Evaluation of the effects of ZnO NPs biosynthesis on Gene Expression for Hemolysin Hla Gene of S. aureus Bacteria Isolated from Diabetic Foot Ulcer

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Abstract

The clinical isolates of *Staph aureus* used in the current study are from (diabetic foot patients) diagnosis results of *S. aureus* gram positive bacteria, beta hemolysis on blood agar, mannitol fermenter and positive catalase and coagulase. Utilizing a scanning electron microscope, fourier transforms infrared (FTIR), X-ray diffraction (XRD), and UV-visible spectrophotometer, were able to characterize the physical-chemical composition of ZnO NPs produced during the biosynthesis process (SEM). According to the XRD results, they were very small crystals with a majority spherical form, measuring 13.5 nm in size. Using the agar well diffusion method and optical densities data at 630 nm, the minimum inhibition concentration, or MIC test, of ZnO NPs at concentrations (31.25 to 500) g/ml against bacterial isolates have been established. Results of the best MIC trial with 125g/ml ZnO NPs concentration revealed a significant difference in the anti-bacterial inhibition for isolates under study at $P<0.01$. Using the RT-qPCR method and the *16SrRNA* gene as a reference, the expression of the *Hla* gene was examined both before and after exposure to ZnO NPs. Cycle threshold (Ct) values for the *Hla* gene and gene expression of the isolates were found to differ significantly at $P<0.05$, according to the data. According to the study's findings, biosynthesized ZnO NPs with a concentration of 125 g/ml had inhibitory effects on bacterial isolates and had the potential to downregulate gene expression.

Keywords: Staphylococcus aureus, Pseudomonas aeruginosa, ZnO NPs, Hemolysin (Hla) gene and gene expression.

1. Introduction

One of the most common endocrine illnesses, it is characterized by decreased glucose uptake and caused by absolute or relative insulin deficiency, resulting in hyperglycemia [1]. Diabetic mellitus is divided into numerous forms, the most prevalent of which are Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), and gestational diabetes, as well as additional types of diabetic mellitus [2]. Among complication of diabetic, the diabetic foot ulcers (DFUs) are a complicated combination of risk factors that include, peripheral vascular disease, peripheral neuropathy, trauma, and reduced infection resistance, and they continue to be a significant cause of lower extremity amputation worldwide [3]. Infections as a result of microbial sources are an important element to consider in diabetic wounds and is crucial and challenging to manage and treat [4]. The normal microflora of the skin consists of various bacterial species; were identified to be clinically infectious [5]; [6]. Damages caused by either direct or indirect trauma in the skin encourages surface bacteria to infest into underlying tissue, as a result of this microbial penetration, the inflammatory cells cause inflammation by releasing protease enzymes and reactive oxygen species to the infection site [7]. The infested bacterial secretes endotoxins and an increased level of these endotoxins are release at the site which increases the pro-inflammatory cytokines as an immune response, this immune response to the bacterial toxin reduces collagen deposition along with decrease in growth factor production which plays a crucial role in wound healing, thus, wound healing is delayed [8]. This study focused on the role cell membrane-associated genes, *Hal* gene for *S. aureus*, that cause the lysis of red blood cells, hemolysin are regulated by regulator gene hemolysin the most important toxin is have activity can induce the lysis of epithelial, erythrocytes cells and cause the apoptosis [9].

2. Materials and Methods

Samples Collections

A swabs were collected under sterile conditions from infected patients with diabetic foot ulcers in general Al-Hashmia hospital Babylon Governorate during (4 months period) from 27 November to the 27 February 2022 and transferred to laboratory for isolate and diagnosis Depending on the [10].

Molecular diagnostic of pathogenic bacteria

The genomic DNA of pathogenic clinical isolates is

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extracted using a commercial purification used kit extraction of Genomic DNA. (Mini Kit Genomic DNA from (Blood-Cultured Cell) Favorgen / Korea), the extraction was done following the manufacture's protocols recommended for bacteria and DNA was stored at -20°C.

Collection and Isolation of *Pseudomonas aeruginosa* for biosynthesis of nanoparticles.

