

Investigation of Antibacterial Properties and Healing Effects of Silver Nanoparticles Synthesized from Nettle Extract for Treating Burn Infections in Mice

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Abstract

Background: Infections in burns caused by *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* are prevalent and may result in severe health issues. Conventional treatments often struggle with bacterial resistance, highlighting the need for alternative approaches. Due to their strong antimicrobial properties, silver nanoparticles have attracted significant attention. The eco-friendly synthesis of these nanoparticles using plant extracts, such as *Urtica dioica*, is emerging as a promising strategy. This study aimed to synthesize silver nanoparticles using *U. dioica* extract through a green method and evaluate their antimicrobial activity and wound healing effects against burn infections.

Methods: Acetonic extracts of *U. dioica* were mixed with silver nitrate to produce silver nanoparticles. Their synthesis was confirmed using X-ray diffraction (XRD) and dynamic light scattering (DLS). The antimicrobial potential of the nanoparticles and plant extract was evaluated using minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) assays. Rats with burn wounds infected by bacteria were treated with ointments containing either the nanoparticles or the plant extract.

Results: Silver nanoparticles synthesized from *U. dioica* showed strong antibacterial effects against all three bacterial strains, while the extract alone was effective only against *S. aureus* and *E. coli*. The nanoparticles also demonstrated superior wound healing effects compared to the extract.

Conclusion: Silver nanoparticles demonstrated superior antimicrobial and wound healing properties compared to *U. dioica* extract, making them a strong candidate for burn infection treatment. However, silver nanoparticles are more effective against a broader range of bacteria and demonstrate enhanced wound healing potential, making them strong candidates for treating burn infections.

Keywords: Microdilution method, Minimum bactericidal concentration (MBC), Minimum inhibitory concentration (MIC), Silver nanoparticles, *Urtica dioica*



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Introduction

Burns are among the significant causes of skin damage, with approximately 300 000 individuals worldwide losing their lives annually due to severe burn injuries (1). Burns can increase the risk of tissue infection, delay skin healing, and lead to blood infections or sepsis (2). Some bacterial species such as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonadaceae* can colonize burn wounds, particularly during extended hospital stays. These bacteria exploit opportunities at wound sites, weakening the immune system and rapidly replicating due to acquired

antibiotic resistance (3). *S. aureus* is a Gram-positive facultative anaerobic bacterium that can sometimes become aerobic (4). This bacterium can be part of the normal flora (5) and is a common agent in hospital-acquired infections, causing burns and surgical infections (6). *E. coli* is a facultative anaerobic Gram-negative bacterium (7) commonly associated with infections shared between humans and animals. The emergence of antibiotic-resistant strains poses a public health concern (8). *P. aeruginosa*, a Gram-negative opportunistic bacterium, significantly impacts immunocompromised



individuals and is linked to burn infections and cystic fibrosis (9). In burn injuries, damaged skin layers and immune cells create a conducive environment for *P. aeruginosa* to establish colonies, potentially leading to infection, septic shock, and even death (10). Infection is a significant challenge (11). Various approaches can be used for treating these issues, with silver nanoparticles serving as an antimicrobial agent for burn-related infections and an alternative to antibiotics (12). Due to their high stability, low reactivity, potent antimicrobial properties, and healing effect, silver nanoparticles have been extensively studied (13). Nanomaterial-based drug delivery carriers with antimicrobial behavior provide novel platforms for treating various inflammatory diseases such as burn infections (14). Moreover, plants serve as valuable sources of antimicrobial agents due to their rich composition of bioactive molecules, including amino acids, vitamins, phytochemicals, and polyphenolic compounds such as flavonoids and phenolic acids, which have gained considerable scientific interest. *U. dioica*, a well-known medicinal herb, contains a variety of essential minerals and antimicrobial constituents like flavonoids and phenolic acids, making it a strong candidate for the development of therapeutic formulations for burn infections (15). Nanotechnology, which focuses on materials structured at the atomic or molecular level, has emerged as a crucial tool in medical sciences, particularly for combating microbial pathogens. Certain naturally occurring antibacterial elements, such as zinc and silver, exhibit significantly improved antimicrobial and wound-healing properties when processed into nanoscale structures (16).

