



Original Article

Asian Pacific Journal of Tropical Biomedicine



apjtb.org

doi: 10.4103/apjtb.apjtb_185_24

Antimicrobial, antibiofilm, angiogenesis, anti-inflammatory, and wound healing activities of zinc nanoparticles green synthesized using *Ferula macrecolea* extractSultan F. Alnomasy[✉]

Department of Medical Laboratories Sciences, College of Applied Medical Sciences, Shaqra University, Al-Quwayyah 19257, Saudi Arabia

ABSTRACT

Objective: To assess the antimicrobial, antibiofilm, anti-inflammatory, angiogenic, and wound healing activities of zinc nanoparticles (ZNPs) green synthesized using *Ferula macrecolea* extract.

Methods: The green synthesis was conducted using the precipitation method. Then, the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibition concentration 50% (MBIC₅₀) of ZNPs against *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*) were evaluated. The effects of ZNPs on the gene expressions of *Staphylococcus* spp. [intracellular adhesion A (*icaA*) and D (*icaD*)] and *P. aeruginosa* (*rhlI* and *rhlR*) were investigated using quantitative real-time PCR. In addition, the effects of ZNPs on wound healing, angiogenesis, and anti-inflammatory markers were assessed.

Results: The green-synthesized ZNPs demonstrated significant antimicrobial efficacy against *S. aureus* and *P. aeruginosa*. The biofilm formation in *S. aureus* and *P. aeruginosa* was also inhibited by ZNPs with MBIC₅₀ values of 3.30 µg/mL and 2.08 µg/mL, respectively. Additionally, ZNPs downregulated the expression of biofilm-related genes *icaA*, *icaD*, *rhlI*, and *rhlR* in the tested bacteria. They also demonstrated promising *in vitro* wound healing effects by promoting fibroblast cell proliferation and wound closure in a dose-dependent manner. A significant increase in the expression of *HLA-G5* and *VEGF-A* genes as well as a marked decrease in the expression of *NF-κB*, *IL-1β*, and *TNF-α* genes were observed in cells treated with ZNPs compared to the control group ($P < 0.05$).

Conclusions: ZNPs display promising antibacterial effects against *S. aureus* and *P. aeruginosa* and wound-healing effects by inhibiting biofilm formation, inducing angiogenesis, and reducing inflammation. However, further studies must be conducted to specify the accurate mechanisms of action and toxicity of ZNPs.

KEYWORDS: Wound healing; Antibacterial; Angiogenesis; Nanomedicine; *Ferula macrecolea*; Inflammation

1. Introduction

Currently, it has been shown that wounds resulting from certain conditions such as diabetes, gastric disorders, and duodenal ulcers have a significant impact on individuals' quality of life[1]. Wounds usually result from skin lacerations caused by physical trauma, burns, infections, or chronic conditions that disrupt the normal structure and function of the skin. Under these conditions, the connective tissue beneath the skin and the integrity of the epithelial

Significance

The process of wound healing is complex, involving stages of inflammation, cellular proliferation, and tissue remodeling, during which intercellular and cell-matrix interactions occur. Our results demonstrate that zinc nanoparticles synthesized using *Ferula macrecolea* extract possess potent antibacterial effects against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, as well as significant wound-healing activities by inhibiting biofilm formation, promoting angiogenesis, and reducing inflammation. Nevertheless, further research is necessary to elucidate the precise mechanisms of action and potential toxicity associated with zinc nanoparticles.

[✉]To whom correspondence may be addressed. E-mail: s.alnomasy@su.edu.sa

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

©2024 Asian Pacific Journal of Tropical Biomedicine Produced by Wolters Kluwer-Medknow.

How to cite this article: Alnomasy SF. Antimicrobial, antibiofilm, angiogenesis, anti-inflammatory, and wound healing activities of zinc nanoparticles green synthesized using *Ferula macrecolea* extract. Asian Pac J Trop Biomed 2024; 14(6): 259-268.

Article history: Received 5 April 2024; Revision 8 May 2024; Accepted 4 June 2024; Available online 28 June 2024

tissue are compromised[1]. The process of wound healing is complex, involving stages of inflammation, cellular proliferation, and tissue remodeling, during which intercellular and cell-matrix interactions occur[2]. Enzymes such as collagenase and elastase play a crucial role in the healing process by breaking down extracellular matrix components, such as elastin, fibrin, and collagen[3]. However, their activity must be regulated by inhibitory mechanisms, as an excessive increase in these enzymes can hinder wound healing[3]. Several factors can complicate the process of wound care. These factors include issues such as inadequate blood circulation, persistent inflammation, neuropathy, infection, insufficient angiogenesis, and limited cell proliferation[3].

At present, there is a growing interest in exploring innovative or alternative therapeutic approaches using nanotechnology, especially nanoparticles synthesized through environmentally friendly methods, for the treatment of both acute and chronic wounds[4]. Nanoparticles (NPs) have shown significant potential in wound healing due to their enhanced adsorption capacity, antimicrobial properties, and drug delivery capabilities[5]. NPs also have the potential to expedite the process of wound healing through mechanisms such as enhancing cell migration, proliferation, and differentiation, as well as regulating the inflammatory response and facilitating angiogenesis[5].

While the effectiveness of different metal NPs in wound healing, such as silver, iron, gold, zinc, and copper, has been established in numerous studies, the outcomes vary due to factors such as the specific metal used, the method of NP synthesis, and the study methodology[6]. Zinc nanoparticles (ZNPs) are known for their antibacterial, antioxidant, and anti-inflammatory properties[7]. Additionally, ZNPs can promote cell migration, re-epithelialization, and angiogenesis[7]. In recent years, several studies have demonstrated the wound-healing effects of ZnONPs green synthesized by extracts from *Ilex paraguariensis*[8], *Nigella sativa*[9], *Prosopis farcta*[10], and *Ziziphora clinopodioides*[11]. However, the results of these studies have faced challenges due to various factors, such as the method of synthesizing NPs, the type of plant utilized, the study design, the concentrations employed, and notably, the lack of clarity regarding the exact mechanisms of action of these NPs[12]. The process of green synthesis offers advantages over conventional chemical synthesis due to its cost-effectiveness, reduction of pollution, and enhancement of environmental and human health safety[13]. The environmentally friendly and highly effective green synthesis of NPs has garnered significant interest within the nanotechnology scientific community. Recent studies have highlighted the potential of green NPs as promising agents for wound healing[12,13]. In the field of green synthesis, numerous plants are currently being used in the eco-friendly production of NPs. Various studies have demonstrated the synthesis of NPs using locally available plants. However, a notable issue is the limited availability of certain plants for large-scale production. Therefore, when