The environmentally isolated bacterial was obtained *Pseudomonas aeruginosa* which was cultivated again for activation and do all the standard diagnostic tests as well to ensure the isolate.

Preparation of Bacterial Suspension for The Purpose of Preparing The nano-extract

The isolate used for research was selected by taking a number of single colonies of the selected isolate and inoculated in 250 ml of sterile brain heart fusion broth medium for 24 h at 35±2°C in a 500 mL Erlenmeyer flask to grow, under shaking at 120 rpm on an incubator orbital shaking, then the sample was filtered after centrifugation using filter paper with a diameter of 0.45 micrometer, then the filter was taken for use in preparing the preparation ZnO NPs [11].

Formation of Zinc Oxide Nanoparticles

Takes 100 mL of the bacterial supernatant, then in a 500mL Erlenmeyer flask, 100 mL of supernatant was mixed with 100 mL of solution (1mM) Zinc acetate $Zn(CH_3COO)_2.H2O_2$ (Sigma), incubated for 96 hours at 32°C with agitation at 150 rpm, ZnO NPs were obtained by centrifuging at 1000 rpm for 10 minutes, after which the precipitant washed once with 96% ethanol and then drying at 60°C in oven, and weight of extracted ZnO NPs [11;12].

3. Results and Discussion

Identification of Bacterial Isolates

The isolated microorganisms from diabetic foot ulcers sources, which were identified using microscopic, macroscopic, and biochemical tests and the results confirmed by VITEK-2 system, and molecular identification, revealed that the most frequently isolated bacteria were *Staphylococcus aureus*, which was consistent with previous findings. [13]. The identity was then verified using the Biomerieux-recommended VITEK 2 compact equipment, and the resulting data was compared with the source supplied by [14; 10].

Genotyping Identification of *Pathogenic bacteria*

In this study, the identification of pathogenic bacteria clinical isolated through DNA of bacterial isolates, were isolates eight S. aureus. Also, purity and concentration were confirmed with Nano-drop, the purity of the nucleic acid in the samples ranged between (1.8-2). Purity of DNA is a good indicator of the extraction process as well as confirming the absence of impurities that could impede the process [15]. the extracted DNA was confirmed and analyzed by the horizontal gel electrophoresis in 1% agarose for 45 min at 100 volts and was exposed to UV light where the DNA appears as compact bands [16]. As shown in Figure (1).

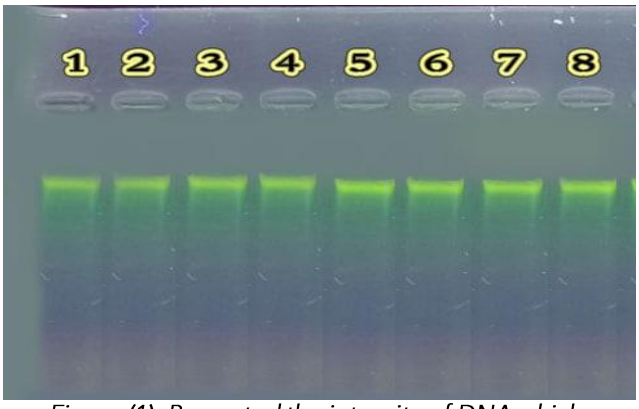

Figure (1): Presented the integrity of DNA which extracted from Pathogenic bacteria isolated from DFUs, S. aureus (1— 8) Agarose 1.3%, Volt 100, Time 45 minutes.

Identification Bacteria for Biosynthesis of ZnO NPs

The characteristics results of the bacteria on the MacConkey medium were pale in color due to their inability to ferment lactose, on the blood agar showed transparent halo evidence of hemolysis and production of hemolysin. While on the nutrient agar they appeared in green, microscopically they were gram negative as well as the shape and arrangement of cells and the biochemical examinations results of *P. aeruginosa* showed that isolate had given negative result for indole, urease test, while citrate was positive, oxidase positive and TSI alkaline/alkaline with no production of H2S and gas, these results agreed with what mentioned by [10]. Similar finding were recorded by [17]. Showed result of the Vitec system was the bacterial isolate identified as a belonging to the *P. aeruginosa*, as mentioned at [18].

