Original Article

Wound healing activity of green synthesized copper nanoparticles through cell proliferation-migration, antimicrobial effects, and nitric oxide triggering

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ABSTRACT

The present experimental study aimed to assess the *in vitro* wound healing and anti-inflammatory effects of green synthesized copper nanoparticles (CuNPs) by the methanol extract of *Ferula macrecolea* (Boiss), as a plant with various pharmacological effects, such as anti-inflammatory and antimicrobial effects, in traditional and modern medicine. The precipitation approach was used for the green synthesis of CuNPs by mixing the methanol and copper sulfate solution. Cell viability and fibroblast proliferation assay were performed by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay. The migration abilities of fibroblast cells were evaluated using the *in vitro* scratch assay for wound healing. The effects of CuNPs on gene expression of inducible nitric oxide synthesis (iNOS) were also examined by real-time polymerase chain reaction (PCR). *In vitro* antibacterial susceptibility test of CuNPs was carried out according to the standards protocol of the National Committee for Clinical Laboratory Standards. The scanning electron microscope analysis revealed that the green synthesized CNP exhibited a globular shape with a size ranging from 15 to 90 nm, while the majority were at 40-60 nm. The results of the MTT assay demonstrated that the calculated 50% cytotoxic concentration (CC_{50}) value of green synthesized CuNPs was 236.3 µg/mL. The optimum concentrations of the CuNPs were selected based on the CC_{50}, which dose-dependently increased the proliferation of fibroblast cells. The CuNPs dose-dependently increased the rate of wound closure after 16 and 24 h. The results of the real-time PCR illustrated that CuNPs caused upregulation in the expression level of the iNOS gene in RAW 264.7 cells. CuNPs showed promising antimicrobial effects against *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa*. The present study highlighted the high potency of green CuNPs synthesized by *F. macrecolea* for wound healing through their antimicrobial properties, proliferation of fibroblast cells, and provoking iNOS.

Keywords: Antibacterial, *In vitro*, Nanotechnology, Treatment, Wound

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1. Introduction

Currently, it has been proven that wounds associated with some conditions, such as diabetes, gastric disorders, and duodenal ulcers, exert severe effects on the quality of life of people (1). Wounds commonly occur due to a cut on the skin after physical injury, burns, infections, or chronic disorders that interrupt the normal anatomy and function of the skin. During wound conditions, the connective tissue under the skin and epithelial tissue integrity are degraded and reduced (1). Wound healing is a complex process, encompassing the inflammation stage, cellular proliferation, and renovation, where cell-to-cell and cell-matrix communications happen (2). For healing development, some enzymes, such as collagenase and elastase, play crucial roles in reducing extracellular matrix factors (e.g., elastin, fibrin, and collagen) (3). Nevertheless, their action must be moderated by inhibiting and increasing the level of these enzymes, which can result in decreased wound healing (3). The search for new or alternative therapy obtained by nanotechnology, such as green synthesized nanoparticles, for healing acute and chronic wounds has received more attention (4). Research has demonstrated that nanoparticles (NPs) have been extensively used in wound healing due to their better adsorption capacity, antimicrobial properties, and drug loading (5). Although wound healing properties of some metal NPs, such as silver (AgNPs), Iron (FenNPs), gold (AuNPs), Zinc (ZnNPs), and copper (CuNPs), have been proven in several studies, there are discrepancies in the results due to some factors, such as the type of metal used, the synthesis method of nanoparticles, and the method of research (6). Among metal NPs, CuNPs are a suitable option in dressings for wound healing since they provoke angiogenesis and skin regeneration, facilitating the healing procedure (7). In light of the aforementioned issues, the present experimental study aimed to evaluate the *in vitro* wound healing effects of CuNPs green synthesized by the methanol extract of *Ferula macrecolea* (Boiss), which is a plant with various pharmacological effects, such as anti-inflammatory and antimicrobial effects, in traditional and modern medicine (8-10).

2. Materials and Methods

2.1. Green synthesis of CuNPs

Aerial parts of *F. macrecolea* were purchased from a plant shop in Tehran, Iran, in April 2022. To extract, 300 g of air-dried and powdered materials were subjected to 70% methanol for three days using a percolation procedure (11). Thereafter, the precipitation approach was used for the green synthesis of CuNPs by mixing the methanol and copper sulfate solution (1 mM) and incubation at 24°C for 12 h (11).