considering the selection of plants for nanomaterial synthesis on a larger scale, it is crucial to assess the feasibility and abundance of local plant resources[14]. The green synthesis process is a significant focal point in nanotechnology due to its high energy consumption and lengthy reaction times. Compared to chemical synthesis, green synthesis typically takes more time. The synthesis of green NPs involves high-temperature reactions that require additional energy input, which could result in negative environmental consequences[14]. Furthermore, the quality of NPs is another crucial factor to consider. Numerous studies have highlighted significant challenges related to particle size control, indicating that the current processes may not be suitable for large-scale industrial production. Achieving precise control over particle size remains a major obstacle in NP synthesis[13,14]. *Ferula macrecolea* (*F. macrecolea*) Boiss from the Apiaceae family is known for its diverse pharmacological effects, including anti-inflammatory and antimicrobial properties, in traditional and modern medicine[14,15]. The present study was designed to assess the antimicrobial, antibiofilm, anti-inflammatory, angiogenic, and wound healing activities of ZNPs green synthesized using *F. macrecolea* extract.

2. Materials and methods

2.1. Chemicals

ZnSO₄ solution (Merck Chemicals, Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM, Merck, Germany), fetal bovine serum (FBS, Sigma-Aldrich, Germany), phosphate buffered saline (PBS, Merck, Germany), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Sigma-Aldrich, Germany), and nutrient agar plates (Sigma-Aldrich, Germany) were used in this study.

2.2. Plant collection

The aerial parts of *F. macrecolea* were obtained from a plant market in Riyadh, Saudi Arabia, in April 2022. After identification by a botanist (voucher No. 2022-4123), 300 g of air-dried and pulverized materials underwent extraction using 70% methanol for 3 d employing the percolation method[11].

2.3. Green synthesis of ZNPs

Biogenic ZNPs were prepared following a method previously described by Albalawi *et al.*[16]. Specifically, 10 mL of *F. macrecolea* methanolic extract (5 mg/mL) was combined with 40 mL of ZnSO₄ solution. Subsequently, this mixture was subjected to microwave exposure in a microwave oven (850 W, 60 s) to reduce the metal

ions, resulting in the formation of a dark gray color indicating the production of ZNPs.

2.4. Physical and chemical characteristics of ZNPs

The physical properties of ZNPs, such as their size and shape, were assessed using a scanning electron microscope (SEM) (Mira3, made in the Czech Republic) and a Zetasizer device (Malvern, UK). The crystalline structure of the ZNPs was determined using a UV-vis spectrophotometer (JENWAY 6405). Additionally, the environmentally friendly synthesis of the NPs using *F. macrocolea* extract was verified through X-ray diffraction (XRD) analysis (2000 APD, Italy). The FTIR spectrophotometer (Shimadzu IR-470, Japan) with a resolution of 40 nm was used to analyze the surface chemistry of biogenic ZNPs in potassium bromide disks.

2.5. Wound healing effects of ZNPs

2.5.1. Cell culture

The normal human skin fibroblast (Hs27) and macrophage (RAW 264.7) cell lines were obtained from the American Type Culture Collection (ATCC) and were cultured in DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C in a 5% CO₂ incubator. The cell concentration was then adjusted to 1×10⁵/mL using a hemocytometer (Hausser Scientific, USA).

2.5.2. Cell viability assay

The cell viability assay was conducted by exposing 0.1 mL of fibroblast cells (1×10⁵ cells/mL) to different concentrations of ZNPs (50–400 µg/mL) in a 96-well plate and incubating them at 24 °C for 48 h. Subsequently, 20 µL of MTT solution (5 mg/mL) was added to the wells, followed by further incubation in 5% CO₂ at 37 °C for 240 min. Upon adding sulfuric acid as the stop solution, the absorbance of the wells was measured at 570 nm using an ELISA plate reader (Biotek ELX-800TS, USA). The 50% cytotoxic concentrations (CC₅₀) were determined using the Probit test in SPSS software version 26.0[17]. The assay was conducted in triplicate.

2.5.3. Fibroblast proliferation assay

The fibroblast proliferation assay was conducted by seeding 0.1 mL of fibroblast cells (1×10⁵ cells/mL) into each well of 96-well plates and incubating them for 24 h. Following the removal of the supernatant, ZNPs (5, 10, 15, and 20 µg/mL) and asiaticoside (used as a positive control) were added to the wells and further incubated for 24 h. Subsequently, MTT solution (5 mg/mL) was added to the wells and incubated in a 5% CO₂ environment at 37 °C for 4 h. The absorbance of the wells was then measured at 570 nm using an ELISA plate reader (Biotek ELX-800TS, USA). The viability was

determined as a percentage of proliferation[17]. The test was carried out in triplicate.

2.5.4. Cell scratch wound healing assay

The migratory abilities of fibroblast cells were evaluated using an *in vitro* cell scratch wound healing assay, as outlined in a previous study[18]. Initially, the cells were cultured at a concentration of 2×10⁴ cells/mL in a 48-well plate until nearly confluent monolayers were formed. Subsequently, a linear wound was created on the cell monolayer using a sterile pipette tip, and any resulting cellular debris was removed by washing the wells with PBS. Following this, ZNPs at concentrations of 5, 10, 15, and 20 µg/mL were added and incubated for 24 h. The cells were then observed using an inverted microscope (Olympus CKX53, Japan) at 16 and 24 h. The ratio of increase in wound closure compared to the pre-treatment value was quantified and reported as cell migration. The cell scratch wound healing assay was performed in triplicate.

2.5.5. Real-time PCR for evaluating the expression level of angiogenesis-related genes

RAW 264.7 cells at a concentration of 1×10⁵ cells/mL were placed in a 24-well plate and incubated at 37 °C. Subsequently, ZNPs at 1/3 CC₅₀, 1/2 CC₅₀, and CC₅₀ concentrations were added and incubated for an additional 24 h. The total RNA was obtained using the kit procedure from Qiagen, Germany, and the quality of the extracted RNA was evaluated using a Nanodrop from Biotek Epoch. A Fermentas kit (USA) was used for cDNA synthesis. cDNA was stored at –20 °C for future use. The SYBR Green real-time PCR analysis was conducted using primers (Table 1) designed for the angiogenesis-related genes including vascular endothelial growth factor A (*VEGF-A*) and human leukocyte antigen-G5 (*HLA-G5*) by denaturation at 96 °C for 10 min, followed by 42 extension cycles and a final cycle at 73 °C for 5 min. The 2^{–ΔΔCt} value was calculated using Bio-Rad iQ5 Optical System Software (USA) to determine the gene expression levels[18]. The quantity of mRNA was assessed using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference for quantifying gene expression[19]. Normalized transcript levels were calculated by dividing the quantities of *VEGF-A* and *HLA-G5* mRNA by the amount of *GAPDH* mRNA in each sample. The reactions for each sample were run in triplicate unless otherwise specified.

