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Anti-biofilm Activity of Zn oxide Nanoparticles Against *Enterococcus faecalis*

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Abstract

Biofilms of bacteria are generally formed due to the activity of bacterial virulence factors, which are one of the most important virulence factors for pathogenic bacteria by helping bacteria to communicate between certain types of bacteria, not only between different genera, but even between prokaryotic cells and eukaryotic cells. Thus, they work to regulate bacterial populations naturally in a specific environmental location. The mechanism used in our current research to combat *Enterococcus faecalis* using zinc oxide nanoparticles prepared physically and which was confirmed through (XRD, SEM, Scherer Equation, and FTIR tests), where it gave an effective result at concentrations (50, 100, 150, 200 mg/ml). Biofilm formation of *Enterococcus faecalis* isolates showed 12% non, 20% weakly, 36% moderate, and 32% strongly biofilm production. Almost number of antibiotics failed to combat multidrug-resistant biofilm-forming bacterial infections, nano products were used to kill bacteria and prevent their growth. Zinc oxide nanoparticles were used to kill *Enterococcus faecalis* and prevent its growth, zinc oxide nanoparticles are not cytotoxic and do not have any harmful effects on human health if used to combat bacteria in any way according to previously conducted laboratory experiments.

Keywords: Anti-biofilm, Zn oxide Nanoparticles, Enterococcus faecalis

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فعالية جزيئات اوكسيد الزنك النانوية ضد بكتريا *Enterococcus faecalis*

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المستخلص

تتكون الأغشية الحيوية للبكتيريا بشكل عام نتيجة لنشاط عوامل الضراوة البكتيرية، والتي تُعد من أهم عوامل الضراوة للبكتيريا المسببة للأمراض، وذلك من خلال مساعدتها على التواصل بين أنواع معينة من البكتيريا، ليس فقط بين الأجناس المختلفة، بل حتى بين الخلايا بدائية النواة والخلايا حقيقية النواة .وبالتالي، تعمل على تنظيم التجمعات البكتيرية بشكل طبيعي في موقع بيئي محدد. ان الآلية المستخدمة في بحثنا الحالي لمكافحة *Enterococcus faecalis*هي استخدام جسيمات أكسيد الزنك النانوية المُحضرة فيزيائيًا، و التي تم تأكيدها من خلال اختبارات (XRD و SEM ومعادلة Scherer Equation و FTIR)، حيث أعطت نتائج فعالة عند تراكيز (50، 100، 150، 200 ملغم/مل).

أظهر تكوين الأغشية الحيوية لعزلات *Enterococcus faecalis* (عدم انتاج بنسبة 12%، وضعيف بنسبة 20%، ومتوسطًا بنسبة 36%، وقويًا بنسبة 32 %) ، فشلت العديد من المضادات الحيوية تقريبًا في مكافحة الالتهابات البكتيرية المقاومة للأدوية المتعددة و المُكوِّنة للأغشية الحيوية، لذا استُخدمت منتجات نانوية لقتل البكتيريا ومنع نموها. و استُخدمت في بحثنا الحالي جسيمات أكسيد الزنك النانوية لقتل بكتيريا المكورات المعوية البرازية *Enterococcus faecalis* و منع نموها، علمًا بأن جسيمات أكسيد الزنك النانوية ليست لها اي تأثيرات سمية للخلايا و لا تُسبب أي آثار ضارة على صحة الإنسان عند استخدامها لمكافحة البكتيريا بأي شكل من الأشكال، وذلك وفقًا لتجارب مختبرية أُجريت سابقًا.

الكلمات المفتاحية: مضاد للأغشية الحيوية، جسيمات أكسيد الزنك النانوية، المكورات المعوية البرازية

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معلومات البحث
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1. Introduction

