The Antimicrobial Effect Of Ethanol And Methanol Silver Nanoparticle (AgNPs) Colloidal On Enterococcus Faecalis Isolated From Endodontic Infections In Najaf Provence. An In Vitro Study

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ABSTRACT

Enterococcus faecalis one of the most common causative agent of endodontic infections and this study aimed to investigate the antibacterial activity of silver nanoparticles (AgNPs) which prepared by ethanol, methanol, and ethanol-methanol v/v as a dispersing solutions. The 3-D AFM image of AgNPs had 55nm average particle size. The roughness average for AgNPs layer was 0.462 nm while the root mean square roughness was 0.57nm. Out of 80 patients with endodontic infections their ages ranging from 7-80 years only five (6.25%) E. faecalis isolated and the antibacterial activity result showed by disc diffusion methods, the best activity is for ethanol-methanol AgNPs colloidal with diameter of inhibition zone 20.8 ± 1.643 mm (Mean ± SD) which is significant compared with control treatment 13.6 ± 1.140 mm, while for ethanol AgNPs colloidal and methanol AgNPs colloidal are 15.4 ± 1.140 mm and 13.2 ± 0.836 mm compared with control treatment 10.6 ± 0.547 mm and 9.0 ± 0.707 mm respectively. The MIC of ethanol-methanol AgNPs colloidal is 20μg/ml and ethanol AgNPs colloidal and methanol AgNPs colloidal are 30, 60 μg/ml and for control treatment are 50, 60, and 80 respectively, all the result are significant in the level of p≤0.05. The result of antibiotic susceptibility showed that 80% of isolate are susceptible for Chloramphenicol, Carbenicillin and Ampiclox while its completely resistant for Metronidazole and Bacitracin.

KEYWORDS: silver nanoparticle, AgNPs, Enterococcus faecalis, endodontic infections, and antibacterial activity

How to cite this article: Al-Jameel DS, Ali Alsasam BM, Abdulridha WM (2020): The antimicrobial effect of ethanol and methanol silver nanoparticles (AgNPs) colloidal on Enterococcus faecalis isolated from endodontic infections in Najaf Province: An in vitro study, Ann Trop Med & Public Health; 23(S12): SP23 12021. DOI: http://doi.org/10.36295/ASRO.2020.231221

INTRODUCTION

Nanomaterial had a long list of applicability in improving human life and its environment. A bulk materials have constant physical properties irrespective of its size, but this is not true at the nanoscale. Several well-characterized bulk materials have been found to have most remarkable properties when studies in the nanoscale. There are many causes for this including the fact that nanoparticles have a very high ratio of surface to volume. Silver nanoparticles (AgNPs) have a most important biological properties as follows: they are effective bactericidal agents against bacteria broad spectrum, including antibiotic resistant strains1. Silver ions (Ag+) work against bacteria in a number of ways; Ag+ may interact with proteins that are necessary for the bacterial breathing and the enzyme thiol groups and the transfer of substances that are significant within the cell and across the cell membrane2. Ag+ may be attached to the bacterial cell wall and then changing the functions of bacterial cell membrane3. Also, Silver (Ag) can prevent enzymatic systems of the respiratory chain and vary DNA (deoxyribonucleic acid) synthesis4,5. Therefore: silver metal and its compounds are the effective in inhibiting bacterial infection of wounds6. The nature of most endodontic diseases is that its infectious, thus certain types of microorganisms causes most pathological changes in the periapical and pulp tissues. The fact that supports the evidence that is the normal oral microbiota is opportunistic and colonize the tissues and make an relationship which is symbiotic with host leading to periodontics and endodontic infections7,8. Biofilm formation is mediated the endodontic infection and treat these infection depends on remove the causative bacteria and elimination of biofilm formed in the root canal9,10. Endodontic treatment is challenged by resistant of several causative agents and one of the recalcitrant candidate is Enterococcus faecalis11. The ability of this bacteria to bind to dentinal tubule collagen and survive within the tubules lead to chronic failure of treatment endodontics12. The nutritional requirement for the growth of this bacteria and the minimal environmental condition needed to survive within root canal as amonoinfectious agent is very low13.