2.5.6. Real-time PCR for evaluating the expression level of inflammation-related genes

To do this, RAW 264.7 cells at a concentration of 2×10⁶/mL were exposed to 0.1 mL lipopolysaccharide (LPS, 0.01 µg/mL; Sigma Aldrich, USA) at a concentration of 20 ng/mL, in the presence and absence of ZNPs at 1/3 CC₅₀, 1/2 CC₅₀, and CC₅₀ in a 24-well

plate for 24 h. The total RNA was acquired using the kit procedure from Qiagen, Germany, and the quality of the extracted RNA was assessed using a nanodrop from Biotek Epoch. A Fermentas kit (USA) was utilized for cDNA synthesis. The SYBR green Real-time PCR analysis was conducted using primers (Table 1) designed for interleukin-1 β (*IL-1 β*), nuclear factor kappa B (*NF- κ B*), tumor necrosis factor-alpha (*TNF- α*) and housekeeping (*GAPDH*) genes by denaturation at 95 °C for 10 min, followed by 40 extension cycles and a final cycle at 72 °C for 5 min.

Table 1. The list of primers used in this study.

Primers	Sequence (5'→3')	Size (bp)
<i>icaA</i>	F: ACACCTTGCTGGCGCAGTCAA R: TCTGGAACCAACATCCAACA	188
<i>icaD</i>	F: ATGGTCAAGCCCAGACAGAG R: AGTATTTTCAATGTTAAAGCAA	198
<i>rhII</i>	F: TTCATCCTCCTTTAGTCTTCCC R: TTCCAGCGATTGAGAGAGC	155
<i>rhIR</i>	F: TGCATTTTATCGATCAGGGC R: CACTTCCTTTTCCAGGACG	133
<i>VEGF-A</i>	F: AGGGCAGAATCATCACGAAGT R: AGGGTCTCGATTGGATGGCA	173
<i>HLA-G5</i>	F: CTGAGATGGAAGCAGTCTT R: GCTCCCTCCTTTTCAATCT	185
<i>TNF-α</i>	F: AGTTCCCAAATGGCCTCCCTCTCA R: GTGGTTTGCTACGACGTGGGCT	205
<i>IL-1β</i>	F: GCAACTGTTCTGAAGTCAACT R: ATCTTTTGGGGTCCGTCAACT	89
<i>NF-κB</i>	F: GCGGGAGAGGGGATTCCCTGCGGCCCG R: CGGGGCCGAGGGAATCCCCTCTCCCGC	169
<i>GAPDH</i>	F: AACTTTGGCATTGTGGAAGG R: ACACATTGGGGGTAGGAACA	223

F: forward; R: reverse.

2.6. Antibacterial effects of ZNPs

2.6.1. Bacteria

Staphylococcus aureus (*S. aureus*) (ATCC 25323), and *Pseudomonas aeruginosa* (*P. aeruginosa*) were obtained from the American Type Culture Collection (ATCC). The frozen bacteria were thawed and incubated at 37 °C for 24 h on nutrient agar plates.

2.6.2. The standard McFarland 0.5 solution

The McFarland 0.5 solution was prepared in accordance with established protocols by combining bacterial colonies with physiological serum and assessing the turbidity of the mixture in comparison to the dilution of the standard 0.5 McFarland solution, as described in previous literature[20].

2.6.3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The *in vitro* antibacterial susceptibility test of ZNPs was conducted following the standard protocol of the National Committee for Clinical Laboratory Standards against common skin bacteria. This

was achieved by determining MIC and MBC using the microdilution method[21]. In summary, 50 μ L of Mueller-Hinton broth (MHB) was dispensed into the designated wells, excluding the control wells. Subsequently, 100 μ L of the synthesized ZNPs and gentamicin were added to the first and second rows (control wells), followed by dilutions from the second to the tenth row. This involved transferring 50 μ L from the second row to the third row, and likewise from the third row to the fourth row, continuing up to the tenth row. Following this, 50 μ L equivalent to half the turbidity of a 24-hour culture of microorganisms, with a McFarland CFU/mL of 1.5×10^8 (as determined by absorbance at 625 nm wavelength), was added to rows two through ten. The plates were then incubated for 24 h at 37 °C. Subsequently, 2,3,5-triphenyltetrazolium chloride salt was introduced into the wells, and those without color development were identified as the MIC. In the following stage, wells without color were subcultured, and the lowest concentration without visible colonies was considered the MBC. The test was conducted in triplicate.

2.7. Effect of ZNPs on inhibition of biofilm production

The study evaluated the inhibitory effects of ZNPs on biofilm formation in the tested bacteria using a method described previously[22]. In brief, the bacterial suspension of the tested bacteria was combined with various concentrations of ZNPs in a 96-well microtiter plate and then incubated for 24 h at 37 °C. The positive control consisted of a bacterial suspension without ZNPs, while the negative control included wells containing brain-heart infusion broth and 2% sucrose. Following the removal of free cells by washing the wells, the crystal violet assay was used to evaluate cell adhesion and biofilm formation. Subsequently, the optical density of the wells was measured at 600 nm using an ELISA reader (Biotek ELX-800TS, USA). The minimum biofilm inhibition concentration (MBIC₅₀) was determined as the lowest concentration of ZNPs that inhibited biofilm production by at least 50%. The test was conducted in triplicate.

2.8. Effect of ZNPs on the expression level of biofilm genes

The effects of ZNPs on the expression of biofilm-related genes in *Staphylococcus* spp. [intracellular adhesion A (*icaA*) and D (*icaD*)] and *P. aeruginosa* (*rhII* and *rhIR*) were investigated using quantitative real-time PCR. In brief, the bacterial suspension was subjected to ZNPs at $\frac{1}{3}$ MIC, $\frac{1}{2}$ MIC and MIC at 37 °C for 24 h. Total RNA was extracted using a commercial kit (Qiagen, Germany), and cDNA was synthesized using the cDNA reverse transcription Kit (Qiagen, Iran). The influence of ZNPs on the expression levels of *icaA*, *icaD*, *rhII*, and *rhIR* genes was assessed using SYBR Green real-time PCR. The primers for these genes, as well as *GAPDH* as the reference gene, are

detailed in Table 1[23]. The thermal reaction involved denaturation for 15 min at 96 °C, annealing for 20 s at 57 °C, and extension for 25 s at 72 °C over 40 cycles. The expression levels were determined using the $2^{-\Delta\Delta Ct}$ method with iQTM5 optical system software (Bio-Rad, Hercules, CA). The reactions for each sample were run in triplicate unless otherwise specified.