Enterococci are opportunistic bacteria that cause infections in individuals with substantial underlying conditions, such as urinary tract infections, endocarditis, and wound infections [1]. *Enterococcus faecalis* has been commonly identified in re-infected, root canal-treated teeth, with prevalence rates ranging from 30% to 90% of cases. Teeth with re-infected root canals are approximately nine times more likely to include *Enterococcus faecalis* compared to those with original infections [2]. Clinical isolates of *enterococci* demonstrate resistance to many frequently employed antimicrobial agents, hence providing a selective advantage in healthcare environments [3]. The inherent antibiotic resistance of *enterococci*, along with their propensity for acquiring and spreading genetically mobile antibiotic resistance components, poses significant obstacles to the management of *enterococcal* infections. Moreover, the capacity of these bacteria to build biofilms enhances their resistance to antibiotics. Various processes have been identified as contributing to increased biofilm resistance to antibiotics, including reduced drug penetration, antibiotic sequestration, and the existence of persister cells. *Enterococci* are frequently present in cats, while canines act as reservoirs for the transmission of these bacteria to humans [4, 5, 6]. The indiscriminate and excessive application of antibacterial agents has led to an increase in drug-resistant *Enterococcus faecalis* strains, leaving the majority of treatments ineffective against this pathogen. Nanoparticles (NPs) are progressively utilized as substitutes for antibiotics, given that zinc oxide (ZnO) has antibacterial efficacy against both Gram-positive and Gram-negative bacteria [7, 8]. Recent research indicate advancements in the application of ZnO nanoparticles as agents against biofilm formation and bacterial growth, yielding a synergistic effect. Investigations into the mechanism of action of ZnO nanoparticles suggest that their enhanced antibacterial efficacy may stem from their capacity to eliminate *Enterococcus faecalis* [9].

2. Materials and Methods

2.1. Materials

X-ray diffraction (XRD)\Broker-Germany, Autoclave\Labtech-Korea, Balance (electrical)\Denver-Canada, Centrifuge\Hitachi-Japan, Digital camera\Sony-Japan, Distillator\GFL-Germany, Fourier Transform Infrared (FT-IR)\Perkin-Elmer 1725x Japan, bulk material of ZnO nanoparticles, Hood\Bio Lab-Korea, (Incubator , Oven)\Memmert-Germany, Gram stains, Light Microscope\Olympus-Japan, Micro and cooling centrifuge\Hermle Labortechnik-Germany, ELISA system\Beekman-Austria, Micropipette\Eppendrof-Germany, pH meter\Orient-USA, Refrigerator\Beko-Korea, Scanning electron microscope\FEI-Netherland, Shaking incubater\Gallenkamp-England, Thermocycler\BioRad-USA, Vortex mixer\Thermolyne-USA, Eppendorf tubes, brain heart broth, Pfizer-specific *Enterococcus* media, 3% H2O2, DNA mini extraction kit, PCR primers, Muller Hinton Agar-Lab /USA, Phosphate Buffer Saline (PBS), Hydrochloric acid (0.25 M), Sodium Hydroxide (NaOH) Solution (1M), D.W, Ethanol.

2.2. Synthesis of ZnO Nanoparticles

ZnO nanoparticles were synthesised using laser ablation of bulk metal, employing methodologies validated by prior research, which successfully produced spherical ZnO nanoparticles with dimensions between 30 nm and 60 nm in an aqueous NaOH solution. The laser ablation technique possesses distinct advantages, facilitating the manufacture of uniformly distributed zinc oxide nanoparticles with constrained shape, size, and purity. The ablation duration and laser wavelength substantially influence the purity and characteristics of zinc oxide nanoparticles [10]. High-intensity laser irradiation of bulk zinc in solution yields zinc oxide nanoparticles characterised by elevated chemical purity and precision in size and morphology.

2.2.1. X ray diffraction of zinc oxide nanoparticles

A significant number of researchers utilise X-ray diffraction (XRD) as an analytical technique for elemental analysis and chemical characterization of materials. The process depends on how an X-ray source and a certain item interact with each other. The atomic makeup of each element is different, which means that each element's electromagnetic emission spectrum has a different set of peaks. This gives the phenomenon its traits and abilities [11], as shown in figure no.1, which shows the ZnO nanoparticles' X-ray diffraction (XRD) pattern.