Characterization of Biosynthesis (Zinc Oxide NPs)

The UV–Visible Spectrophotometer

The highest absorption peaks for ZnO-NPs was observed at 379 nm, this result indicates the successful biosynthesis of ZnO NPs by bacterial filtrate, the successful biosynthesis of ZnO-NPs by was confirmed by the color change to white precipitate The results of this study are in agreement with the highest absorption was observed when biosynthesizing zinc particles from bacteria P. aeruginosa was 380 nm. it was identical to the result of [11].

X-ray Diffraction Characterization

The XRD pattern showed to bacterial biosynthesis intense diffraction peaks at 2 values of respectively,

these results confirm that the material is zinc oxide and that it is of high purity, when compared to X-ray diffraction, our observations were consistent with the crystalline character of biologically generated ZnO-NPs, and the presence of bacterial metabolites including proteins and chemical compounds that coated the ZnO NPs surface was strongly indicated [11; 19]. The average ZnO NP size that matched the highest diffraction peak was determined to be 15.8 nm.

Scanning Electron Microscope (SEM)

The results showed the phenotypic features of ZnO NPs by the bacterial filtrate, regular structures spherical. In addition to the particle size. Our study succeeded in achieving good results with the smallest sizes of ZnO NPs, which were less than what was found [20]. Also [21], indicated in his research that the average size ranged between (50-120) nm, and [22], he indicated his results for ZnO NPs biosynthesized by with an average size of (20-40)nm. The current study succeeded in achieving the best and most accurate results at the level of the small sizes of ZnO NPs, and the originality of good results for biosynthesis.

Fourier Transformed Infrared Spectroscopy (FTIR)

The formation of ZnO-NPs by bacteria was discovered using FTIR. The stretching NH of amines was represented by the overlap of the vibration mode of the -OH group with that wave number, whereas the stretching O-H of carboxylic acid was represented by peaks at wave numbers between 3000 and 3700 cm-1 [23]. While the faint signal at 2590 cm-1 was connected to the stretching SH thiol group, the medium peak at 1620 cm-1 was assigned to the bending primary amine (NH) overlapped with carboxylate salts or amide. [24]. Moreover, with respect to CO2 and carbonates (CO32) adsorption at the NPs surface, a medium peak at 1430 cm-1 was identified as stretching C=O of carboxylic salts. [23] Strong peaks at 1032 cm-1 and 895 cm-1 may correspond to sulfoxide stretching S=O and alkene bending C=C, respectively. [24; 25]. As previously reported, the successful production of ZnO-NPs was confirmed at peaks of 400 to 700 cm-1[26]. The involvement of several groups, including the C=O, O-H, NH, and SH thiol groups present in bacterial reducing, capping, and stabilizing activities, was validated by the FT-IR analysis of ZnO-NPs.

Real Time qPCR of Hla Gene Expression

Demonstrated q RT-PCR using eight isolates of S. aureus from a diabetic foot ulcer patient before and after treatment with ZnO NPs, the results of the Hla gene, which gave high expression for all isolates and with different degrees of the bacterial biosynthesis product ZnO NPs. The treatment with the bacterial biosynthesis product ZnO NPs, as it gave a low expression with an increase in Ct value and it was statistically P<0.01 evidence of occupational differences that were between the isolates before and after treatment as in Table (1) and Figures (2,3) showing the pattern of gene amplification. Their differences depend on the range of the Ct values as untreated and treated. By comparing the results on the extent of the Ct value of the gene when treating bacterial isolates with ZnO NPs. As a result of the biological product's possession of biologically active functional groups such as the amine group, the carboxyl group, and others, and merely touching the surface of the cell, the cell generates electrical charges that damage its membranes and disrupts the genes of the cell membranes, and its influence stops cellular activities and thus the death of the cell. As indicated [27].