2.9. Statistical analysis

The data were expressed as mean $\pm$ standard deviation and analyzed by one-way ANOVA test using SPSS software 26.0. A significance level of $P < 0.05$ was employed to determine statistically significant differences.

3. Results

3.1. Characterization of ZNPs

Figure 1A shows the confirmation of the ZNPs synthesis using UV-vis spectral analysis, which demonstrated an absorption peak in the range of 280–320 nm. SEM analysis showed that the ZNPs had a solid appearance with a spherical shape (Figure 1B). ZNPs ranged in size from 10 to 70 nm with an average size of 45 nm, while displaying a zeta potential of -18.7 mV (Figure 1C). The FTIR results displayed absorption bands at 3315, 1526, and 715

cm^{-1} , which were associated with the stretching of the O-H bands, carbonyl group (C=O), and the presence of Zn ions, respectively (Figure 1D). The XRD findings revealed diffraction peaks at (100), (003), (101), (102), (110), (103), and (112), which were observed at 27.3, 31.7, 33.4, 44.3, 52.0, and 69.1 degrees, respectively (Figure 1E).

3.2. Wound healing effects of ZNPs

The results of the MTT assay showed that ZNPs dose-dependently reduced the viability of fibroblast cells (93.10%, 85.90%, 71.30%, and 44.60% at 50, 100, 200, and 400 $\mu\text{g/mL}$ respectively). The calculated CC_{50} value for ZNPs synthesized using environmentally friendly green methods was 362.4 $\mu\text{g/mL}$. The concentrations of ZNPs selected for further investigation were based on the CC_{50} value. It was observed that these concentrations resulted in a dose-dependent increase in the proliferation of fibroblast cells. ZNPs at concentrations of 5, 10, 15, and 20 $\mu\text{g/mL}$ increased the fibroblast proliferation rate by 3.56%, 9.30%, 14.10%, and 26.40%, respectively.

Moreover, the results of the scratch test demonstrated that the application of ZNPs led to a dose-dependent enhancement in wound healing. After a 16-hour exposure, the rate of wound closure increased in a dose-dependent manner by ZNP treatment. A significant enhancement was observed at concentrations of 15 and 20 $\mu\text{g/mL}$ compared to the control group. A similar trend was also

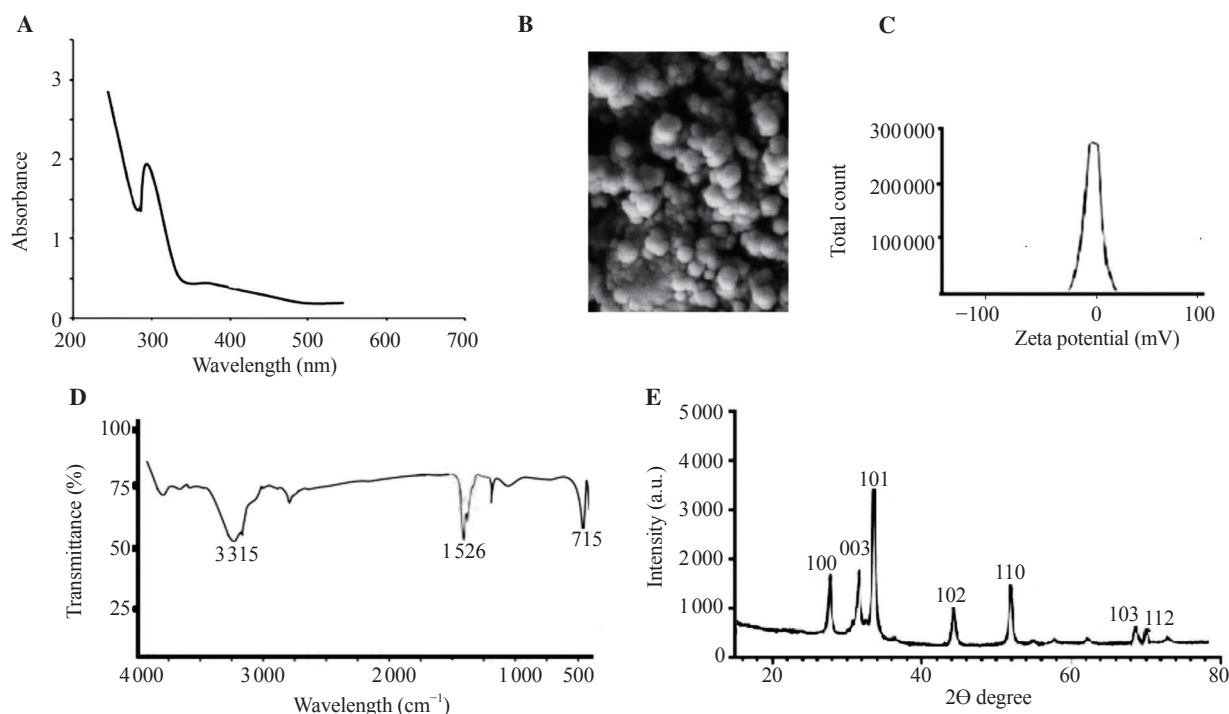


Figure 1. UV-vis (A), scanning electron microscope (B), zeta potential (C), Fourier transform infrared spectroscopy (D) and X-ray diffraction (E) analyses of green synthesized zinc nanoparticles.

observed in the ZNPs-treated group after 24 h of exposure, with 10, 15, and 20 µg/mL showing a significant improvement in wound closure compared to the control group (Figure 2).

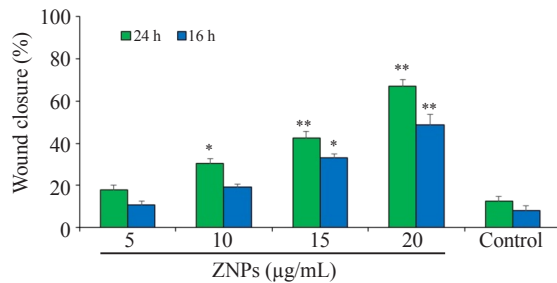


Figure 2. Effects of zinc nanoparticles on wound closure in human skin fibroblast Hs27. The data are presented as mean $\pm$ standard deviation ($n=3$) and analyzed by one way ANOVA. * $P<0.05$ and ** $P<0.01$ compared with the control group. ZNPs: zinc nanoparticles.