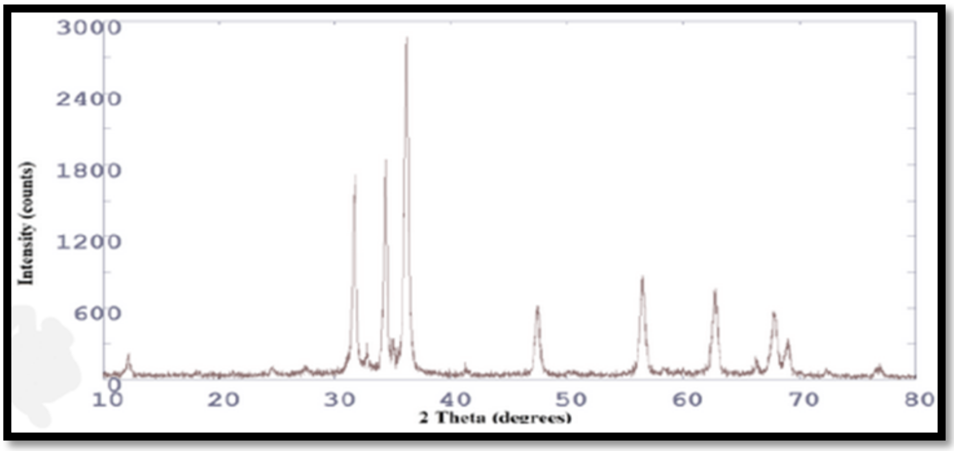

Figure (1): X ray diffraction of zinc oxide nanoparticles

The use of the Scherrer Equation, as depicted in figure no.2 was to calculate the average of ZnO nanoparticles size by analyze of the peak amount in X-ray diffraction (XRD) picture.

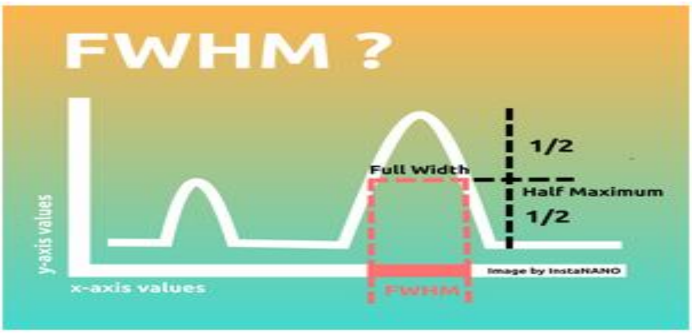

Figure (2): XRD Crystallite (grain) ZnO nanoparticle size Calculator (Scherrer Equation)

https://instanano.com/all/characterization/xrd/crystallite-size

2.2.2. Scanning Electron Microscope (SEM) analysis of ZnO nanoparticle

Spherical ZnO nanoparticles were identified using scanning electron microscopy (SEM) analysis, with a size range of 30 to 60 nm. In Scanning Electron Microscopy (SEM), as depicted in figure 3, it is crucial for a specimen to be entirely dry prior to analysis, due to the specimen chamber operating under high vacuum conditions. Nonetheless, the examination of living cells, tissues, and entire soft-bodied organisms necessitates chemical fixation to maintain and stabilize their structural integrity [12]. as show figure (3).

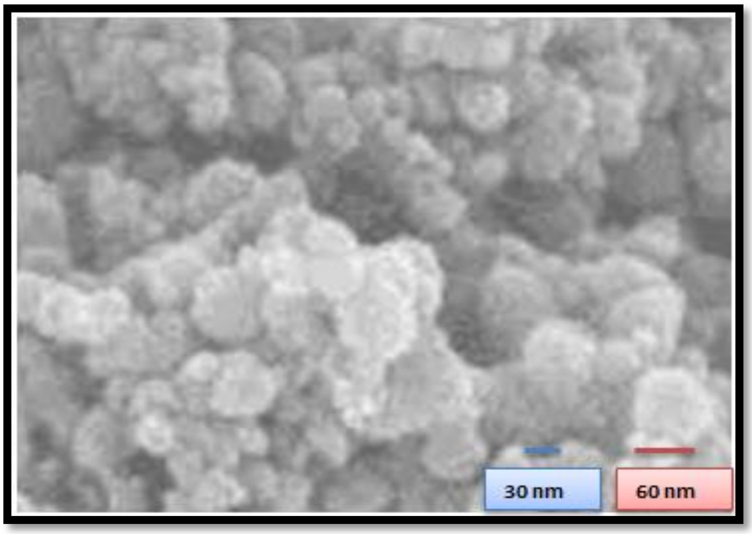

Figure (3): Scanning Electron Microscope (SEM) of ZnO nanoparticles (particle size ranging between 30nm to 60nm)

3. Isolation and Identification of Bacteria

3.1. Collection of Samples

From September 2023 to April 2024, twenty root canal specimens were collected when patients visited dental clinics in the Kerbala governorate. Using clean paper points, each sample was taken out and put into a sterile Eppendorf tube with 1 ml of sterile brain heart broth, then transported to the microbiology laboratory for examination.