2.2. Determining the physical and chemical characteristics

Physical characterizations of CuNPs, including size and shape, were evaluated by scanning electron microscope (SEM) (Mira3, Made in Czech). The surface plasmon resonance of CuNPs was measured employing a UV-vis spectrophotometer (Shimadzu UV2550, Japan). The green synthesis of nanoparticles by *F. macrecolea* extract was also confirmed by an X-ray diffraction (XRD, 2000 APD, Italy) analysis.

2.3. Cell culture

The normal human skin fibroblast (Hs27) and macrophage (RAW 264.7) cell lines were obtained from the American Type Culture Collection (ATCC). They were cultivated in Dulbecco's Modified Eagle Medium (Merk, Germany) enhanced with 10% fetal bovine serum (Sigma-Aldrich, Germany) and 1% pen-strep (Sigma-Aldrich, Germany) at 37°C in an incubator with 5% CO_2. The cells were then adjusted to1×10^5/mL using a hemocytometer.

2.4. Cell viability assay

The assay was performed by exposing the 0.1 mL of fibroblast cells (1×10^5/mL) with CuNPs at 10-300 µg/mL concentrations in a 96-well plate and kept at 24°C for 48 h. Following that, 20 µL of MTT solution (Sigma-Aldrich, Germany) was added to wells and kept again in 5% CO_2 at 37°C for 240 min. After adding sulfuric acid as the stop solution, the absorbance of wells was recorded at 570 nm utilizing an enzyme-linked immunosorbent assay (ELISA) plate reader (12, 13). The 50% cytotoxic concentrations (CC_{50}) were calculated using the Probit test in SPSS software (version 26.0).

2.5. Fibroblast proliferation assay

Firstly, 0.1 mL of fibroblast cells (1×10^5/mL) was seeded into each 96-well plate and incubated for 24 h. After removing the supernatant, CuNPs and asiaticoside (positive control) were added to the wells and further incubated for 24 h. Subsequently, MTT solution (5 mg/mL) was added to wells and kept again in 5% CO_2 at 37°C for 4 h. After adding sulfuric acid as the stop solution, the absorbance of wells was recorded at 570 nm utilizing an ELISA plate reader (BIOTEK ELX-800TS, USA) (19). The viability was calculated as the percentage of proliferation (12, 13).

2.6. Cell scratch wound healing assay

According to the previous study, the migration abilities of fibroblast cells were evaluated using a cell scratch *in vitro* wound healing assay (14). At first, the cells (2×10^4 cells/mL) were cultured into a 48-well plate pending the formation of confluent cell monolayers. After producing a linear wound on the cell monolayer using a sterile pipette tip, the artifact cellular debris was discarded by washing the wells with phosphate-buffered saline. Thereafter, CuNPs at ½ CC_{50} and CC_{50} concentrations were added and incubated for 24 h. The cells were then visualized using an inverted microscope at 0 and 24 h. The ratio increases in wound closure were measured compared to the value achieved before treatment and reported as cell migration.

2.7. Effects of CuNPs on iNOS expression gene

RAW 264.7 cells (4×10^5/mL) were kept in a 24-well plate and incubated for 48°C. Following that, the CuNPs at ⅓ CC_{50}, ½ CC_{50}, and CC_{50} concentrations were again incubated for 24 h. Total RNA was isolated via the commercial kit procedures (Qiagen, Germany). The

obtained RNAs were transcribed utilizing the Fermentas kit, USA. Following that, the products were evaluated using SYBR green real-time polymerase chain reaction (PCR) based on the primers of iNOS F: GCC TCG CTC TGG AAA GA, iNOS R: TCC ATG CAG ACA ACC TT, β-actin F: GTGACGTTGACATCCGTAAAGA, and β-actin R: GCCGGACTCATCGTACTCC (15). To this end, basic denaturation was defined at 95°C for 10 min, 40 extension cycles, and a separate cycle at 72°C for 5 min. To end, $2^{-\Delta\Delta Ct}$ was estimated utilizing Bio-Rad iQ5 Optical System Software, USA.

2.8. Antimicrobial effects of CuNPs

Staphylococcus aureus (ATCC 25323), *Staphylococcus epidermidis*, and *Pseudomonas aeruginosa* were provided by the ATCC. The frozen bacteria were defrosted and cultured at 37°C for 24 h on nutrient agar plates (Sigma-Aldrich, Germany) and then adjusted in sterile saline to gain a bacterial suspension of 0.5 McFarland unit. *In vitro* antibacterial susceptibility test of CuNPs was carried out according to the standards protocol of the National Committee for Clinical Laboratory Standards against common skin bacteria by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) by the microdilution method (16).

