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ORIGINAL ARTICLE

Evaluation in Vitro and in Vivo Toxicity of Synthesized Zinc Oxide Nanoparticles Against Streptococcus Oralis

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ABSTRACT
In the current study, an antibacterial activity of synthesized zinc oxide nanoparticle (ZnO NPs) at different concentrations (16,32,64,256,512) µg/ml has been evaluated in vitro (well- diffusion method) against Streptococcus spp. that cause dental caries. The results demonstrated that ZnO had anti-Streptococcus activity especially at 64 µg/ml. Moreover, several methods have been performed such as EDS, atomic force microscope (AFM) to characterize ZnO NPs features such as EDS, atomic force microscope (AFM), X-ray diffraction (XRD), and TEM to ensure the features and size of ZnO nanoparticles. These techniques revealed that the average size of ZnO was about 30.52nm with hexagonal shape, and the UV-visible results demonstrated the big was determined at 340.8 mm, and the function of the ZnO NPs caused toxicity with inside the IC50 values of the HdFn cells. These data proved the biological activity of ZnO NPs against Gram-positive pathogens with less toxic effect on liver and spleen cells of rats.
Keywords: Zinc oxide nanoparticles, Streptococcus oralis , HdFn cells, Liver cells

INTRODUCTION
Nano scale substances regularly exhibit conduct that is intermediate among that of microscopic solid and that of an atomic or molecular system [1]. Zinc oxide nanoparticles (ZnO NPS), as one of the maximum crucial metal oxide nanoparticles, are popularly used in multidisciplinary fields because of their strange physical and chemical properties [2]. Last decades, ZnO NPs have emerged as one of the maximum famous metal nanoparticles in biological field because of their terrific biocompatibility, economic, and weak toxicity. ZnO NPs have emerged a promising capability in biomedicine, specifically within of anticancer and antibacterial fields [3]. Dental caries: the expression of dental caries used to explain results, signal and signs of localization chemical dissolution of the teeth surfuce because of metabolic activities taking place in biofilm coating the affected area. The destruction process can have a potential effect on enamel, dentin and cement [4]. Nanoparticle toxicology an emergent field thatworks about setting up the danger of nanoparticles, and consequently their potential risk, in view of the increased use and probability of exposure [5]. Nanotoxicology is rising as a significant subdiscipline of nanotechnology. It indicates to the consideration of the interactions of nanostructures with biological system with the emphasis on elucidating the connection between the physical and chemical properties (e.g., size, shape, surface chemistry, composition, and aggregation) of nanostructures with induction of toxic biological response [6]. Candida albicans that is a yeast grows certainly in the human body, lives within the gastrointestinal tract, respiratory tract, female reproductive tract and at the skin [7].

METHODS
Isolation and Identification of S.oralis: Streptococcus. oralis was isolated from patients that had dental carries that and presented to the College of Science/University of Baghdad. S. oralis was identified by biochemical methods and confirmed by VITIC 2 system. Bacterial isolate was incubated into both (Blood and Mitis Salivarius agar), incubated under anaerobic conditions, and differentiated from other bacteria via biochemical reactions.
Preparation of stock solution of Zn.2H2O(CH3CO2)2: A stock solution of Zinc acetate Zn.2H2O(CH3CO2)2 was prepared by dissolve 0.01gram of Zn.2H2O(CH3CO2)2 in 50 milliliter deionized water.
Zn NPS biosynthesis: To synthesis zinc oxide nanoparticles, (7 ml) of the Streptococcus oralis filtrate was added to 3 ml of 1 mM Zn.2H2O(CH3CO2)2 (512) at room temperature 37C° [8].
MIC determination: Double serial dilutions of ZnONPS were prepared by using MHB, and dilution at (16, 32, 64, 256, 512) µg/ml was added to different tubes of bacterial broth of S. oralis and incubated for 24 hours at 37° C.. Figure 1 showed steps of zinc oxide nanoparticles synthesis by biological method.