Objectives

This study aimed to compare the antimicrobial and healing properties of silver nanoparticles synthesized from the acetonic extract of *U. dioica* with those of the extract itself in treating burn infections caused by *S. aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* in mice. The macrodilution method was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Furthermore, the silver nanoparticles were characterized through material analysis techniques.

Materials and Methods

Preparation of Acetonic Extract of Nettle

First, *U. dioica* was collected and dried. Next, 40 g of ground sample was weighed using a laboratory scale and placed into a 500 mL Erlenmeyer flask. Then, acetone was added at a ratio of 1:10 (mass of powdered plant: acetone volume), and the flask was sealed with parafilm and stored in a refrigerator at 4 °C for 48 hours. After 48 hours, the solutions were filtered through 4 layers of sterile gauze and centrifuged for 4 minutes at 2500 rpm. Finally, the extracts were diluted and poured into Petri dishes to dry at room temperature, away from sunlight (17).

Synthesis of Silver Nanoparticles

Silver nanoparticles were synthesized using a green method. First, a 0.1 M solution of silver nitrate was prepared in a beaker. Then, 4 mL of the filtered extract was added dropwise to the solution using a PTFE filter to purify the extract from suspended particles. After stirring for 1-2 hours at 80 °C, signs of synthesis were observed, the color of the solution changed to black, and the solution status changed from liquid to foam. The pH of the solution was adjusted by adding 5 mL of NaOH. The synthesized solution was dried in an 80 °C oven for 48 hours. Afterwards, the nanoparticle films were stored in a refrigerator for material and antimicrobial analysis (18). It is necessary to mention that alternative methods, such as co-precipitation and chemical methods, also exhibit antimicrobial activity (19).

Material Characterization

Silver nanoparticles (Ag^+), ranging in size from 20 to 80 nm, were successfully synthesized using the acetonic solution derived from *U. dioica*. Their structural and optical properties were analyzed through UV-visible spectroscopy, X-ray diffraction (XRD) analysis, and Zeta potential measurements. Following characterization, the antimicrobial and wound-healing capabilities of the synthesized nanoparticles were evaluated (19).

Preparation of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* Strains

Staphylococcus aureus strain ATCC292113, *E. coli* strain ATCC25922, and *P. aeruginosa* strain ATCC1542 were obtained from the Microbial Bank of the Nanobiotechnology Research Center at Islamic Azad University, Zanjan branch. These pathogenic bacteria were sub-cultured on Mueller Hinton Agar medium.

Determination of MBC and MIC

MBC and MIC values were determined for three antimicrobial agents: silver nanoparticles, acetonic extract of *U. dioica* combined with nanoparticles, and the extract alone. First, stock solutions for each agent were prepared at a concentration of 500 mg/mL by dissolving the extract film in dimethyl sulfoxide (DMSO) (Merck, Germany). After adding Mueller-Hinton broth (Merck) to each well plate (SPL), serial dilutions were prepared, starting with a concentration of 250 mg/mL. The first step involved adding 500 μ L of the stock solution to 500 μ L of Mueller-Hinton broth, followed by 500 μ L of bacterial suspension. The concentration in each subsequent well was halved compared to the previous one, with the dilution carried out using an Eppendorf pipette, transferring 500 μ L from one well to the next. Then, the cellular concentrations of bacterial cultures were adjusted to a cell density of 1.5×10^8 cells/mL (0.5 McFarland standard) (Iran Baharafshan). Afterwards, they were added to their respective well plates, which were subsequently incubated (Memmert) at 37 °C for 24 hours. After incubation, all the well plates were

examined using positive and negative controls. Finally, the MBC and MIC values were determined by culturing the bacteria on the plates containing Mueller Hinton agar medium (20).