MATERIALS AND METHODS

Annals of Tropical Medicine & Public Health http://doi.org/10.36295/ASRO.2020.231221
This study achieved in the microbiology lab. In the dept. of basic science- college of dentistry/ Kufa university in Najaf city- Iraq. Over a period from January to May 2016, 80 swap sample collected from patients with endodontic infection attended to dental clinics in the university hospital and 40 sample from healthy persons as a control group.

**Identification of isolates:** Identification of Bacterial Isolates *E. faecalis* isolates were identified to the level of species using the traditional morphological and biochemical and genetic tests, according to the methods of 14-16.

**Preparation of AgNPs colloidal:** Silver nanoparticles (AgNPs) powder with CAS no. 7440-22-4, assay 99.0%, radius 50nm and spherical shape provided by EPRUI was dispersed in a solution contain of ethanol and methanol (1:1). A concentration of 0.0334mg/mL AgNPs fluid suspensions was prepared by dispersing AgNPs in the combination without any surfactant, suspension was homogenized by using sonication (GT Sonic, Germany) for 4 hours to overcome the aggregation of AgNPs. To facilitate the dispersion of AgNPs in the combination, the water of ultrasonic path was heated to 50°C.

**Physical Investigations of AgNPs colloidal:** The morphological properties of AgNPs were previously investigated at room temperature by means of Angstrom AA 3000 atomic force microscopy.

**Preparation of AgNPs dried filter paper discs:** Whatman filter paper no. 1 is used to prepare discs approximately 6 mm in diameter, which are placed in a Petri dish and sterilized in a hot air oven. Dispense 0.005 ml (5 microliter) of solution using sterile micropipette tips.

**Minimum inhibitory concentrations:** MIC have been achieved by the technique of micro broth dilution according to clinical laboratory standard guidelines 17. *E. faecalis* growth adjusted to 1×10^5 cell/ml then 10 μl of bacterial growth inoculated to the each of the 8 test tubes contain 100 μl of dilution of AgNPs ranging from 10-80 μg/ml.

**Antibiotic susceptibility test:** In vitro susceptibility tests were performed on Mueller-Hinton agar (Himedia, India) by the disk diffusion method as described by the Clinical and Laboratory Standards Institute (CLSI) standards. After streaking on Mueller-Hinton agar, the antibiotic disks was added after 15 minutes on the plates by sterile forceps. Then, the plates were incubated at 37°C for overnight (18-24 hours). The results were read according to CLSI standards. The antibiotics used in this study are: Clindamycin, (DA: 10μg), Oxacillin, (OX: 5μg), Nitrofurantoin, (F: 300μg), Chloramphenicol, (C: 10μg), Amoxicillin, (AX: 25μg), Carbencillin, (PY: 25μg), Metronidazole, (MET: 30μg), Lincomycin, (L: 10μg), Ampicillin and cloxacillin (APX: 25μg and 5 μg), and Bacitracin, (B: 10 units).

**Statistical analysis:** Statistical analysis was done by Statistical Package for Social Science (SPSS) version 25 and the variance was analyzed by general linear model by univariate, the result considered significant by using Post-Hoc LSD multiple comparison tests in the level of (p<0.05).

**Morphological analysis of AgNPs:** Ultrasound technique provide a very operative technique for minimized the size of particles and dispersing of nanoparticles in solution. This procedure produced a gray and homogenized suspension denoted to very good dispersion of AgNPs in a combination of methanol and ethanol after 4 hrs. of sonication at 50 O°C, the suspension may contain more separated AgNPs and a less number of agglomeration. The physical investigations of AgNPs revealed that, AgNPs fluid suspensions dropped on a glass substrate by drop casting method had a good a uniform surface homogeneity and gives a good indication for formation which has nanospikes with regular distribution of the AgNPs had 55nm average grain size. The roughness average for AgNPs layer was 0.462 nm while the root mean square roughness was 0.57nm (Figure 1).