3.3. Effects of ZNPs on the expression level of angiogenesis-related genes

As shown in Figure 3, the gene expressions of *HLA-G5* and *VEGF-A* were significantly upregulated in cells treated with ZNPs at $\frac{1}{2}$ CC_{50} ($P<0.05$) and CC_{50} ($P<0.01$) compared to the control group. However, no noticeable changes were found in ZNPs at $\frac{1}{3}$ CC_{50} ($P>0.05$).

3.4. Effects of ZNPs on the expression level of inflammation-related genes

As depicted in Figure 4, ZNPs dose-dependently downregulated the expression levels of inflammatory genes *NF- κ B*, *IL-1 β* , and *TNF- α* in LPS-induced RAW 264.7 cells compared to the control group. The significant downregulation of inflammation-related genes *IL-1 β* and *TNF- α* was observed after treating cells with $\frac{1}{2}$ CC_{50} ($P<0.05$),

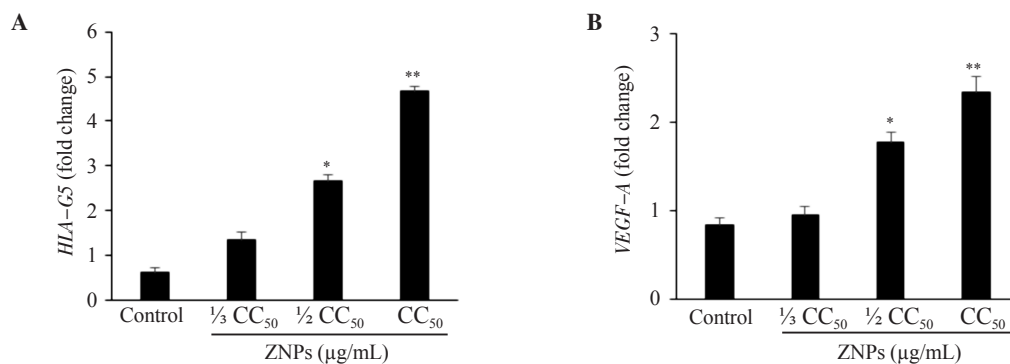


Figure 3. Effects of zinc nanoparticles on the expression level of angiogenesis-related genes [A: human leukocyte antigen-G5 (*HLA-G5*); B: vascular endothelial growth factor A (*VEGF-A*)]. The data are presented as mean $\pm$ standard deviation ($n=3$) and analyzed by one way ANOVA. * $P<0.05$ and ** $P<0.01$ compared with the control group.

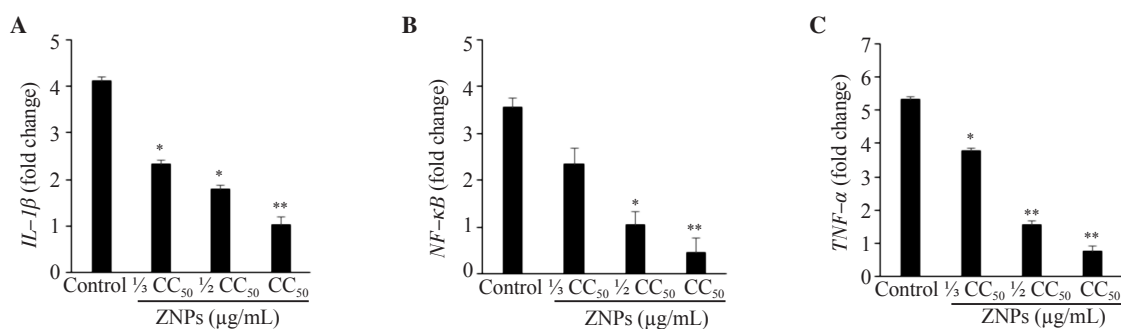


Figure 4. Effects of green synthesized zinc nanoparticles on the expression level of inflammation-related genes of interleukin-1 β (*IL-1 β*), nuclear factor kappa B (*NF- κ B*), and tumor necrosis factor alpha (*TNF- α*). The data are presented as mean $\pm$ standard deviation ($n=3$) and analyzed by one way ANOVA. * $P<0.05$ and ** $P<0.01$ compared with the control group.

Table 2. Antibacterial and antibiofilm effects of green synthesized zinc nanoparticles against the bacteria involved in wound healing by determining the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibition concentration 50% (MBIC₅₀) (µg/mL, $n=3$).

Treatment	<i>Staphylococcus aureus</i>			<i>Pseudomonas aeruginosa</i>		
	MIC	MBC	MBIC ₅₀	MIC	MBC	MBIC ₅₀
ZNP	6.60 $\pm$ 2.80	10.00 $\pm$ 0.00	3.30 $\pm$ 1.40	5.00 $\pm$ 0.00	5.00 $\pm$ 0.00	2.08 $\pm$ 0.72
Gentamicin	3.30 $\pm$ 1.40	3.30 $\pm$ 1.40	1.60 $\pm$ 0.72	3.30 $\pm$ 1.40	3.30 $\pm$ 1.40	1.60 $\pm$ 0.72

$\frac{1}{2}$ CC_{50} ($P<0.05$), and CC_{50} ($P<0.01$) compared to control cells. On the other hand, cells treated with ZNPs at $\frac{1}{2}$ CC_{50} ($P<0.05$) and CC_{50} ($P<0.01$) led to a significant decline in *NF- κ B* gene expression compared to control cells.

3.5. Antimicrobial and antibiofilm effects of ZNPs

As indicated in Table 2, the MIC and MBC values of ZNPs ranged from 5 μ g/mL to 10 μ g/mL. Notably, *P. aeruginosa* was the most sensitive strain to ZNPs, with the lowest MIC and MBC values of 5.0 μ g/mL. In terms of biofilm inhibition, the results indicated that ZNPs inhibited the biofilm formation in *S. aureus* and *P. aeruginosa* with $MBIC_{50}$ values of 3.30 and 2.08 μ g/mL, respectively.