Animal Model

A total of 45 adult laboratory rats weighing approximately 200 ± 10 g were used in this study. The rats were obtained from the Faculty of Medical Sciences and transferred to the animal laboratory at the Nanobiotechnology Research Center of Islamic Azad University, Zanjan Branch. They were allowed to acclimate for 48 hours under controlled conditions with a 12-hour light/dark cycle, a temperature of 24°C - 25°C , and a humidity of about 51%. The rats were provided with standard food and water. Afterwards, the study included three main categories based on bacterial strains. The first category was *S. aureus*, which consisted of five subgroups: (1) control group, three mice that received no treatment, (2) acetonic extract group, three mice that were treated with acetonic extract, (3) silver nanoparticles group, three mice that were treated with silver nanoparticles, (4) Eucerin group, three mice that received a moisturizing ointment without antimicrobial properties, and (5) silver nanoparticles + acetonic extract group, three mice that were treated with a combination of silver nanoparticles and acetonic extract. The second category was *E. coli*, and the third was *P. aeruginosa*, both of which followed the same subgrouping as the *S. aureus* category. To induce burns, the rats were anesthetized by the intraperitoneal administration of thiopental (4 mg/kg). Then, the hair on the back of their necks was shaved, and a molten circle-shaped metal with a diameter of 1 cm was placed on the shaved area for 3-4 seconds. After the rats regained consciousness, they were housed in clean and disinfected cages. On the following day, the wounds were inoculated with bacterial suspensions containing 6×10^8 cells/mL. After 24 hours, treatment was initiated using 1 g of Eucerin ointment for 6 days (0.5 g of ointment per day, administered three times). On the 4th day, samples were collected from each wound using the punching method

and cultured in blood agar for colony counting (21). This research was conducted in accordance with international standards. The study was also approved by the Ethics Committee of Islamic Azad University, Zanjan branch (IR.IAU.Z.REC.1400.001).

Statistical Analysis

The gathered data were evaluated using a one-way ANOVA and the LSD test. A significance level of $P < 0.01$ was considered for all comparisons (22).

Results

UV-Visible Spectrophotometry

The UV-Visible spectrophotometry analysis of the acetonic extract of *U. dioica* containing silver nitrate is shown in Figure 1B. The distinct absorption peak in the spectrum verifies the effective production of silver nanoparticles. Spectrophotometric measurements were conducted after the nitrate-containing extract was kept under laboratory conditions for 24 hours. In contrast, the wavelength pattern of the acetonic extract alone exhibited a downward trend, as illustrated in Figure 1A. These results confirm the successful fabrication of silver nanoparticles utilizing the acetonic extract of *U. dioica*, as evidenced by the characteristic absorption peak. Additionally, the data in Text Box 1 illustrate the presence of nanoparticles. It is noteworthy that the wavelength associated with the extract fluctuated due to the presence of naturally occurring impurities (Figure 1).

X-Ray Diffraction Analysis

The XRD analysis of silver nanoparticles derived from the acetonic extract of *U. dioica* revealed corresponding peaks at angles of 29° , 38° , 48° , and 65° (Figure 2). These peaks are consistent with the standard XRD pattern of silver, confirming the successful synthesis of silver nanoparticles. Additionally, the obtained pattern indicates that the synthesized particles possess a specific size distribution, which can be further analyzed for potential applications. This section summarizes the XRD data, emphasizing