2.9. Statistical analysis

All experiments were performed in triplicate and were indicated as mean ± standard error of the mean. Data were then analyzed in SPSS software (version 26.0) via a one-way ANOVA test. A p-value less than 0.05 was considered statistically significant.

3.1. Determining the physical and chemical characteristics

Figure 1 illustrates the physical and chemical characteristics of green synthesized CuNPs. The maximum absorption of CNP acquired from the UV-Vis was observed at 423 nm, which displayed the presence of CNP. As for physical properties, the SEM analysis revealed that the green synthesized CNP exhibited a globular shape with a size ranging from 15-90 nm, while the majority were at 40-60 nm. Considering the X-ray diffraction analysis, we observed the presence of a diffraction peak at 47.4º, 50.7º, and 71.3º corresponding to 111, 200, and 220, respectively, which is similar to the standard sample of CuNPs and confirms their crystal structure.

3.2. Cell viability and proliferation assay

The results of the MTT assay demonstrated that the calculated CC_{50} value of green synthesized CuNPs was 236.3 µg/mL. The optimum concentrations of CuNPs were selected based on CC_{50} value, whereas they dose-dependently increased the proliferation of fibroblast cells (Table 1).

3.3. Wound healing assay

As depicted in figure 2, the scratch test revealed that CuNPs dose-dependently increased the rate of wound closure. After 16 h exposure, the CuNPs dose-dependently increased the rate of wound closure; nonetheless, a significant increase was observed at concentrations of 15 and 20 µg/mL, compared to the control group. After 24 h exposure, the CuNPs dose-dependently increased the rate of wound closure; however, a significant increase was detected at concentrations of 10, 15, and 20 µg/mL, compared to the control group.

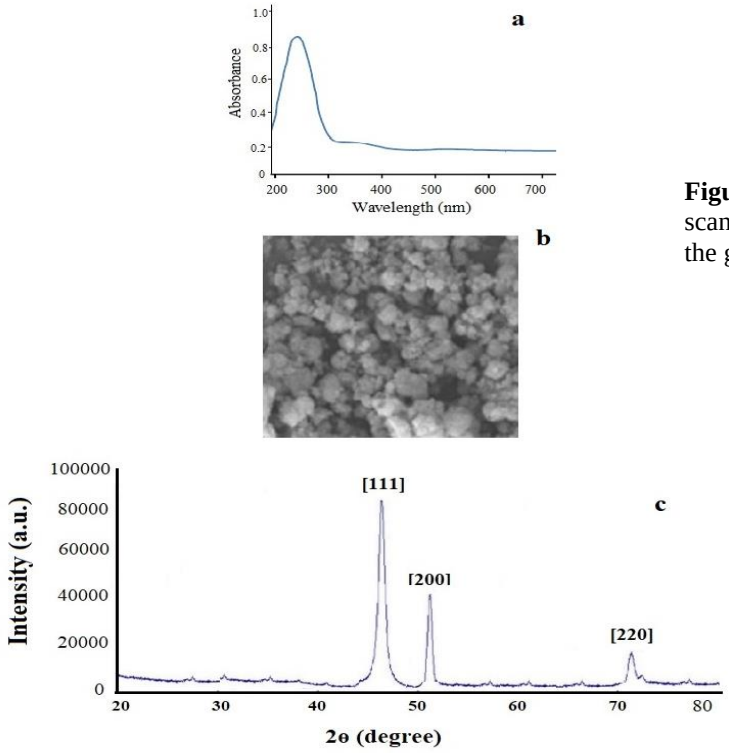

Figure 1. The findings of the analysis of UV-Vis (a) and scanning electron microscope (b) and X-ray diffraction (c) for the green synthesized copper nanoparticles.

Table 1. Effects of copper nanoparticles on cell viability and proliferation in skin fibroblast cells.