3.6. Effect on the expression level of biofilm genes

As shown in Figure 5, a dose-dependent downregulation of *icaA*, *icaD*, *rhlI*, and *rhlR* gene expression was found in the ZNPs-treated bacteria. ZNP at $\frac{1}{2}$ MIC and MIC caused a significant decrease in *icaA* and *icaD* gene expression in *S. aureus* ($P<0.01$); whereas, ZNP at $\frac{1}{3}$ MIC ($P<0.05$), $\frac{1}{2}$ MIC ($P<0.01$), and MIC ($P<0.01$) significantly downregulated *P. aeruginosa* biofilm-related genes (*rhlI* and *rhlR*).

4. Discussion

Nowadays, the use of plant extracts as reducing and capping agents in the environmentally friendly synthesis of ZNPs has emerged as a prominent area of investigation in recent studies[13]. This method offers numerous benefits, such as simplicity, environmental friendliness, safety, cost-effectiveness, and reproducibility, leading

to the achievement of high stability in the resulting ZNPs[13]. In the present study, ZNPs were green synthesized using *F. macrocolea* extract. They ranged in size from 10 to 70 nm, with an average size of 45 nm. Reportedly, studies have shown that the stability and biological functions of NPs are affected by their size[24]. Smaller NPs generally exhibit higher levels of stability and a reduced tendency to aggregate[24]. Due to their diminutive sizes, NPs are more adept at traversing cell membranes and have the potential to enhance the pharmacokinetics, pharmacodynamics, and biodistribution of therapeutic compounds[24].

The surface charge of NPs significantly affects their stability, biocompatibility, and catalytic activity[25]. This factor has been extensively investigated within the realm of NPs to prevent their aggregation. Conversely, the electric charge of particles is a crucial factor in their interactions with cells and microorganisms, as well as their ability to attract ions during water purification processes[25]. While metal NPs have exhibited various zeta potential values, previous studies have suggested that biogenic synthesis can enhance the zeta potential of metal NPs, resulting in improved stability and better control of aggregation and agglomeration[26]. It is well-established that NPs are commonly stabilized through electrostatic interactions. Zeta potential values above 30 mV or below -30 mV indicate effective stabilization[26].

Numerous methodologies have been established for synthesizing ZNPs, including sol-gel, hydrothermal, spray pyrolysis, microwave-assisted techniques, chemical vapor deposition, ultrasonic conditions, and precipitation methods. These conventional approaches are characterized by their high energy requirements and the use of toxic and hazardous substances, which pose potential biological hazards. Conversely, biological pathways are increasingly favored due to their simplicity, environmental friendliness, safety, and cost-effectiveness, often requiring only a single step for synthesis[27].

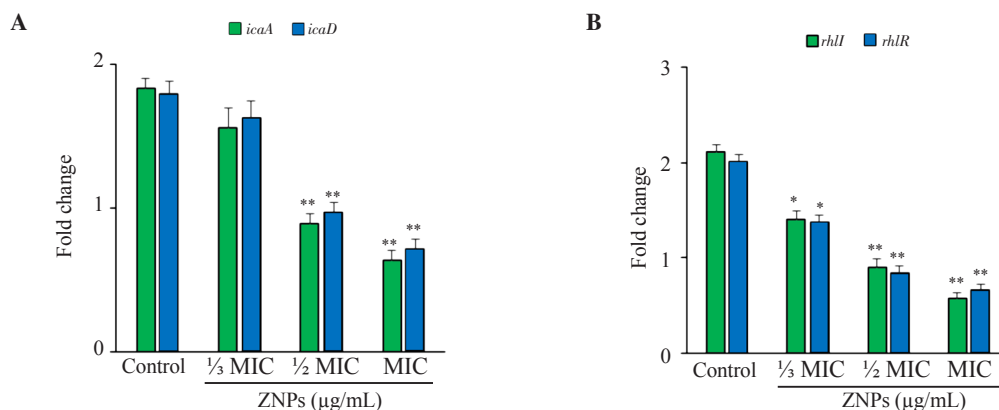


Figure 5. Effects of green synthesized zinc nanoparticles on the expression level of biofilm-related genes in (A) *Staphylococcus aureus* [intracellular adhesion A (*icaA*) and D (*icaD*)] and (B) *Pseudomonas aeruginosa* (*rhlI* and *rhlR*). The data are presented as mean $\pm$ standard deviation ($n=3$) and analyzed by one way ANOVA. * $P<0.05$ and ** $P<0.01$ compared with the control group.

Several studies have reported the green synthesis of ZNPs using extracts derived from various plants, such as *Azadirachta indica*, *Aloe vera*, *Agathosma betulina*, *Ocimum basilicum*, *Cocos nucifera*, and *Spathodea campanulata*, resulting in particle sizes ranging from 9 to 140 nm[27]. The variation in size of green-synthesized ZNPs is likely attributed to factors such as the method of green synthesis, type of plants, the plant part used, and the solvents and catalysts employed in green processes[27].

It has been proven that the wound healing process consists of four sequential yet interconnected stages: hemostasis, inflammation, proliferation, and remodeling[3]. Various cellular components, enzymes, cytokines, proteins, and hormones are involved in the repair of damaged tissues. Following an injury, hemostasis initiates the formation of blood clots and constriction of blood vessels to control blood flow, followed by the release of proinflammatory cytokines and growth factors[28]. These growth factors promote inflammation by recruiting macrophages, neutrophils, and lymphocytes through epithelial cells[28,29]. Angiogenesis is then stimulated by growth factors, leading to re-epithelialization through the proliferation of fibroblasts and keratinocytes[30]. Subsequently, fibroblasts differentiate into myofibroblasts, contributing to the synthesis of extracellular matrix at a later stage[30]. The results indicated that the green synthesized ZNPs exhibited potent *in vitro* wound healing activity by increasing the proliferation of fibroblast cells and accelerating the rate of wound closure in a dose-dependent manner after 16 and 24 h. In recent years, several studies have demonstrated the wound-healing effects of ZNPs synthesized using extracts from *Ilex paraguariensis*, *Nigella sativa*, *Prosopis farcta*, and *Ziziphora clinopodioides*[8–11]. VEGF is a key driver of angiogenesis, stimulating endothelial cells, acting as a chemotactic agent, and enhancing vascular permeability[31]. Conversely, HLA-G5 is crucial for immune tolerance in the body, playing a significant role in immunomodulation[32]. Our findings revealed a significant increase in the expression of both *HLA-G5* and *VEGF-A* genes in cells treated with ZNPs compared to the control group. This suggests that ZNPs stimulated angiogenesis and promoted wound healing.