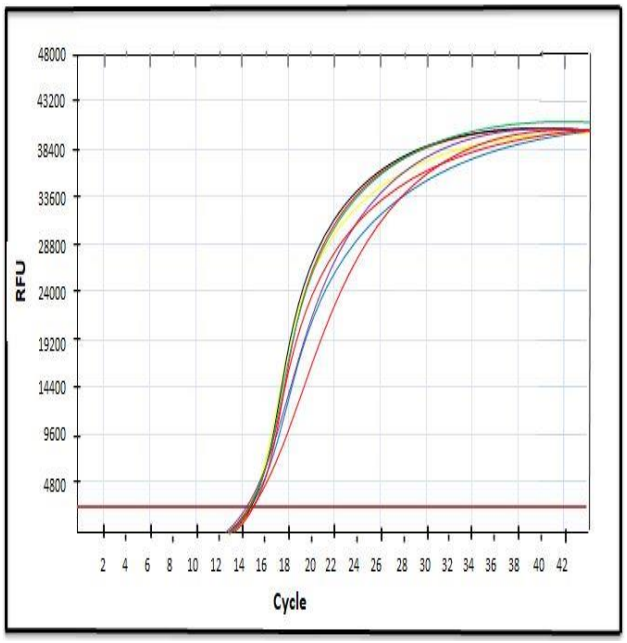

Figure (2): The Amplification of Hla gene before treatment with ZnO NPs.

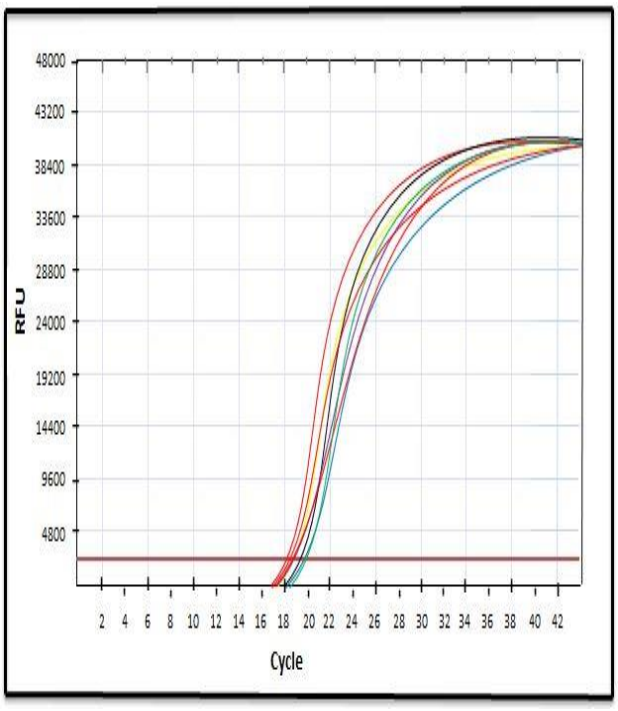

Figure (3): The Amplification of 16SrRNA Gene after treatment with ZnO NPs biosynthesis by P. aeruginosa.

Table (1): Show The Gene Expression of *Hla* gene Before and After Treatment with ZnO NPs *P. aeruginosa*							
No. of isolates	Treatment ZnO NPs p.aeruginosa	Ct (Hla) of Target Gene	Ct of reference (HK) gene	ΔCt Target (Ct (Hla) - Ct (HK)	ΔΔCt (ΔCt – Ave ΔCt)	2-ΔΔCt Fold of gene expression	P-value
S1	Untreated	14.79	14.92	-0.13	-0.076	1.054 ±0.17	0.0072 **
	Treated	18.11	14.92	3.19	3.243	0.105 ±0.042	
S2	Untreated	14.83	14.88	-0.05	0.003	0.997 ±0.11	0.0078 **
	Treated	19.75	14.87	4.88	4.933	0.032 ±0.006	
S3	Untreated	14.81	14.81	0	0.053	0.963 ±0.10	0.0093 **
	Treated	18.96	14.03	4.93	1.686	0.31 ±0.08	
S4	Untreated	14.75	14.77	-0.02	0.033	0.976 ±0.09	0.0077 **
	Treated	18.76	14.25	4.51	4.563	0.042 ±0.01	
S5	Untreated	14.79	14.80	-0.01	0.043	0.970 ±0.11	0.0082 **
	Treated	19.30	14.67	4.63	4.683	0.038 ±0.005	
S6	Untreated	14.66	14.73	-0.07	-0.016	1.011 ±0.12	0.0075 **
	Treated	19.91	14.54	5.37	5.423	0.023 ±0.008	
S7	Untreated	14.69	14.71	-0.02	0.033	0.976 ±0.10	0.0075 **
	Treated	18.97	14.22	4.75	4.803	0.035 ±0.007	
S8	Untreated	14.55	14.68	-0.13	-0.0762	1.054 ±0.14	0.0069 **
	Treated	18.32	14.16	4.16	4.213	0.053 ±0.007	
** (P≤0.01).							