Cell cytotoxicity			Fibroblast proliferation assay	
Concentration (µg/mL)	**Viability (%)**	**IC_{50}(µg/mL)**	**Concentration (µg/mL)**	**Proliferation (%)**
Non-treated	97.6± 0.51	-	5	2.33± 0.71
50	89.4± 3.2	236.3	10	8.6± 1.58
100	76.4± 3.5	-	15	12.6± 3.5
200	56.6± 5.12	-	20	29.3± 2.21
300	36.5± 4.46	-	-	-

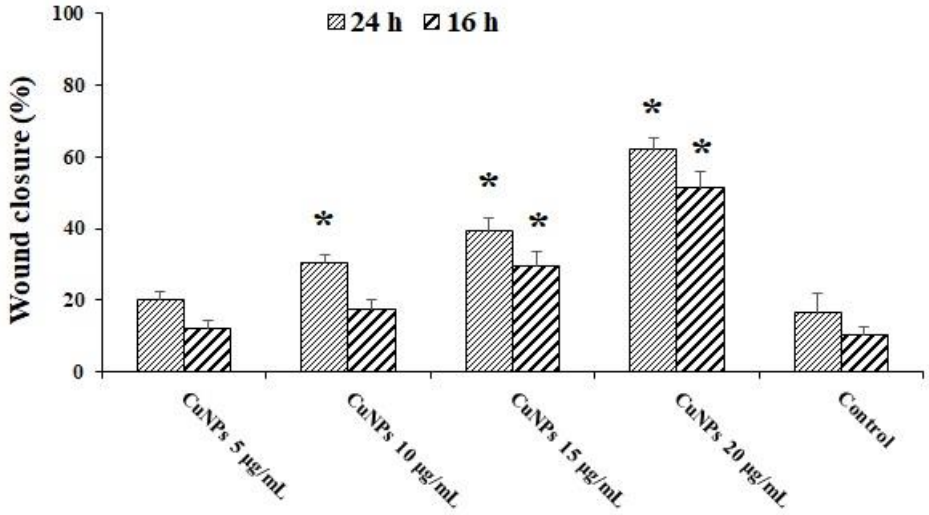

Figure 2. Effect of copper nanoparticles on the migration rate of scratched human skin fibroblast (Hs27). (Mean±sd) * significant difference compared to control.

3.4. Effects of CuNPs on iNOS expression gene

Figure 3 shows the effects of green synthesized CuNPs on the expression gene level of iNOS. The results of the real-time PCR indicated that CuNPs cause upregulation in the expression level of the iNOS gene in RAW 264.7 cells as a dose-dependent response. In this regard, at CC_{50} concentration, the maximum upregulation of iNOS gene expression was observed (P<0.05).

3.5. Antimicrobial effects of CuNPs

As displayed in table 2, CuNPs demonstrated promising antimicrobial effects against *S. aureus*, *S. epidermidis*, and *P. aeruginosa*. CuNPs illustrated that MIC and MBC values ranged from 11.3 to 17.3 µg/mL. The lowest MIC and MBC were reported for *P. aeruginosa* with MIC and MIC values of 2.66 and 3.33 µg/mL, respectively, indicating that *P. aeruginosa* was the most sensitive strain to CuNPs.

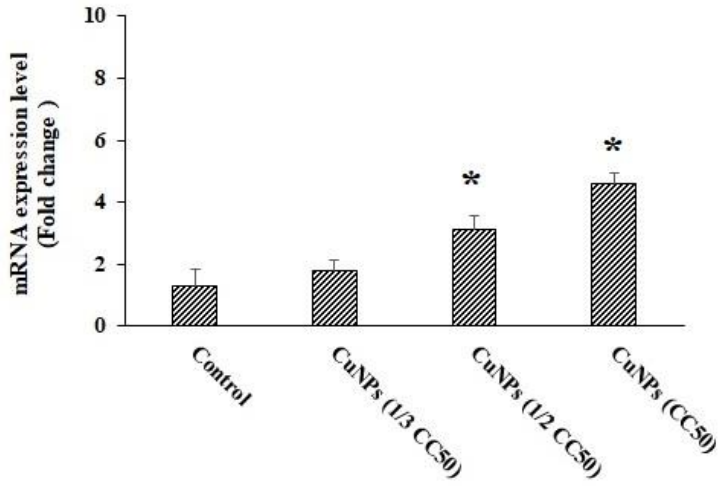

Figure 3. Effects of copper nanoparticles on iNOS expression genes in the RAW 264.7 cells by Real-time PCR. CC_{50}: The 50% cytotoxic concentrations; * p < 0.001 compare to untreated cells.