3.2. Bacterial culture and microscopic examination

Samples were put in-to Pfizer-specific *Enterococcus* media, which is a medium that only grows *Enterococci spp*., and left to grow without oxygen at 37°C for 24 hours. The shapes, sizes, and colors of the separates were carefully studied .

To prove the identity of the isolated *Enterococcus* spp., biochemical tests were done on a single pure colony. The isolates that had been dyed with gram stain were looked at under a light microscope to find out their shape, how well they stained, and other details.

3.3. Biochemical Tests

Bacteria were moved from Pfizer selective *Enterococcus* plates to a glass slide, and a drop of 3% H2O2 was added to several colonies. Quick effervescence shows that the results were good. For the growth test at 15°C and 40°C, bacterial isolates were kept in Brain Heart Infusion broth without oxygen for 24 hours at each temperature, growth was considered a positive finding [13, 14].

3.4. Molecular detection:

For bacterial isolates, a molecular method was used. Following the manufacturer's directions, a small extraction kit was used to get genomic DNA from pure bacterial colonies. The bacterial DNA was then amplified using polymerase chain reaction (PCR). Primers F: TCAAGTACAGTTAGTCTTTATTAG and R: ACGATTCAAAGCTAACTGAATCAGT were used for this work. They made a product size of 940 bp. These primers came in a lyophilized form. They were reconstituted in clean deionised distilled water according to the manufacturer's instructions to make a final concentration of 100 pmol/µl. They were then put in a deep freezer until they were used for PCR amplification [14].

3.5. Biofilm formation detection

We tested how well *Enterococcus faecalis* isolates could make biofilm using the crystal violet staining method in Styrofoam microtiter plates. 490 nm was used to measure the optical density (OD). The *Enterococcus faecalis* culture that was grown overnight was sub-cultured in Tryptic Soy Broth (TSB) that had 1% glucose added to it and kept at 37°C for 18 hours. The growth was slowed down until it reached 1.5 x 10^8 CFU/ml after being incubated. Each bacterial strain suspension of 200 microlitres was put into its own well on a 96-well polystyrene plate with a flat bottom. For 24 hours, the plate was kept at 37°C. After that, 200 µl of clean phosphate-buffered saline (pH 7.2) was used three times to rinse the wells. After heating a biofilm at 60°C for 15 minutes and letting it set, 200 µl of a 0.1% (wt/vol) crystal violet solution was added to each well and left to work for another 15 minutes. Rinsing with pure water got rid of the extra crystal violet, and the paper was left to dry overnight. When 200 µl of 96% ethanol was added, the bound crystal violet was freed [15]. Spectrophotometry was used to measure absorbance at 630 nm (A630), which was directly linked to biofilm growth. Only TSB was in the negative control well. The test for biofilm development was done according to these rules:

- < OD Non
- ODc < ODt < 2ODc Weak
- 2ODc < ODt < 4ODc Moderate
- 4ODc < ODt High

4. Results : as show table 1,2,3,4,5.

Table (1): The result of applied Scherrer Equation is equal to (47.94 n.m), in the link

Scherrer Equation Details	Amount (measurements)
Peak Position (2θ)	**1300**
FWHM (2θ)	**0.5**
X-Ray Wavelenth	**0.15418**
Result	**47.94 nm**

The measurement of ZnO nanoparticle diameters from SEM analysis indicates no significant size variations, demonstrating normality within the natural nanoparticle range, which is conducive to penetrating bacterial cell walls.

Table (2): The size of ZnO nanoparticles was determined using (SEM)

Diameter of ZnO nanoparticles n.m .
40.25
57.53
44.36
53.64
34.72
47.38
32.45
46.74

Table (3): Show content of PCR tube

Chemical compound	Volume
Master mix	5 µ l
DNA	3 µ l
Sense primer	1 µ l
Antisense primer	1 µ l
Deionizer D.W	10 µl
Total	20 µl

Table (4): PCR condition for genetic detection

Step	Temperature C°	Time mine	Cycles
Initial	94	5	1

Denaturation	94	4	30
Annealing	56	1	30
Elongation	72	2	30
Final Elongation	72	7	1

Table (5): Biofilm formation of *Enterococcus faecalis*

Biofilm formation	Percentage
Non	12%
Weak	20%
Moderate	36%
Strong	32%

4. Discussion

Enterococcus faecalis isolates showed positive growth on Pfizer selective agar, showing up as round, grey colonies 2 mm in diameter with a black spot in the middle and black zones around it. All of the isolates were looked at under a microscope. Figure no.4 shows that the isolate had Gram-positive ovoid or spherical cells grouped alone, in pairs, or in short chains. Along with that, the bacteria isolates did not react with catalase and were able to grow in 6.5% NaCl, at pH 9.6, and at temperatures between 10 and 45ºC. as show figure (4).