Figure 1: A(bacteria activation in broth), B (countergauge bacteria to obtain suspension and remove cell), C (different concentration of ZnONPs)

Different techniques that have been used:-
1 UV visible absorption spectroscopy
2 X-Ray diffraction test (XRD)
3 Atomic force microscopy (AFM)
4 Transsmtion electron microscopy (TEM)
5 Scanning electron microscopy (SEM)
6 Energy dispersive X-Ray spectrometry (EDS)

Activity of ZnO NPs in vitro: Well diffusion assay (WDA) has been used to evaluate the biological activity of ZnO NPs in vitro. Serial concentrations of ZnO NPs were used (16, 32, 64, 256, 512) µg/ml to evaluate the minimal inhibitory concentration (MIC) of ZnO NPs against S. oralis growth that has been cultured on Muller Hinton agar at 37 ° C. Then, the inhibitory zone was measured after 48 hrs. [9].
Cytotoxicity assay:
Cell Line Maintenance: When the cells withinside the vessel shaped confluent monolayer, The normal fibroblast cells and HdFn cells from human colon through the usage of microtiter plates at a cocentration of range (0.5-2.5) mg ml. [10]
MTT Protocol (11): The cytotoxic impact of ZnO NPs synthesis against S. oralis was executed through the usage of MTT Kit.
Animal study: To evalute whether ZnONPs has toxic effect on body tissue, histopathological study was perforemed on albino male mice, weighing (20 -25)g and aged (5-6) weeks, that had been received and housed in plastic cages in the animal house of the Baghdad Research Center, University of Baghdad for 7 days acclimation period. . Mice received intraperitionelly 0.1 ml volume of zinc oxide nanoparticles at does 64µg/ml for 7 Days. After that, all mice were sacrificed by diethyl ether and samples of liver and spleen were collected . and stored in 10% formalin.

RESULTS
Bacterial isolates: Bacterial samples have been streaked on selective Mitis Salivarius Agar to obtaine bacterial colonies of oral S. oralisunder anaerobic conditions (jar under co_2 at 37°C for 24 hrs). Batcerial idrentification was confirmed optochin sensitivity and by Vitech 2 system [10] shown in figure below

Fig 2: S.oralis growth on Mitis Salivarius Agar at 37c anaerobic jar for 24hrs

It has been taken in consideration the first time to apply S.oralis in preparation to synthesize of zinc oxide nanoparticles . The research of the formation of nanoparticles through turning the coloration from yellow to brown, After several times of centrifugation and washing steps , the final precipitate has changed into a brown color after drying the product in microwave to obtain nanopartical powder , as has been previously achieved by (11) shown in figure below.

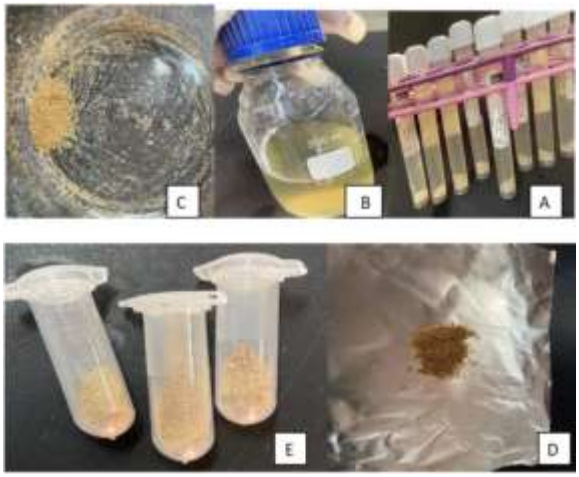

Figure 3: ZnONPs solution biosynthesized by S.oralis A: Zinc acetate solution with S.oralis before synthesis. B: Zinc acetate with S.oralis after synthesis(after 72hrs). C: Synthesis ZnONPs as precipitate white particles. D: After centrifugation. E: After preparation of ZnONPs as a powder.

UV–Vis Spectral Analysis: The Uv-vis spectrophotometer was the step to characterize the biosynthesized ZnONPs. The results confirmed that biosynthesized ZnONPs exhibited a maximum peak at (294nm) nm as shown in figure (4).

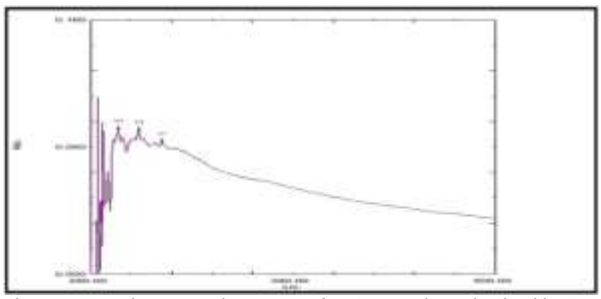

Figure 4: UV–Vis spectrophotometry of ZnO NPs Biosynthesized by S. oralis

X-ray Diffraction (XRD): The XRD spectra of ZnO nanoparticle powder, was shown in figure (8),