**Bacterial isolate and identification:** Out of 80 endodontic infections patients only 5(6.25%) bacterial isolates identified as *E. faecalis* while 40 control sample show no totally no growth of *E. faecalis*. After identification of bacteria the five isolates used in the disc diffusion antibacterial activity of AgNPs and MIC as well to antibiotics susceptibility test.

**Antibacterial activity of AgNPs colloidal:** The result show that the ethanol-methanol AgNPs colloidal is the highest activity in inhibition of bacterial growth (20.8 ± 1.643) Mean ± SD, followed by ethanol AgNPs colloidal (15.4 ± 1.140) and the least activity by methanol AgNPs colloidal (13.2 ± 0.836), all the result show significant differences in the level of (p≤0.05) compared...
with control groups where the results are $13.6 \pm 1.140$, $10.6 \pm 0.547$, and $9.0 \pm 0.707$ for ethanol-methanol v/v, ethanol, and methanol respectively. All the results have been shown in table (1) and the figures (2,3,4).

Table 1: the antibacterial activity of silver nanoparticle by disc diffusion method where three solvent treatment ethanol, methanol, and mixed ethanol methanol v/v, all colloidal solvent applied on the growth of E. faecalis in the Mueller-Hinton agar compared with control treatment which contain solvent only without nanoparticle.

<table>
<thead>
<tr>
<th>Type of colloidal Solvent (nanoparticle)</th>
<th>Inhibition zone for nanoparticle treatment (mm)</th>
<th>Inhibition zone for control treatment (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Methanol (AgNPs)</td>
<td>$13.2 \pm 0.836^*$</td>
<td>$9.0 \pm 0.707$</td>
</tr>
<tr>
<td>Ethanol (AgNPs)</td>
<td>$15.4 \pm 1.140^*$</td>
<td>$10.6 \pm 0.547$</td>
</tr>
<tr>
<td>Ethanol - Methanol (AgNPs) v/v</td>
<td>$20.8 \pm 1.643^*$</td>
<td>$13.6 \pm 1.140$</td>
</tr>
</tbody>
</table>

* Result are significant in the level of (p<0.05) compared with control treatment.

Figure (2): Growth of E. faecalis in the Mueller-Hinton agar with two disc, the left disc loaded by methanol solution as a control, the right disc loaded with MethanolAgNPs colloidal. The diameter of inhibition zone of MethanolAgNPs colloidal loaded disc is higher than that of control treatment.

Figure (3): Growth of E. faecalis in the Mueller-Hinton agar with two disc, the left disc loaded by ethanol solution as a control, the right disc loaded with ethanol AgNPs colloidal. The diameter of inhibition zone of ethanol AgNPs colloidal loaded disc is higher than that of control treatment with growth of bacterial isolates.
Figure (4): Growth of *E. faecalis* in the Mueller-Hinton agar with two disc, the left disc loaded by ethanol-methanol AgNPs colloidal solution as a control, the right disc loaded with AgNPs colloidal. The diameter of inhibition zone of AgNPs colloidal loaded disc is higher than that of control treatment.

**Minimum inhibitory concentration test (MIC):** The results of MIC test are significant in the level of $(p \leq 0.05)$ where ethanol-methanol AgNPs colloidal is 20 μg/ml and ethanol AgNPs colloidal is 30 μg/ml and finally methanol AgNPs colloidal is 60 μg/ml compared with control treatments which are 50, 60, and 80 for ethanol-methanol v/v, ethanol, and methanol respectively.

Table 2: the MIC of silver nanoparticle by micro broth dilution technique where three solvent treatment ethanol, methanol, and mixed ethanol methanol v/v, all colloidal solvent applied on the growth of *E. faecalis* compared with control treatment which contain solvent only without nanoparticle.