Figure (4): *Enterococcus* under microscope as Gram positive cocci

Enterococcus faecalis was identified at the molecular level using a PCR test. Gene confirmation was achieved using 1.5% agarose gel electrophoresis that was stained with ethidium bromide and run at 75 volts. For fifty minutes and were shot with a UV light source [16]. The results showed that *Enterococcus faecalis* gene bonds found a 940 bp area when compared to the DNA ladder, as show figure (5).

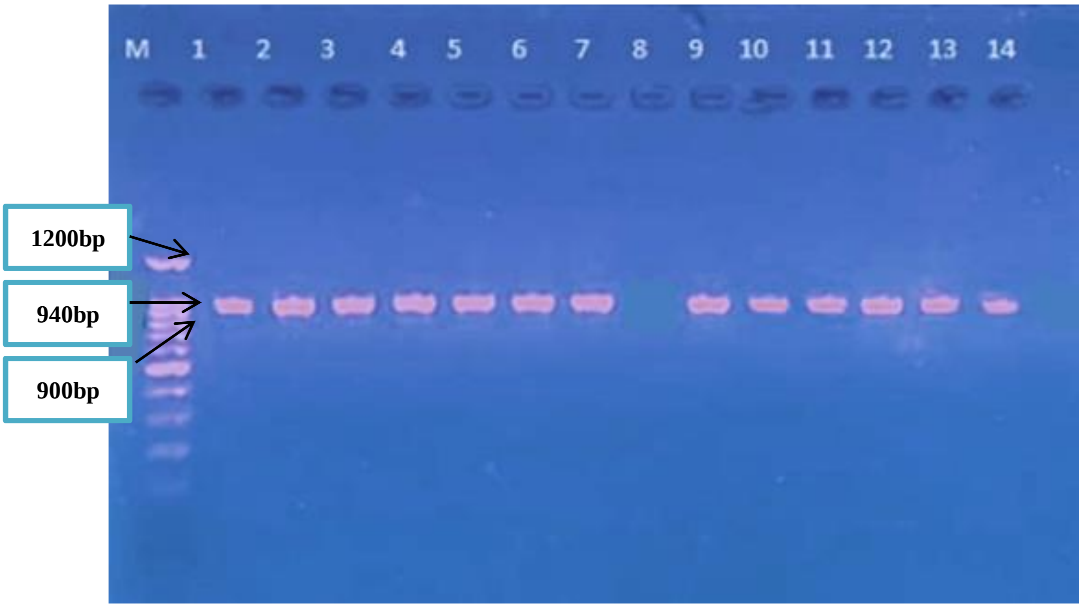

Figure (5): Agarose gel electrophoresis of a specific PCR product measuring 940 bp was conducted using 1.5% agarose gel at 90 V for 1 hour in 1x TBE buffer, followed by visualization under a transilluminator with UV light after staining with Red Safe. Lane L:bp DNA ladder

The capacity of *Enterococcus faecalis* isolates to produce biofilm was assessed using pre-sterilized 96-well polystyrene micro-titer plates, with absorbance measured at 630 nm to quantify the extent of biofilm formation by the isolates adhering to the micro-titer well surface. The isolates of *Enterococcus faecalis* exhibited varying capacities to produce biofilm under identical experimental settings. Approximately 12% of the evaluated isolates exhibited no biofilm formation, 20% had weak biofilm production, 36% displayed moderate production, and 32% were classified as high biofilm producers. This study demonstrates considerable differences in the biofilm production capability of *Enterococcus faecalis*, as illustrated in figure 6. given that *Enterococcus faecalis* exhibits significant resistance to numerous antibiotics, alternative therapeutic options must be considered [17].