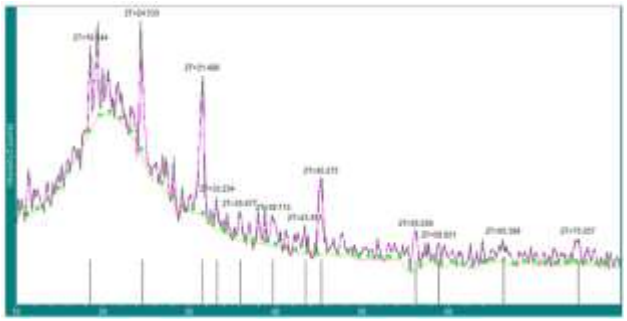

Figure 8: XRD analysis of synthesized ZnO NPs

(AFM) analysis: The atomic force microscopy has been used as a confirmatory technique to characterize the biosynthesis of ZnONPs through detecting their average diameter which similarly to the morphology in each 2D and 3D. The outcomes achieved in this study confirmed that the biosynthesized ZnONPs via S. oralis had average size of 24nm as proven in table and figure (5).

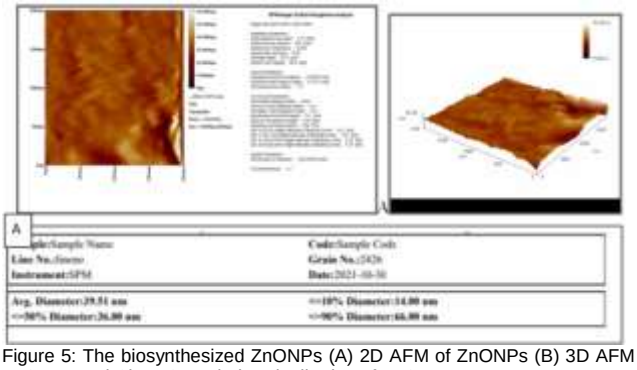

Figure 5: The biosynthesized ZnONPs (A) 2D AFM of ZnONPs (B) 3D AFM ZnONPs and Chart Granularity Distribution of ZnONPs.

Transmission Electron Microscopy analysis (TEM) of ZnO NPs: The TEM micrographs showed the spherical to hexagonal shape of the ZnO NPS as proven in figure (6). The diameter of the nanoparticles turned into measured to be 24 nm. From the TEM images, shown figure below.

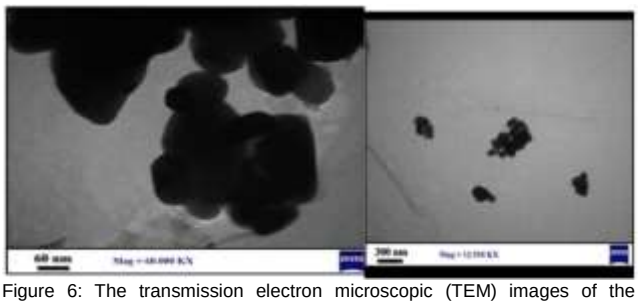

Figure 6: The transmission electron microscopic (TEM) images of the ZnONPs

Scanning Electron Microscope (SEM): The biosynthesized ZnONPs has been tested under the SEM to confirm the expected size and morphology of ZnO NPs shown in figure (7).

Energy dispersive X-Ray spectrometry (EDX): Figure below shown EDX analysis for the nanoparticle which emphasis that the product is ZnO .

Minimum Inhibitory Concentration (MIC) of Zinc Oxide nanoparticles: Well diffusion method was used to detect the

activity of Zinc Oxide nanoparticles against (Streptococcus spp). ZnO NPs at different concentrations (512, 256, 128, 64,32 and 16)µg/ml showed antimicrobial activity and inhibition zone reached (14) mm.

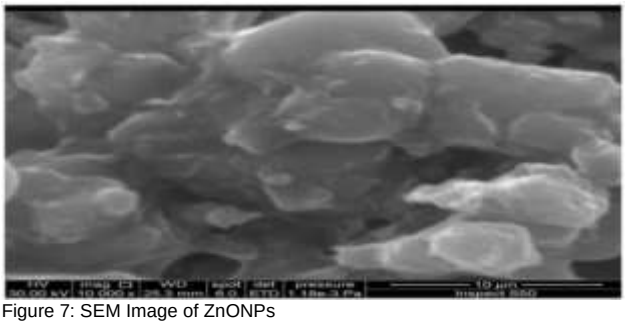

Figure 7: SEM Image of ZnONPs

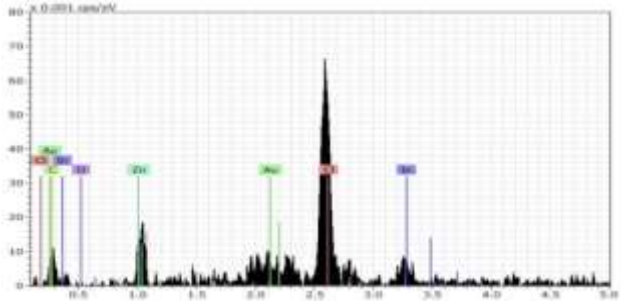

Figure 8: Energy dispersive X-Ray spectrometry (EDX)