<table>
<thead>
<tr>
<th>Type of colloidal Solvent (nanoparticle)</th>
<th>MIC for nanoparticle treatment μg/ml</th>
<th>MIC for control treatment μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol (AgNPs)</td>
<td>30*</td>
<td>60</td>
</tr>
<tr>
<td>Methanol (AgNPs)</td>
<td>60*</td>
<td>80</td>
</tr>
<tr>
<td>Ethanol - Methanol (AgNPs) v/v</td>
<td>20*</td>
<td>50</td>
</tr>
</tbody>
</table>

*Result are significant in the level of $(p<0.05)$ compared with control treatment.

**Antibiotics susceptibility test:** This test was achieved by disc diffusion method and aimed to investigate the susceptibility and resistant of AgNPs sensitive 5 isolates of *E. faecalis* for antibiotics and the results showed that Chloramphenicol, Carbenicillin and Ampiclox are the highest level of susceptibility with about (80%) of isolates then Amoxicillin (60%) and for Clindamycin, Nitrofurantoin, and Lincomycin which are (40%) then Oxacillin with (20%) susceptibility and finally Metronidazole and Bacitracin for which are all five isolates are completely resistant. All the levels of susceptibility and resistant are illustrated in figure (5).
The use of AgNPs for the biomedical purposes are increased according to their activity as a bactericidal and fungicidal. Further using of AgNPs is in the treatment of cancer, delivery vectors for drugs, theragnostic agents, and antibacterial agents. AgNPs used as endodontic sealers where have demonstrating significant antimicrobial effect against Staphylococcus aureus, Candida albicans, as well as Enterococcus faecalis. In this study the disc diffusion inhibitory diameter is 20.8 ± 1.643 mm and its significant compared with control treatment 13.6 ± 1.140 mm for Ethanol - Methanol AgNPs colloidal it’s may be according to the synergistic effect of alcohol and silver nanoparticle. The activity of AgNPs as antimicrobial endodontic infection is against resident bacteria and recurrent infections, so that it considered as a contributor in the therapy of endodontic infections. The combination of AgNPs with other nanoparticles like chitosan nanoparticle (CsNPs) have been used in the avoiding contamination in rate skin injuries for in vivo and in vitro tests.

In this study the result of MIC for Ethanol - Methanol AgNPs colloidal is 20 μg/ml and the MIC of AgNPs-dervied from Fusariumsemitectum against E. faecalis is 30 μg/ml while the MIC forAgNPs of fungal-derived for E. faecalis embedded in biofilm was 30 μg/ml. The mechanism of the AgNPs activity against bacteria is according to produce a wide area that contact with electrical charge, leading to penetration membranes and bacterial death. Another possible mechanisms of AgNPs as antimicrobial agents is two theories suggested to resolve the antimicrobial activity. First theory depends on silver as a soft acid, and naturally the acids react with basis, and in the bacterial cell the sulfur and phosphorus, both are soft basis and there is great tendency to react with AgNPs (soft acid) leading to salt formation resulting in death of bacterial cell. Interaction of AgNPs with phosphorus on the DNA leading to impaired replication for both DNA and microbes. The second theory depends on positive charge for silver ion, the electrostatic attraction between silver nanoparticles (positive charge) and microorganism cell membrane negatively charged and thus the microbe terminate. The AgNPs cytotoxicity have been reported in animal models for kidney, liver, small intestine, and brain followed by oral exposition.

CONCLUSION
The antibacterial activity of silver nanoparticle against E. faecalis is best by using combination of ethanol – methanol v/v as adespresing solution furthermore it can be used as antibacterial agent in industrial dental product and for medication industry.

CONFLICTS OF INTEREST
The authors declare that there are no conflicts of interest.

FUNDING
None

ACKNOWLEDGMENTS
All the diagnosis of bacterial isolates supported by the microbiology lab / College of Dentistry / university of kufa.

AUTHORS' CONTRIBUTION
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

DATA AVAILABILITY
Annals of Tropical Medicine & Public Health  http://doi.org/10.36295/ASRO.2020.231221
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES