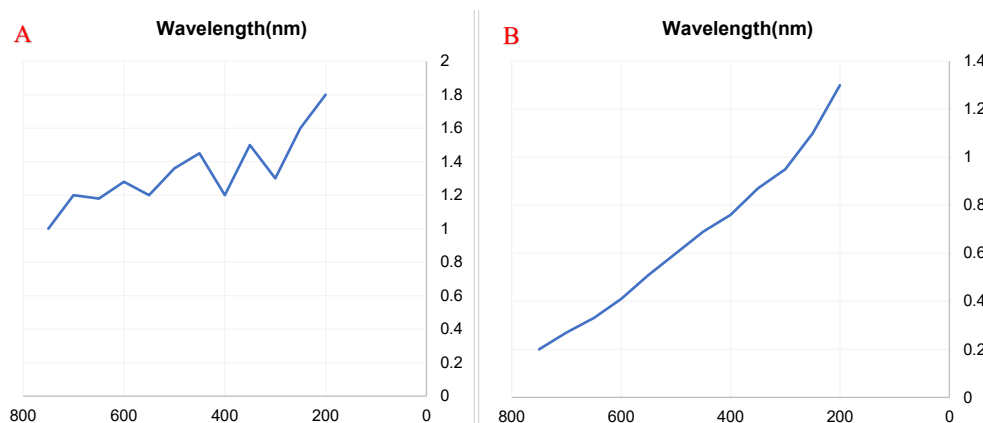


Figure 1. Average Absorbance of the Acetonic Extract of *Urtica dioica* Following a 24-hour Period (A), Average Absorbance of Silver Nanoparticles Synthesized from Acetonic Extract of *U. dioica* after 24 Hours (B). This figure illustrates the mean absorbance of silver nanoparticles synthesized from the acetonic extract, alongside the absorbance spectrum of the extract itself

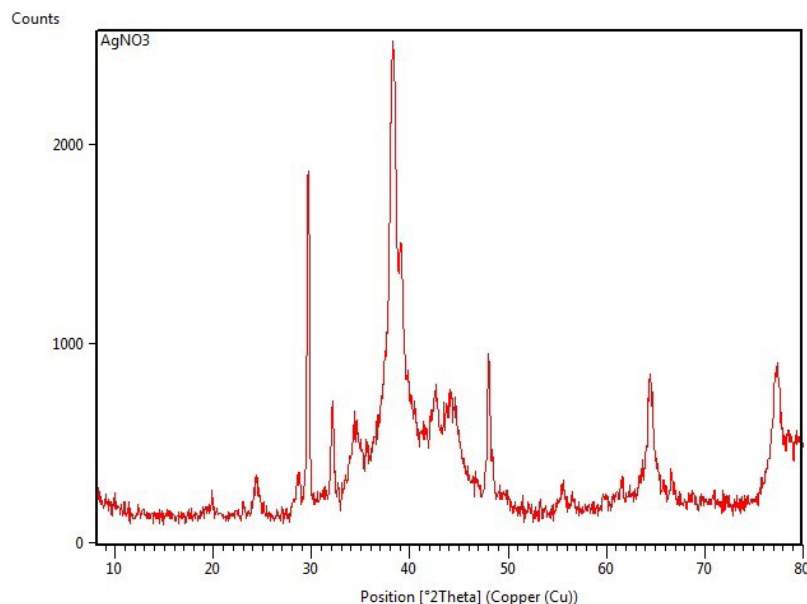


Figure 2. The XRD figure of silver nano particles there are four main peaks which are same with standard graph of silver nanoparticles. These peaks positions include 29, 38, 48 and 65.

the key findings that validate the synthesis of silver nanoparticles with the acetonc extract of *U. dioica*. It is worth mentioning that these peaks were reached by measuring crystallography features and these are the same as those of standard graphs.

Zeta Potential of Nanoparticles

The zeta potential data revealed that the synthesized silver nanoparticles exhibited a zeta potential of -50.05 mV at a temperature of 25.1°C (A) and -47.00 mV at 25.2°C (B). The dynamic light scattering (DLS) test determines the hardness, amount, and size of the synthesized nanoparticles. Additionally, this test can identify the size of nanoparticles. The quality of the data obtained from this analysis is evaluated by analyzing the autocorrelation function (ACF = g^2) and its goodness of fit based on the characteristics of static light scattering and particle size using machine intervals (Frequency Undersize). Key quality indicators in this analysis include: (a) The ACF line and curve must reach 1.0. (b) The ACF sum of squares (SOS) for monodisperse samples must be less than 100 nm. Based on the described criteria, particles with a size of 70–80 nm and an ACF of 1.0 indicate high-quality nanoparticles with proper medical correlation (C). These values indicate low mobility, suggesting that the synthesized particles are stable and resistant to agglomeration. This revised section highlights the key findings from the zeta potential analysis, emphasizing the stability of the synthesized silver nanoparticles. The results demonstrate that nanoparticles exhibit minimal agglomeration, making them suitable for potential applications in various fields.

Determination of MBC and MIC

MBC and MIC values were determined for three antimicrobial agents tested against *S. aureus*, *E. coli*, and *P. aeruginosa*. These agents included silver nanoparticles

(AgNPs), the acetonc extract of *U. dioica*, and AgNPs combined with the acetonc extract of *U. dioica*.

Silver Nanoparticles

AgNPs were effective at the lowest concentrations against all three bacterial species, including *S. aureus*, *E. coli*, and *P. aeruginosa*.

Silver Nanoparticles Combined with Acetonc Extract of *Urtica dioica*

The combination of AgNPs and acetonc extract showed effectiveness at minimal concentrations against *S. aureus* and *E. coli*, while it exhibited a moderate effect on *P. aeruginosa*.

Acetonc Extract of *Urtica Dioica*

The acetonc extract of *U. dioica* alone demonstrated efficacy at medium concentrations against *S. aureus* and *E. coli*, and at higher concentrations against *P. aeruginosa*.

The results are summarized in Table 1 for *S. aureus*, *E. coli*, and *P. aeruginosa*, respectively. These findings indicate that AgNPs and AgNPs combined with the acetonc extract are more effective against the tested bacterial species compared to the acetonc extract alone.

Animal Model

In the animal model, the results were expressed in colony forming units (CFUs). The groups treated with silver nanoparticles against all bacteria showed a significant difference compared to the control group ($P < 0.01$). In addition, a significant difference was observed between these groups and those that received both nanoparticles and plant extract, as well as those treated with the acetonc extract alone ($P < 0.01$), as presented in Table 2.

The healing process in the extract group was observed to occur within a short time, and a similar effect was seen

Table 1. MBC and MIC of the Antimicrobial Agents against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*

Bacteria	Antimicrobial Agents	MBC	MIC
<i>Staphylococcus aureus</i>	Silver nanoparticles	3.9 mg/mL	1.9 mg/mL
<i>Staphylococcus aureus</i>	Silver NPs with extract	3.9 mg/mL	1.9 mg/mL
<i>Staphylococcus aureus</i>	Acetonic extract	15.6 mg/mL	7.8 mg/mL
<i>Escherichia coli</i>	Silver nanoparticles	3.9 mg/mL	1.9 mg/mL
<i>Escherichia coli</i>	Silver NPs with extract	3.9 mg/mL	1.9 mg/mL
<i>Escherichia coli</i>	Acetonic extract	15.6 mg/mL	7.8 mg/mL
<i>Pseudomonas aeruginosa</i>	Silver nanoparticles	3.9 mg/mL	1.9 mg/mL
<i>Pseudomonas aeruginosa</i>	Silver NPs with extract	15.6 mg/mL	7.8 mg/mL
<i>Pseudomonas aeruginosa</i>	Acetonic extract	31.2 mg/mL	15/6 mg/mL

Table 2. Effect of the Antimicrobial Agents on the Number of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* Colonies