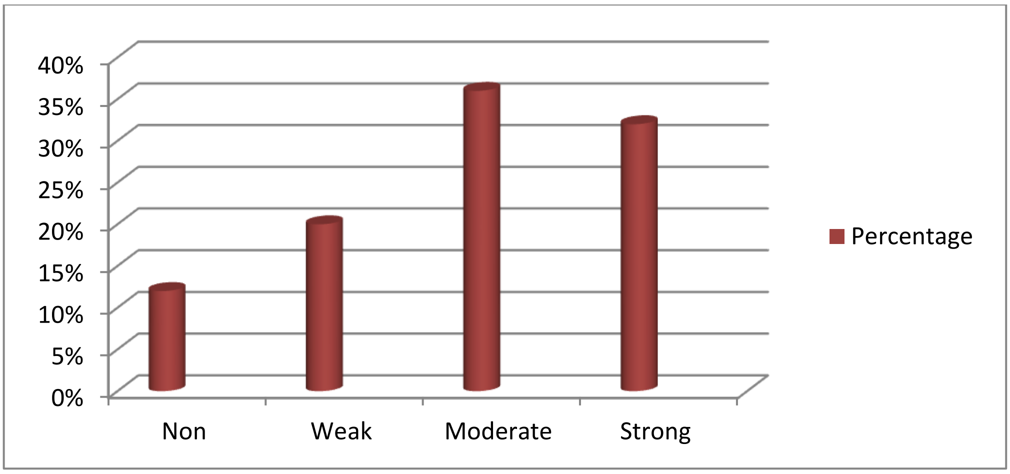

Figure no.6: different levels of biofilm formation from *Enterococcus faecalis* isolates

Among the most significant therapeutic possibilities are nanoparticles, particularly zinc nanoparticles. Zinc oxide nanoparticles have been examined for several biological applications owing to their reduced toxicity to humans [18, 19]. Zinc oxide nanoparticles exhibit antibacterial properties against several pathogenic microorganisms in humans and animals [20].

The zinc oxide nanoparticles produced during the manufacturing process have specifications that enable them to work as an anti-biofilm. The average of zinc oxide nanoparticles size (47.94 nm) that shown in table no.1 as a result in (Scherrer Equation) calculation, and their round shape that sown in figure no.3 may be one of the reasons that enable them to prevent the growth of bacteria and to combat the biofilms produced by *Enterococcus faecalis*.

The use of zinc oxide nanoparticles as an antibacterial agent that prevents the production of biofilms in the study is at different concentrations estimated at (50, 100, 150, 200 mg/ml) respectively. The concentration of (200 mg/ml) recorded the highest value that can be used as an anti-biofilm, followed by the concentration of (150 mg/ml), then comes the concentration of (100 mg/ml) with the least effect, while the concentration of (50 mg/ml) has no effect on preventing the growth of bacteria or the production of biofilms, as shown in the figure (7).

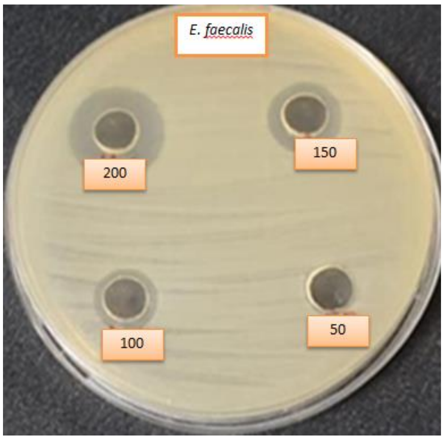

Figure (7): Zone of inhibition on *Enterococcus faecalis* cultured, that affected by zinc oxide nanoparticles

Kishen et al. [21] demonstrated the antibacterial efficacy of zinc oxide nanoparticles against *Enterococcus faecalis* solution. Shrestha et al. [2] assessed zinc oxide nanoparticles on both biofilm and planktonic *Enterococcus faecalis*, demonstrating bacterial eradication in suspension and a reduction in biofilm thickness, consistent with the findings of this work.

Conclusion

The current research indicates that nanoparticles can serve as an antibacterial agent, effectively inhibiting the growth of *Enterococcus faecalis* and preventing biofilm formation, a critical virulence factor utilized by bacteria to infect hosts, whether in the root canal or other body sites. Biofilm formation significantly enhances bacterial activity and can lead to several problems and associated diseases that are challenging to manage. Due to bacteria's significant resistance to most antibiotics, the application of zinc nanoparticles may effectively inhibit bacterial proliferation or eradicate them as an antibacterial agent, given its role as an anti-biofilm against the virulence factors produced by bacteria.

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