecalis, S.mutans, C.albicans* respectively.

The MBC/MFC of Chi-IONP were 250µg/ml, 500µg/ml and 125µg/ml for *E.faecalis, S.mutans* and *C.albicans* respectively.

There is no standard way to make a comparison to the chitosan MIC\MBC due to several differences in laboratory circumstances such as differences in methods followed to assess the MIC\MBC and differences in material properties as degree of acetylation, pH and molecular weight [56].

CONCLUSION

Chi-IONP was a powerful antimicrobial agent against the tested microorganisms, and this test results shows antifungal activity of Chi-IONP higher than NaOCl 5.25% and comparable antibacterial effect to NaOCl 5.25%.

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