Group	<i>Staphylococcus aureus</i> Mean ± SD (10 ⁵)	<i>Escherichia coli</i> Mean ± SD (10 ⁵)	<i>Pseudomonas aeruginosa</i> Mean ± SD (10 ⁵)
Treatment with silver nanoparticles	3.66 × 10 ⁵ ± 1.15*	4.66 × 10 ⁵ ± 0.57*	7.66 × 10 ⁵ ± 1.52*
Treatment with silver NPs and extract	5 × 10 ⁵ ± 2.64*	5.66 × 10 ⁵ ± 1.52*	10.66 × 10 ⁵ ± 2.08*
Treatment with acetonic extract	8.33 × 10 ⁵ ± 8.52*	9.66 × 10 ⁵ ± 1.52*	19.33 × 10 ⁵ ± 1.52*
Treatment with Eucerin	20.33 × 10 ⁵ ± 2.51*	21 × 10 ⁵ ± 2*	25.66 × 10 ⁵ ± 2.08*
Control and no treatment	275 × 10 ⁶ ± 2.65	294.33 ± 10 ⁶ ± 7.37	295 × 10 ⁶ ± 4.35

* Indicates a significant difference with the control and no treatment groups ($P < 0.01$).

in the groups treated with silver nanoparticles after 6 days. In contrast, the control groups required 21 days for healing, while the groups treated with a combination of silver nanoparticles and extract took 8 days to heal.

The antimicrobial activity and healing effects in the groups treated with silver nanoparticles were significantly more effective than other treatment groups in this study.

Discussion

Burn wounds are vulnerable to opportunistic colonization by both endogenous and exogenous microorganisms. Factors like the patient's age, the severity of the burn, and the depth of the wound, combined with microbial variables such as the species and quantity of microorganisms, their enzyme and toxin production, and motility, all affect the risk of infection in burn wounds. These infections can be categorized based on the type of organism, the depth of invasion, and the response of the tissue. Common pathogens responsible for burn wound infections include *E. coli*, *S. aureus*, and *P. aeruginosa* (23,24). Both green and chemical methods are commonly used to synthesize silver nanoparticles; however, the green method is more eco-friendly as it utilizes plant extracts. Ahmad et al studied the green synthesis of silver nanoparticles and their antimicrobial activity. In the present study, the methods of synthesis, characterization of nanoparticles, and antimicrobial activity were aligned with the findings of the study conducted by Ahmad (25). The results from animal studies and the antimicrobial activity of silver nanoparticles against burn infections caused by *S. aureus* and *E. coli* were consistent with the study conducted by Wasef et al, which investigated the effects of silver nanoparticles on burn wound healing in a mouse model

(26). However, evaluating the cytotoxicity of nanoparticles in medical applications is crucial in assessing their safety (27). One of the limitations of this study is the absence of cytotoxicity assays such as the MTT assay or other related tests. Although our findings, consistent with those of Wasef et al, confirm the antimicrobial properties of the nanoparticles, further investigations are necessary to assess their potential biomedical applications comprehensively. According to the study conducted by Jagtap et al, additional cytotoxicity evaluations are recommended to ensure the safety and efficiency of these nanoparticles in biomedical environments (26,28). The XRD analysis confirmed the crystalline nature of the synthesized nanoparticles, as indicated by the characteristic diffraction peaks. A comparison with previous studies, such as those conducted by Abdulsahib et al and Hassan Afandy et al, demonstrated a strong similarity in peak positions and intensities, suggesting comparable crystallinity. Minor variations observed in the diffraction patterns may stem from differences in synthesis parameters, precursor concentrations, and particle size distributions. These results support the reliability of our XRD findings and further confirm the successful formation of crystalline nanoparticles (19,29). However, the UV-Vis spectrophotometry results in the present study differ from those of previous studies. In the study conducted by Abdulsahib et al, the peak was recorded at 400 nm (19), while in the study conducted by Ansari et al, the absorption spectrum was observed in the range of 420–440 nm (30). Additionally, in the study by Tesfaye et al, the absorption spectrum was found in the range of 411–430 nm (31). In contrast, in the current study, the peak was observed below 400 nm. This effect has been investigated in the studies by