Table (2): The Ct Value of (Housekeeping16SrRNA gene) before and after the Treatment with (ZnO NPs)		
No. Of isolates	The Ct value of *(HK)* gene before treatment	The Ct value of *(HK)* gene after treatment
HK-1	14.92	14.92
HK-2	14.88	14.87
HK-3	14.81	14.03
HK-4	14.77	14.25
HK-5	14.80	14.67
HK-6	14.73	14.54
HK-7	14.71	14.22
HK-8	14.68	14.16
NS: Non-Significant		

Real Time qPCR of 16SrRNA Gene Expression

In the present study, the internal control gene *16SrRNA* was used, the range of Ct values for this gene when not treated with ZnO NPs was from (14.55 - 14.68) in all (8) *S. aureus* isolates from DFUs, the isolates treated with synthesized ZnO NPs, the Ct value did not change at a high rang from (14.03-14.99), as showed in Table (2), the statistical analysis showed a non-significant difference between isolates before and after treatment. Figures (4,5) showing the pattern of gene amplification.

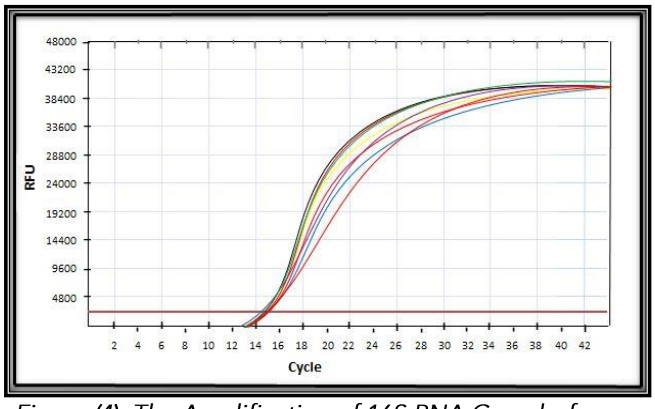

Figure (4): The Amplification of 16SrRNA Gene before treatment with ZnO NPs.

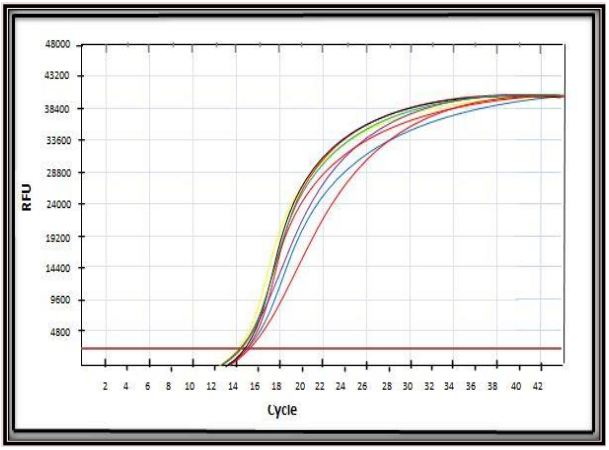

Figure (5): The Amplification of 16SrRNA Gene after treatment with ZnO NPs.