Several studies have emphasized the crucial role of inflammation in the body's defense against pathogens and in clearing dead tissues at injury sites. Prolonged inflammation, however, can have negative effects by disrupting normal wound-healing processes and contributing to excessive scarring. Therefore, managing and reducing inflammation can be beneficial for wound healing[33]. Our findings indicated that ZNPs dose-dependently downregulated the expression levels of inflammatory genes *NF-κB*, *IL-1β*, and *TNF-α* in LPS-induced RAW 264.7 cells. This suggests that ZNPs could be an effective tool for controlling inflammation and ultimately enhancing wound healing.

Reportedly, microbial pathogens in a chronic wound create a

biofilm structure by aggregating within an extracellular matrix. This formation enhances their resistance to host immune responses and antimicrobial agents. This biofilm configuration hinders wound healing by promoting an inadequate inflammatory response from the host and causing harm to host tissues; therefore, addressing the biofilm is crucial in the management of chronic wounds[3]. Quorum sensing in *P. aeruginosa* is responsible for regulating various virulence factors. Two distinct quorum sensing systems, known as Las and Rhl, have been identified in *P. aeruginosa*. The Rhl system involves the transcriptional activators RhlR and RhlI, which play a crucial role in the formation and development of biofilms[34,35]. On the other hand, in *S. aureus* bacteria, the coexpression of *icaA* with *icaD* leads to a significant increase in the formation and development of biofilms[36]. Our results showed that ZNPs demonstrated significant antimicrobial efficacy against *S. aureus* and *P. aeruginosa*, with MIC and MBC values of ZNPs ranging from 5 µg/mL to 10 µg/mL. The results also found that ZNPs inhibited the biofilm formation in *S. aureus* and *P. aeruginosa* and significantly downregulated the expression of biofilm formation-related genes *icaA*, *icaD*, *rhlI*, and *rhlR* in the tested bacteria. Previous studies have also revealed that ZNPs exhibit antimicrobial effects *via* various possible mechanisms, such as inhibiting biofilm formation, generating reactive oxygen species, oxidizing proteins, peroxidizing lipids, and degrading DNA[37]. The main limitations of the study include the absence of animal studies, the need for further investigation into the detailed mechanisms of ZNPs, and their systemic toxicities.

In conclusion, the obtained results showed that ZNPs displayed promising antibacterial effects against *S. aureus* and *P. aeruginosa*, which are the main causes of wound infection. The current study also revealed that green-synthesized ZNPs have potent wound-healing effects by inhibiting biofilm formation, inducing angiogenesis, and reducing inflammation. Therefore, ZNPs can be considered an active wound dressing due to its positive effects on controlling infection, regulating inflammation, and promoting angiogenesis in wounds. Further research is warranted to explore the efficacy of green-synthesized ZNPs in animal models of wound healing and antimicrobial applications. Additionally, investigations need to comprehensively elucidate the mechanisms of cytotoxicity associated with these NPs and assess their long-term safety and efficacy for wound healing.

Conflict of interest statement

The author declares no competing interest.

Funding

The study received no extramural funding.

Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

References

- [1] Gonzalez AC, Costa TF, Andrade ZA, Medrado AR. Wound healing - A literature review. *An Bras Dermatol* 2016; **91**(5): 614-620.
- [2] Murphy PS, Evans GR. Advances in wound healing: A review of current wound healing products. *Plast Surg Int* 2012; **2012**. doi: 10.1155/2012/190436.
- [3] Guo S, Dipietro LA. Factors affecting wound healing. *J Dent Res* 2010; **89**(3): 219-229.
- [4] Rowan MP, Cancio LC, Elster EA, Burmeister DM, Rose LF, Natesan S, et al. Burn wound healing and treatment: Review and advancements. *Crit Care* 2015; **19**: 243. doi: 10.1186/s13054-015-0961-2.
- [5] Alrashoudi RH, Abudawood M, Mateen A, Tabassum H, Alghumlas NI, Fatima S, et al. Characterization and antimicrobial, antioxidant, and anti-proliferative activities of green synthesized magnesium oxide nanoparticles with shoot extracts of *Plicosepalus curviflorus*. *Asian Pac J Trop Biomed* 2023; **13**(7): 315-324.
- [6] Albalawi AE, Alanazi AD, Baharvand P, Sepahvand M, Mahmoudvand H. High potency of organic and inorganic nanoparticles to treat cystic echinococcosis: An evidence-based review. *Nanomaterials* 2020; **10**(12). doi: 10.3390/nano10122538.
- [7] Saadatmand M, Al-Awsi GR, Alanazi AD, Sepahvand A, Shakibaie M, Shojaei S, et al. Green synthesis of zinc nanoparticles using *Lavandula angustifolia* Vera. extract by microwave method and its prophylactic effects on *Toxoplasma gondii* infection. *Saudi J Biol Sci* 2021; **28**(11): 6454-6460.
- [8] Bandeira M, Chee BS, Frassini R, Nugent M, Giovanela M, Roesch-Ely M, et al. Antimicrobial PAA/PAH electrospun fiber containing green synthesized zinc oxide nanoparticles for wound healing. *Materials* 2021; **14**(11). doi: 10.3390/ma14112889.
- [9] Bai X, Jarubula R. Development of novel green synthesized zinc oxide nanoparticles with antibacterial activity and effect on diabetic wound healing process of excisional skin wounds in nursing care during sports training. *Inorg Chem Commun* 2023; **150**. doi: 10.1016/j.inoche.2023.110453.
- [10] Khatami M, Varma RS, Zafarnia N, Yaghoobi H, Sarani M, Kumar VG. Applications of green synthesized Ag, ZnO and Ag/ZnO nanoparticles for making clinical antimicrobial wound-healing bandages. *Sustain Chem Pharm* 2018; **10**: 9-15.
- [11] Mahdavi B, Saneei S, Qorbani M, Zhaleh M, Zangeneh A, Zangeneh MM, et al. *Ziziphora clinopodioides* Lam leaves aqueous extract mediated synthesis of zinc nanoparticles and their antibacterial, antifungal, cytotoxicity, antioxidant, and cutaneous wound healing properties under *in vitro* and *in vivo* conditions. *Appl Organ Chem* 2019; **33**(11): e5164.
- [12] Balalakshitha M, Kolanjinathan K, Aishwarya T, Vinothini B. A review on diabetic wound infection and its treatment using zinc oxide nanoparticle synthesized by green chemistry techniques. *BEPLS* 2021; **10**: 1-22.
- [13] Hussain I, Singh NB, Singh A, Singh H, Singh SC. Green synthesis of nanoparticles and its potential application. *Biotechnol Lett* 2016; **38**: 545-560.
- [14] Sharma P, Kaushal A. Green nanoparticle formation toward wound healing, and its application in drug delivery approaches. *European J Med Chem Rep* 2022; **6**. doi: 10.1016/j.ejmcr.2022.100088.
- [15] Mahmoudvand H, Yadegari JG, Khalaf AK, Hashemi MJ, Dastyarhaghighi S, Salimikia I. Chemical composition, antileishmanial, and cytotoxic effects *Ferula macrecolea* essential oil against *Leishmania tropica*. *Parasite Epidemiol Control* 2022; **19**. doi: 10.1016/j.parepi.2022.e00270.
- [16] Albalawi AE, Alanazi AD, Alyousif MS, Sepahvand A, Ebrahimi K, Niazi M, et al. The high potency of green synthesized copper nanoparticles to prevent the *Toxoplasma gondii* infection in mice. *Acta Parasitol* 2021; **66**(4): 1472-1479.
- [17] Albalawi AE, Abdel-Shafy S, Khudair Khalaf A, Alanazi AD, Baharvand P, Ebrahimi K, et al. Therapeutic potential of green synthesized copper nanoparticles alone or combined with meglumine antimoniate (glucantime®) in cutaneous leishmaniasis. *Nanomaterials* 2021; **11**(4). doi: 10.3390/nano11040891.
- [18] Fronza M, Heinzmann B, Hamburger M, Laufer S, Merfort I. Determination of the wound healing effect of *Calendula* extracts using the scratch assay with 3T3 fibroblasts. *J Ethnopharmacol* 2009; **126**(3): 463-467.
- [19] Kwon N, Lee KE, Singh M, Kang SG. Suitable primers for *GAPDH* reference gene amplification in quantitative RT-PCR analysis of human gene expression. *Gene Rep* 2021; **24**. doi: 10.1016/j.genrep.2021.101272.
- [20] Kordestani M, Rashidipour M, Mahmoudvand H, Jalali S, Kooshki F. Antibacterial and cytotoxicity of chitosan nanocomposite loaded with thymol against some cariogenic bacteria. *J Herbm Pharm* 2023; **12**(2): 223-227.
- [21] Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing; Twenty-second informational supplement*. Wayne, PA: CLSI; 2012.
- [22] Mehdipour A, Ehsani A, Samadi N, Ehsani M, Sharifinejad N. The antimicrobial and antibiofilm effects of three herbal extracts on *Streptococcus mutans* compared with Chlorhexidine 0.2% (*in vitro* study).