Ansari et al and Tesfaye et al, where various factors such as different temperatures, synthesis times, pH, solvent concentration, plant extract volume, and nanoparticle synthesis time were found to influence the absorption spectrum in different ranges (30,31). Zeta potential plays a crucial role in determining the stability of nanoparticles, as it directly affects their tendency to aggregate or remain dispersed (32). In the present study, the synthesized silver nanoparticles exhibited a zeta potential of -50.05 mV (Figure 3A) and $+47$ mV (Figure 3B), indicating strong repulsion between particles. These findings are consistent with the results reported by Alzubaidi et al, who recorded zeta potential values of -44.5 and $+231.8$ mV (33), and by Netala et al, who reported values of $+44$ mV and -19.65 mV (34). The differences in the reported values between this study and previous ones may be due to variations in factors such as nanoparticle size, solvent type, or temperature. The results of this study, along with previous studies, indicate that the synthesized nanoparticles have diverse applications in biomedical fields (33,34). Antimicrobial tests were performed with high sensitivity, in line with the study conducted by Kowalska-Krochmal et al (20) on microdilution and macro-serial dilution. Studies

have shown that smaller sample sizes reduce statistical power, making results less reliable (35). Additionally, variations in the pathogen spectrum studied may lead to inconsistencies and hinder the generalization of results in clinical settings (36). The concentration of the bacterial inoculum and the volume of the medium were consistent with the studies by Andrews (37) and Wiegand et al (38). However, inadequate sample size and pathogen spectrum can affect the clinical relevance and reproducibility of findings. Studies have shown that smaller sample sizes reduce statistical power, making results less reliable (35). Additionally, variations in the pathogen spectrum studied may lead to inconsistencies and hinder the generalization of results in clinical settings (36). Therefore, increasing sample sizes and broadening the pathogen spectrum would enhance the reliability and applicability of nanoparticle-based studies. Dağlıoğlu et al studied the biosynthesis of silver nanoparticles derived from Nettle leaf extract, and the materials analysis was consistent with the current study. However, the antimicrobial results differed, with the current study showing more promising results. In general, both studies indicate that Nettle-mediated biosynthesized AgNPs exhibit strong antibacterial