4. Conclusion

The Gram-positive bacteria such as S. aureus causes diabetic foot ulcer infections, but Gram positive were dominant S. aureus. Nano-products is preparing from bacteria Pseudomonas aeruginosa and using them to inhibit a treatment diabetic foot ulcer, efficiency of ZnO to inhibit the pathogenic bacteria and inhibitory effects on bacterial isolates and had the potential to down regulate Hla gene expression in this study. Our study succeeded where it was

distinguished with biosynthesis ZnO NPs in the present study have smaller size when compared with the previous studies, which is preferable to achieve better antimicrobial effects.

References

1-Yong, J, Johnson, J. D, Arvan, P, Han, J, & Kaufman, R. J. (2021). Therapeutic opportunities for pancreatic β-cell ER stress in diabetes mellitus. Nature Reviews Endocrinology, 17(8), 455-467.

2-Solis-Herrera, C, Triplitt, C, Reasner, C, DeFronzo, R. A, & Cersosimo, E. (2018). Classification of diabetes mellitus. Endotext [Internet].

3-Albadri, A. T. J. (2021). Characterization and Molecular study to detect Multidrug Resistance Bacteria Isolated from Patients with Diabetic Foot Ulcers in Wasit Province (Doctoral dissertation, Ministry of Higher Education).

4-Balalakshitha, M, Kolanjinathan, K, Aishwarya, T, & Vinothini, B. (2021). A Review on Diabetic Wound Infection and Its Treatment Using Zinc Oxide Nanoparticle Synthesized by Green Chemistry Techniques. Bull. Env. Pharmacol. Life Sci, 10, 01-22. (Patel et al,2022)

5-Patel, B. K, Patel, K. H, Huang, R. Y, Lee, C. N, & Moochhala, S. M. (2022). The Gut-Skin Microbiota Axis and Its Role in Diabetic Wound Healing—A Review Based on Current Literature. International Journal of Molecular Sciences, 23(4), 2375.

6-Han, G, & Ceilley, R. (2017). Chronic wound healing: a review of current management and treatments. Advances in therapy, 34(3), 599-610.

7-Liu, Y. F, Ni, P. W, Huang, Y, & Xie, T. (2022). Therapeutic strategies for chronic wound infection. Chinese Journal of Traumatology, 25(01), 11-16.

8-Da, L. C, Huang, Y. Z, Xie, H. Q, Zheng, B. H, Huang, Y. C, & Du, S. R. (2021). Membranous Extracellular Matrix-Based Scaffolds for Skin Wound Healing. Pharmaceutics, 13(11), 1796.

9-Shahid, A, & Rafiq, A. (2021). Effects of Staphylococcus aureus hemolysin toxins on blood cells and association with skin and soft tissue infections. Abasyn Journal of Life Sciences, 4(1), 152-160.

10-MacFaddin, J. (2000). Deoxy ribonuclease (DNase) and thermos nuclease) Tnase) tests. Biochemical Tests for Identification of Medical Bacteria, 3rd ed, ed. by MacFaddin, JF, Lippincott Williams and Wilkins, Philadelphia, PA, 136-159.

11-Abdo, A. M, Fouda, A, Eid, A. M, Fahmy, N. M, Elsayed, A. M, Khalil, A. M. A, ... & Soliman, A. M. (2021). Green Synthesis of Zinc Oxide Nanoparticles (ZnO-NPs) by Pseudomonas aeruginosa and Their Activity against Pathogenic Microbes and Common House Mosquito, Culex pipiens. Materials 2021, 14, 6983.

12-Barsainya, M, & Singh, D. P. (2018). Green synthesis of zinc oxide nanoparticles by Pseudomonas aeruginosa and their broad-spectrum antimicrobial effects. J. Pure Appl. Microbiol, 12(4), 2123-2134.

13-Stensen, D. B, Cañadas, R. A. N, Småbrekke, L, Olsen, K, Nielsen, C. S, Svendsen, K, ... & Furberg, A. S. (2022). Social network analysis of Staphylococcus aureus carriage in a general youth population. International Journal of Infectious Diseases.

14-Collee, J. G. (1996). A dreadful challenge. The Lancet, 347(9006), 917-918.