Fig 9: Minimum Inhibitory Concentration (MIC) of Zinc Oxide con. 1) 512 µg/ml 2) 265 µg/ml 3) 128 µg/ml on MHA at 37°C for 24 hrs

Toxicity effect of ZnO NPs on the cell line: The MTT results , at different concentrations of ZnO NPs (16,32,64,256,512) µg/ml via biological method shown in figure and table below

	CAF		HdFn	
Concen.	Mean	SD	Mean	SD
32.00	72.88	4.79	78.22	6.67
16.00	74.69	2.55	86.07	3.08
8.00	89.20	2.41	90.08	1.05
4.00	93.98	0.53	93.87	1.10
2.00	94.68	0.60	94.64	0.48
1.00	95.14	1.22	95.10	0.29

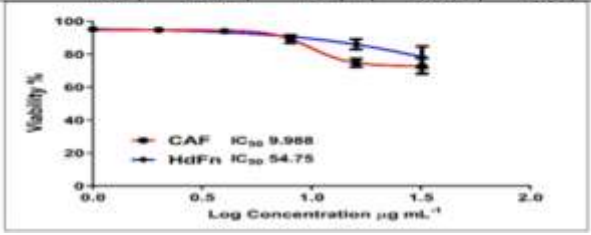

Figure 10: The MTT assay results of synthesized ZnO NPs on HdFn cells and normal CAF cell.

Histopathological examination: Nanoparticles at concentration 64 µg/ml of ZnO NPs demonstrated several histopathological alterations in spleen and liver.. The liver sections appeared with severe congestion of central veins as shown in figure 11 (determine the histopathological changes in the liver 7 day post-zinc oxide nanoparticles management discovered moderate vascular degeneration of the hepatocytes and disorganization of the hepatic cords

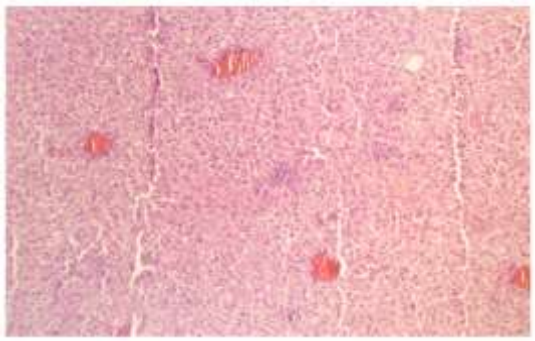

Figure11: The liver segment 7 day post management confirmed sever congestion of significant veins (H & E; 100%).

While the spleen showed moderate hyperplasia of the white pulp and proliferation of megakaryocytes in the red pulp(figure 12).

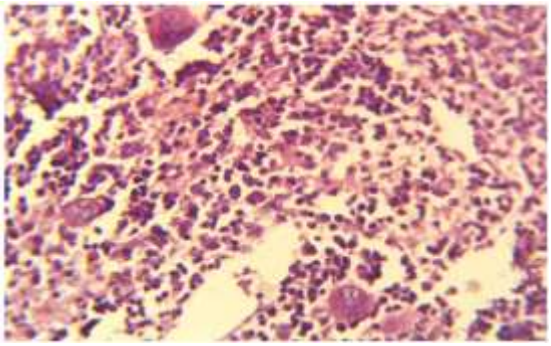

Figure12: Spleen at day 7 confirmed proliferation of megakaryocytes within the purple pulp (H & E; 400×)

DISCUSSION

Biosynthesis of ZnO Nanoparticles: This study describes a comparison of the biological and chemical synthesis of ZnONP at both the basic and application levels. Green synthesis of nanoparticles (NPs) is an environmentally friendly and inexpensive method, but it may serve as a new biostimulant [12]. We investigated the efficiency of ZnONP synthesized under s.oralis. When zinc nitrate was added to the extract, the color changed from pale yellow to dark brown, indicating the formation of ZnONP. The results are consistent with the following ZnO NPs, a green synthetic method using an Agathosma betulina (Buchu) extract that acts as a capping or reducing agent and as a precursor of zinc nitrate [13] The biological approach is physical. It is a promising non-toxic alternative to target and chemical synthesis. Microorganisms (bacteria, fungi, algae, bacteriophage) are used for the biological synthesis of ZnO NP, but the method of biological synthesis of ZnONP is not yet known [14].