- J Med Life* 2022; **15**(4): 526-532.
- [23]Kot B, Sytykiewicz H, Sprawka I. Expression of the biofilm-associated genes in methicillin-resistant *Staphylococcus aureus* in biofilm and planktonic conditions. *Int J Mol Sci* 2018; **19**(11). doi: 10.3390/ijms19113487.
- [24]Fakhari S, Jamzad M, Kabiri Fard H. Green synthesis of zinc oxide nanoparticles: A comparison. *Green Chem Lett Rev* 2019; **12**(1): 19-24.
- [25]Fröhlich E. The role of surface charge in cellular uptake and cytotoxicity of medical nanoparticles. *Int J Nanomed* 2012; **7**: 5577-5591.
- [26]Honary S, Zahir F. Effect of zeta potential on the properties of nano-drug delivery systems-a review (Part 1). *Tropical J Pharm Res* 2013; **12**(2): 255-264.
- [27]Agarwal H, Kumar SV, Rajeshkumar S. A review on green synthesis of zinc oxide nanoparticles—An eco-friendly approach. *Resour Effic Technol* 2017; **3**(4): 406-413.
- [28]Asif M, Chaudhry AS, Ashar A, Rashid HB, Saleem MH, Aslam HB, et al. Zinc oxide nanoparticles accelerate the healing of methicillin-resistant *Staphylococcus aureus* (MRSA)-infected wounds in rabbits. *Asian Pac J Trop Biomed* 2023; **13**(11): 488-496.
- [29]Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound healing: A cellular perspective. *Physiol Rev* 2019; **99**(1): 665-706.
- [30]Veith AP, Henderson K, Spencer A, Sligar AD, Baker AB. Therapeutic strategies for enhancing angiogenesis in wound healing. *Adv Drug Deliv Rev* 2019; **146**: 97-125.
- [31]Cherreddy KK, Her CH, Comune M, Moia C, Lopes A, Porporato PE, et al. PLGA nanoparticles loaded with host defense peptide LL37 promote wound healing. *J Control Release* 2014; **194**: 138-147.
- [32]Su T, Wang H, Yao Y. Novel nucleic acid aptamer gold (Au)-nanoparticles (AuNPs-AptHLA-G5-1 and AuNPs-AptHLA-G5-2) to detect the soluble human leukocyte antigen G5 subtype (HLA-G5) in liquid samples. *Ann Transl Med* 2021; **9**(18): 1416. doi: 10.21037/atm-21-3334.
- [33]Shukla SK, Sharma AK, Gupta V, Yashavardhan MH. Pharmacological control of inflammation in wound healing. *J Tissue Viability* 2019; **28**(4): 218-222.
- [34]Rasamiravaka T, Labtani Q, Duez P, El Jaziri M. The formation of biofilms by *Pseudomonas aeruginosa*: A review of the natural and synthetic compounds interfering with control mechanisms. *Biomed Res Int* 2015; **2015**. doi: 10.1155/2015/759348.
- [35]Cheraghipour K, Shakib P, Khalaf AK, Mehrnia M, Moridnia A, Amiri S, et al. Unveiling therapeutic effects of *Thymbra spicata* L. on cystic echinococcosis. *Jundishapur J Nat Pharm Prod* 2024; **19**(2). doi: 10.5812/jjnpp-146063.
- [36]Peng Q, Tang X, Dong W, Sun N, Yuan W. A review of biofilm formation of *Staphylococcus aureus* and its regulation mechanism. *Antibiotics* 2022; **12**(1). doi: 10.3390/antibiotics12010012.
- [37]Correa MG, Martínez FB, Vidal CP, Streitt C, Escrig J, de Dicastillo CL. Antimicrobial metal-based nanoparticles: A review on their synthesis, types and antimicrobial action. *Beilstein J Nanotechnol* 2020; **11**(1): 1450-1469.

Publisher's note

The Publisher of the *Journal* remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Edited by Liang Q, Tan BJ