15-Wilfinger, C, Castellani, C, Raith, J, Pilhatsch, A, Höllwarth, M. E, & Weinberg, A. M. (2009). Nonoperative treatment of tibial spine fractures in children-38 patients with a minimum follow-up of 1 year. Journal of orthopaedic trauma, 23(7), 519-524.

16-Brennan, C, Momota, H, Hambardzumyan, D, Ozawa, T, Tandon, A, Pedraza, A, & Holland, E. (2009). Glioblastoma subclasses can be defined by activity among signal transduction pathways and associated genomic alterations. PloS one, 4(11), e7752.

17-Al-Saeedi, M, Steinebrunner, N, Kudsi, H, Halama, N, Mogler, C, Büchler, M. W, ... & Müller, M. (2018). Neutralization of CD95 ligand protects the liver against ischemia-reperfusion injury and prevents acute liver failure. Cell death & disease, 9(2), 1-9.

18-Raheema, R. H. (2016). Experimental study for the effect of Trigonellafoenum-graecum (fenugreek) seeds extract on some biochemical and histo pathological study in induced diarrhea in mice by Klebsiella pneumoniae. IOSR Journal of Pharmacy, 6(2), 04-13.

19-Hassan, S. E. D, Fouda, A, Radwan, A. A, Salem, S. S, Barghoth, M. G, Awad, M. A, ... & El-Gamal, M. S. (2019). Endophytic actinomycetes Streptomyces spp mediated biosynthesis of copper oxide nanoparticles as a promising tool for biotechnological applications. JBIC Journal of Biological Inorganic Chemistry, 24(3), 377-393.

20-Kulkarni, S. S, & Shirsat, M. D. (2015). Optical and structural properties of zinc oxide nanoparticles. International Journal of Advanced Research in Physical Science, 2(1), 14-18.

21-Pavani, P. G, Sadhana, K, & Mouli, V. C. (2011). Optical, physical and structural studies of boro-zinc tellurite glasses. Physica B: Condensed Matter, 406(6-7), 1242-1247.

22-Mandal, A. K, Katuwal, S, Tettey, F, Gupta, A, Bhattarai, S, Jaisi, S, ... & Parajuli, N. (2022). Current Research on Zinc Oxide Nanoparticles: Synthesis, Characterization, and Biomedical Applications. Nanomaterials, 12(17), 3066.

23-Koupadi, K, Boyatzis, S. C, Roumpou, M, Kalogeropoulos, N, & Kotzamani, D. (2021). Organic Remains in Early Christian Egyptian Metal Vessels: Investigation with Fourier Transform Infrared Spectroscopy and Gas Chromatography–Mass Spectrometry. Heritage, 4(4), 3611-3629.

24-Hamza, M. F, Salih, K. A, Adel, A. H, Zayed, Y. E, Wei, Y, Liang, J, & Guibal, E. (2021). Sulfonic-functionalized algal/PEI beads for scandium, cerium and holmium sorption from aqueous solutions (synthetic and industrial samples). Chemical Engineering Journal, 403, 126399.

25-Fouda, A, Abdel-Maksoud, G, Saad, H. A, Gobouri, A. A, Mohammedsaleh, Z. M, & Abdel-Haleem El-Sadany, M. (2021). The efficacy of silver nitrate (AgNO3) as a coating agent to protect paper against high deteriorating microbes. Catalysts, 11(3), 310.
26-Ebadi, M, Zolfaghari, M. R, Aghaei, S. S, Zargar, M, Shafiei, M, Zahiri, H. S, & Noghabi, K. A. (2019). A bio-inspired strategy for the synthesis of zinc oxide nanoparticles (ZnO NPs) using the cell extract of cyanobacterium Nostoc sp. EA03: from biological function to toxicity evaluation. RSC advances, 9(41), 23508-23525.
27-Sarwar, S, Chakraborti, S, Bera, S, Sheikh, I. A, Hoque, K. M, & Chakrabarti, P. (2016). The antimicrobial activity of ZnO nanoparticles against Vibrio cholerae: Variation in response depends on biotype. Nanomedicine: Nanotechnology, Biology and Medicine, 12(6), 1499-1509.