Table 2. Antibacterial effects of green synthesized copper nanoparticles against the bacteria involved in wound healing through the determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Drug	*Staphylococcus aureus*		*Staphylococcus epidermidis*		*Pseudomonas aeruginosa*	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
CuNPs	12.6±1.15	15.3±2.3	11.3 ± 1.15	13.1 ± 3.15	14.1 ± 3.15	17.1 ± 3.15
Gentamicin	2.66±1.15	3.33±1.15	2.66±1.15	3.33±1.15	4.0±1.17	4.0±1.17

4. Discussion

At present, there is a growing interest in exploring novel or alternative therapies obtained by nanotechnology, such as green synthesized nanoparticles, for the treatment of both acute and chronic wounds (6). Among metal NPs, CuNPs are suitable agents for wound healing since they provoke angiogenesis and skin regeneration, facilitating the healing procedure (7). Here, we evaluated the *in vitro* wound healing and anti-inflammatory effects of CuNPs green synthesized by the methanol extract *F. macrecolea* (Boiss). The SEM analysis revealed that green synthesized CNP exhibited a globular shape with a size ranging from 15-90 nm, while the majority were at 40-60 nm. The results of this study pointed out that green synthesized CNP exhibited a globular shape with a size ranging from 15-90 nm, while the majority were at 40-60 nm. It has been demonstrated that the stability and biological properties of nanoparticles depend on their size, whereas the therapeutic activity of nanoparticles is substantially increased by reducing their size to below 100 nm. Due to a greater surface-to-volume ratio, these particles are more stable and aggregate less frequently. We synthesized the CNPs smaller than 100 nm and suitable for antiparasitic application (17). The MTT assay demonstrated that the calculated CC_{50} value of green synthesized CuNPs was 236.3 µg/mL. The optimum concentrations of the CuNPs, selected based on the CC_{50} value, exhibited a dose-dependent enhancement in the proliferation of fibroblast cells. Previously, Alizadeh et al. (2019) reported that chemical synthetic CNP at the concentrations of 1-10 µM had no cytotoxicity on the cultured fibroblast, endothelial, and keratinocyte cells, whereas, at 1 µM concentration, it increased endothelial cell migration and proliferation (18). It was found that CuNPs dose-dependently increased the rate of wound closure after 16 and 24 h exposure. Hu et al. (2018) reported that copper-doped borate bioactive glass/poly (lactic-co-glycolic acid) dressing loaded with vitamin E stimulates endothelial cell migration, proliferation, and fibroblast cell proliferation (19). Another study conducted by Alizadeh (2019) demonstrated that a 1 mM concentration of 80 nm CuNPs accelerated wound healing over a shorter time via the formation of granulation tissue and higher new blood vessels (18). Nitric oxide (NO) plays a critical role in the regulation of various wound-healing processes, including inflammatory response, cell proliferation, collagen formation, and angiogenesis (20). The critical role of NO in wound healing has garnered significant research attention towards NO-based wound healing therapy (21). The results of the real-time PCR pinpointed that CuNPs caused an upregulation in the expression level of the iNOS gene in RAW 264.7 cells, signifying that CuNPs are probably able to provoke the wound healing process by increasing the iNOS gene and subsequently enhancing the NO production. The results of this study demonstrated the promising antimicrobial effects of CuNPs against *S. aureus*, *S. epidermidis*, and *P. aeruginosa*, with MIC and MBC values ranging from 11.3 to 17.3 µg/mL. The lowest MIC and MBC were reported for *P. aeruginosa* with MIC and MIC values of 2.66 and 3.33 µg/mL, respectively, indicating that *P. aeruginosa* was the most sensitive strain to CuNPs. Nowadays, the antimicrobial effects of CuNPs against various Gram-negative (*Escherichia coli*, *Acinetobacter baumannii*, *Helicobacter pylori*, *Salmonella* spp., and *Shigella* spp.), Gram-positive (*Streptococcus* spp., *Staphylococcus* spp., and *Bacillus* spp.), and antibiotic resistance (*Pseudomonas aeruginosa*-carbapenem resistant, *Haemophilus influenzae*-ampicillin-resistant) strains have been reported (22). Studies have pointed out that copper nanoparticles exhibited antimicrobial activities by triggering reactive oxygen species, disrupting cell walls, enhancing plasma membrane permeability, and inhibiting protein and DNA synthesis (22, 23). Among the notable limitations of this study are the lack of animal studies and the failure to investigate the exact mechanisms of wound healing. The present study highlighted the high potency of green CuNPs synthesized by *F. macrecolea* for wound healing through their antimicrobial properties, proliferation of fibroblast cells, and provoking NO synthesis.

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Authors' Contribution

MK designed the experiments; SH performed experiments and collected data; MK supervised, directed, and managed the study; SH prepared the methodology. All authors read the published version of the manuscript.

Ethics

This study was approved by the Ethical Committee of the Faculty of Pharmacy, Eastern Mediterranean University (2021-15702032).

Conflict of Interest

The authors declare no conflict of interest in this study.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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