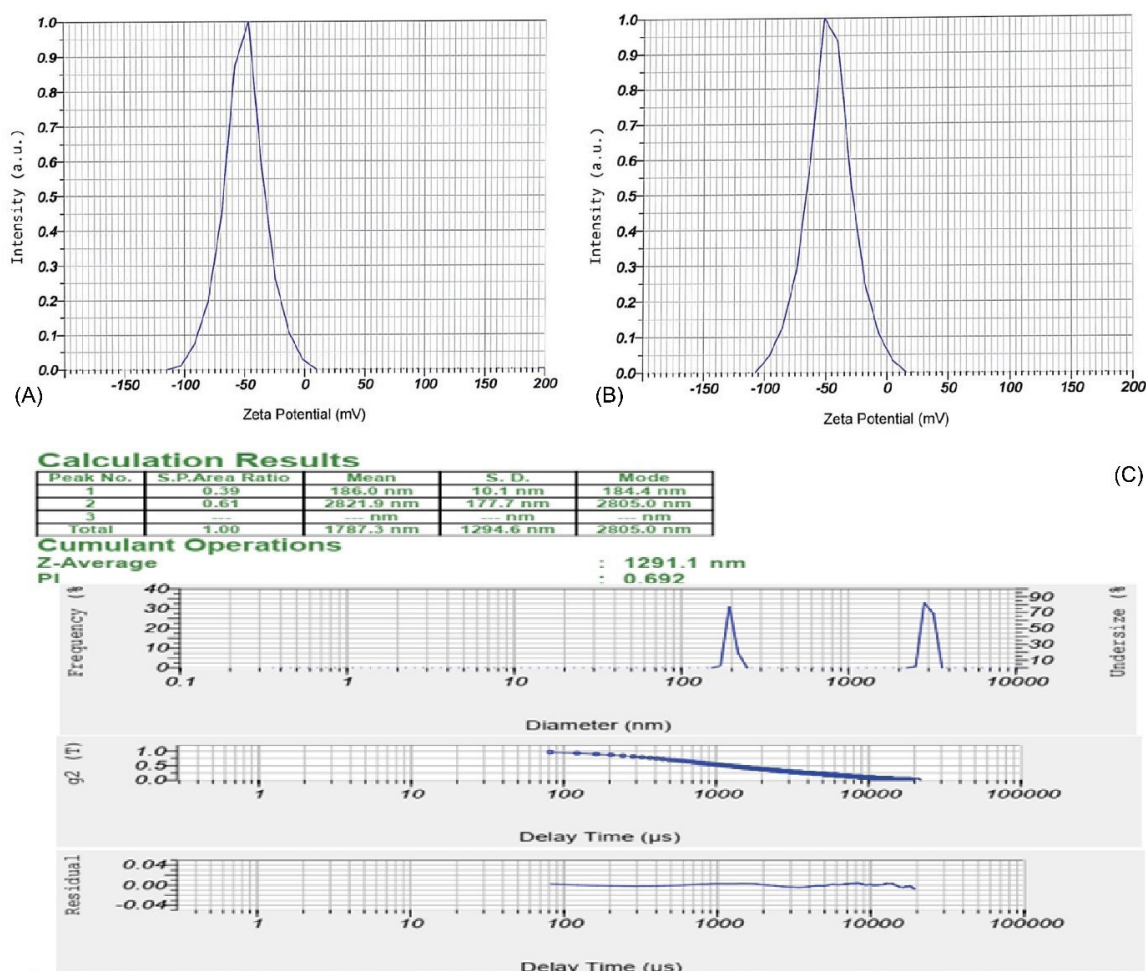


Figure 3. Zeta Potential of Silver Nanoparticles at Temperatures of 25.1 °C (A) and 25.2 °C (B). These figures display the zeta potential of the synthesized silver nanoparticles at various temperatures, indicating stability and resistance to agglomeration across both values. The data provide a concise description of the zeta potential of the synthesized nanoparticles, showing minimal agglomeration and suitability for various applications. DLS diagram indicates the abundance, hardness, and size of nanoparticles, where the maximum size of the particles is 70 - 80 nm (C).

activity against microbial agents (39). Zeinali Aghdam et al researched the biosynthesis of silver nanoparticles using Nettle extract, and their materials and microbial analysis methods were similar to those used in our study. However, the methods used for the synthesis of silver nanoparticles differed, with the current study employing an optimized synthesis method. Both studies demonstrate the antimicrobial and antioxidant activity of Nettle-derived AgNPs against potential pathogens (40). Hashem and Salem (41) and Ebrahiminezhad et al (42) explored the potential of Nettle for the synthesis of various metal nanoparticles, including selenium and iron nanoparticles. In the study conducted by Wasef et al, the results show that silver nanoparticles obtained from the green method have a potential antibacterial effect on pathogens, and the general results obtained in this research are consistent with the current study. Furthermore, silver nanoparticles have a prominent clinical effect on infection control (26). The results of this study support the antimicrobial activity and healing effects of metal nanoparticles on different types of wounds, such as burn wounds and surgical wounds. Metal nanoparticles with antimicrobial properties include gold, silver, copper, and iron. In conclusion, silver nanoparticles were synthesized from the acetonic extract of *U. dioica* using the green method, and the results of metal and antimicrobial analyses demonstrated the efficacy of silver nanoparticles against resistant microorganisms.

Conclusion

The findings of this study indicate that silver nanoparticles synthesized from *U. dioica* extract possess significant antimicrobial and wound healing properties against *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa*, outperforming the acetonic extract of *U. dioica* alone. Therefore, green-synthesized silver nanoparticles represent a promising approach for treating burn infections.

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Authors' Contribution

Conceptualization: Nader Kazemi.

Data curation: Nader Kazemi.

Formal analysis: Mahdi Arfaei.

Methodology: Nader Kazemi.

Supervision: Masoud Kaboutari.

Writing—original draft: Mahdi Arfaei.

Writing—review & editing: Masoud Kaboutari.

Competing Interests

The authors declare that they have no conflict of interests.

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