UV-VIS absorption: UV-visible results show that a large band is observed at 294 nm. Other consequences according gto [15], (Rauf et al., 2017) confirmed that the UV-Vis spectrum of ZnONPs was at the spectrum (373) nm Whereas the absorption peak was obtained at 260 nm. Balogun et al., (2020), [16].

XRD (x ray diffraction): The result of XRD proven formation of hexagonal shape of the ZnO NPS via revealing distinguished peaks corresponded to the diffraction peaks "(18.5), (24.5), (31.4), (33.2), (35.8), (45.2) , (39.7), and (56) proven in figure (8) whichagreed with [17] (Santhoshkumar et al 2016).

AFM (atomic force microscopy): According to (18) (Getie et al., 2017), the averaged crystallite size of Zinc oxide nanoparticles that were synthesized from the zinc acetate dihydrate turned into approximately 28.09 mm and 31.86 mm at the same time as average particle size is 37 nm in accordance to (19) (Rajabairavi et al., 2017).

TEM (transmission electron microscopy): From the TEM images, the samples shapes started from spherical to hexagonal;and the spherical shapes regarded in the samples pattern that showed the ZnO nanoparticles were poly crystalline. Other consequences in accordance to [20] (Khan et al. 2015)

SEM scanning electron microscopy: The morphology of the nanoparticles was irregular and the ranged-size was (24) nm that still verified with the AFM results, bulks of the debris have been abnormal in form figure (7). Results in accordance to [21] (Jha and Prasad,2018), the nanoparticles with tubules and other irregular shape. Scanning electron microscopy additionally showed the morphology and size of nanoparticles [22] (Datta et al,. 2017).

EDX Energy dispersive X-Ray spectrometry: In the biological synthesis of ZnONP, the spectrum did not show pure nanozinc oxide due to the presence of bacterial suspension components. These results are consistent with Nagarajan S. et al. [23] ZnONP was prepared by a biological method,

MIC (minimum inhibitory concentration): The results show that using the MIC of biosynthesized ZnO NP found by sufficient diffusion, ZnONP at various concentrations of 0.25 mg / ml and 64 µg / ml (separation of different types of bacteria from the dental caries). It was shown that the activity of zinc oxide nanoparticles with respect to the strain was measured. The highest antibacterial activity of the green synthetic ZnONP inhibition zone as a detection method against test bacteria. In a previous study by [24], MIC was observed to inhibit ZnONP biosynthesis against Staphylococcus aureus in a 12.7 mm inhibition zone. The variation in the antibacterial activity of ZnO-NP, as indicated by the nanoparticle MIC, may be due to differences in the genus and species of the bacteria tested. [25] ZnO-NP showed antibacterial activity against Gram-positive bacteria. (Staphylococcus aureus) and Gram-negative bacteria (E. Kori and Salmonella) [26]. One study showed the inhibitory activity of synthetic ZnONP against MDR in K. pneumoniae, E. coli, P. aeroginosa, and S. aureus. The mechanism of ZnO NP antibacterial activity may involve Zn2 + release and ROS formation and cell membrane damage [27]. Increasing the concentration of nanoparticles creates a large inhibition zone, indicating improved antibacterial activity.

Toxicity assay: The MTT results (Figure 10), showed that different concentrations of ZnO NPs (16,32,64,256,512) µg/ml via biological method decreased the number of HdFn cells, and the ZnO nanoparticles were attached to the toxic oxygen species of photocatalytic activity[28], and these results indicate that the ZnO NPs may have toxic effect through reduction HdFn cell viability as a result of cell dying such by damage to the cell membrane[29]

Histopathological examination: Utilizing of Zinc oxide nanoparticles has been significantly increased an multi- industrial merchandise, in addition to in biological toxicological impact on humans. The end results were identical with the results of Lin, W. et al [30]. The harm of orgain became detected histopathological examinations however decrease than ZnONPs education via way of means of biological method effect exert their toxic on human liver cells[31] Sharma, V. et al.

CONCLUSION

The environmentally friendly natur green synthesize ZnO-NPs from bacterial suspension and many characterization are compared to traditionally synthesized These NPs can be sized and shaped by SEM and AFM additionally other included X-ray diffraction and Zeta potential also have study show toxic more effect on the some organ (liver and spleen cells) in vivo at same concentration that causes congestion liver and damage some cell in the red pulp in spleen. exposure to nanoparticles maybe depended to dose and method prepration.

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