



Original Research Paper

Bioengineered Silver Nanoparticle-Loaded Chitosan Hydrogel for Enhanced Wound Healing: Characterization, Antimicrobial Activity, and In Vivo Assessment in Rats

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Abstract

Background: Chronic and infected wounds require advanced topical therapies capable of providing effective antimicrobial protection while accelerating tissue regeneration. Green-synthesized silver nanoparticles (AgNPs) incorporated into biocompatible hydrogels represent a promising strategy for enhancing wound healing.

Objective: The present study aimed to evaluate the physicochemical characteristics, antimicrobial efficacy, in vivo wound healing potential, and stability of a neem-mediated green-synthesized silver nanoparticle-loaded chitosan hydrogel developed using an optimized Quality by Design (QbD) approach.

Methods: Green-synthesized AgNPs were prepared using *Azadirachta indica* (Neem) leaf extract and optimized through a Box–Behnken Design. The optimized nanoparticles were incorporated into a chitosan hydrogel and evaluated for pH, viscosity, spreadability, occlusive properties, and silver nanoparticle content. Antibacterial activity was assessed against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* using the agar well diffusion method. Wound healing efficacy was investigated in Wistar rats using an excision wound model with four experimental groups: untreated control, marketed silver nitrate gel, optimized AgNP-loaded chitosan hydrogel, and neem hydrogel. Wound contraction was monitored for 21 days, followed by histopathological evaluation. Accelerated stability studies were conducted according to ICH guidelines.

Results: The optimized hydrogel exhibited a skin-compatible pH (5.87 ± 0.35), suitable viscosity (4208 ± 101.01 cPs), good spreadability (12.11 ± 3.11 g·cm/s), high occlusion factor ($76.69 \pm 5.18\%$), and excellent AgNP content ($98.64 \pm 0.71\%$). The formulation demonstrated superior antibacterial activity against all tested pathogens, producing inhibition zones of 24.8 ± 0.7 mm against *S. aureus*, 23.5 ± 0.8 mm against *E. coli*, and 22.6 ± 0.5 mm against *P. aeruginosa*, outperforming the marketed silver gel. In vivo studies showed significantly enhanced wound healing, with the optimized AgNP hydrogel achieving $97.01 \pm 0.17\%$ wound contraction by Day 21 compared with $90.01 \pm 0.33\%$ for the marketed gel, $81.92 \pm 0.17\%$ for neem hydrogel, and $78.78 \pm 1.75\%$ for the untreated control ($p < 0.001$). Histopathological observations confirmed enhanced re-epithelialization and tissue remodeling in the AgNP-treated group. The hydrogel remained physically and chemically stable during three months of accelerated storage, retaining more than 97% AgNP content.

Conclusion: The optimized green-synthesized silver nanoparticle-loaded chitosan hydrogel demonstrated excellent physicochemical characteristics, broad-spectrum antimicrobial activity, superior wound healing efficacy, and satisfactory stability. The synergistic combination of neem phytochemicals, silver nanoparticles, and chitosan significantly accelerated wound repair compared with the marketed silver gel, highlighting its potential as a safe, effective, and sustainable topical wound dressing for future clinical translation.

Keywords: Green synthesis silver nanoparticles, Neem extract, *In vivo* wound healing, antibacterial activity, Chitosan based hydrogel

Introduction

Wound healing is a dynamic and tightly regulated biological process that restores the structural and functional integrity of damaged tissues through four

overlapping phases: hemostasis, inflammation, proliferation, and remodeling.¹ The healing process can be compromised by microbial infection, excessive inflammation, oxidative stress, and impaired angiogenesis, resulting in delayed healing

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and chronic wounds. Chronic and infected wounds remain a major clinical challenge, particularly in patients with diabetes, burns, vascular disorders, and immunocompromised conditions, emphasizing the need for advanced wound dressings capable of promoting rapid tissue regeneration while preventing microbial contamination^{2,3}.

Silver-based topical formulations have long been considered the gold standard for the management of infected wounds because of their broad-spectrum antimicrobial activity. Among various silver formulations, silver nanoparticles (AgNPs) have attracted considerable attention due to their nanoscale dimensions, large surface area, and enhanced biological activity.⁴ AgNPs exhibit potent antibacterial effects against both Gram-positive and Gram-negative microorganisms by disrupting bacterial cell membranes, generating reactive oxygen species, and interfering with intracellular metabolic pathways.^{5,6} Beyond their antimicrobial properties, AgNPs have also been shown to reduce inflammation, stimulate fibroblast proliferation, enhance collagen deposition, promote angiogenesis, and accelerate tissue remodeling, making them promising candidates for wound healing applications.⁷

The method employed for nanoparticle synthesis significantly influences their physicochemical characteristics and biological performance. Conventional chemical synthesis methods frequently utilize hazardous reducing agents that may leave toxic residues, thereby limiting biomedical applications.^{8,7,9} In contrast, green synthesis has emerged as a sustainable and environmentally friendly alternative by employing plant-derived phytochemicals as natural reducing and stabilizing agents.^{10,11,12,13} *Azadirachta indica* (Neem) is widely recognized for its antimicrobial, anti-inflammatory, antioxidant, and wound healing properties. Bioactive constituents such as flavonoids, polyphenols, terpenoids, and phenolic compounds present in neem extract facilitate the reduction of silver ions into stable nanoparticles while simultaneously contributing additional therapeutic benefits.^{14,15,16,17}

Hydrogels are considered one of the most effective topical drug delivery systems for wound management because they maintain a moist environment, absorb wound exudate, permit oxygen diffusion, and provide sustained release of therapeutic agents.^{18,19} Chitosan, a naturally derived polysaccharide obtained by deacetylation of chitin, has gained considerable attention owing to its excellent biocompatibility, biodegradability, mucoadhesive nature, intrinsic antimicrobial activity, and ability to promote hemostasis and tissue regeneration. Incorporation of green-

synthesized silver nanoparticles into a chitosan hydrogel offers a multifunctional wound dressing capable of combining the biological properties of chitosan with the antimicrobial and regenerative effects of AgNPs, thereby enhancing the wound healing process.^{20,21}

The development and optimization of a neem-mediated silver nanoparticle-loaded chitosan hydrogel using a Quality by Design (QbD) approach and Box–Behnken Design (BBD) have been reported previously, where the optimized formulation demonstrated desirable physicochemical characteristics and promising *in vitro* performance.^{22,23} However, comprehensive preclinical evidence supporting its therapeutic efficacy in wound healing remains limited. Therefore, the present study aimed to investigate the *in vivo* wound healing efficacy of the previously optimized AgNP-loaded chitosan hydrogel using an excision wound model in Wistar rats. The therapeutic performance of the optimized formulation was compared with that of a marketed silver-based wound healing gel by evaluating wound contraction, epithelialization period, and histopathological changes. Furthermore, accelerated stability studies were performed in accordance with ICH guidelines to assess the physical stability of the optimized hydrogel and establish its suitability as a topical wound healing formulation.

Materials & Methods

Materials

Silver nitrate (Sisco Research Laboratories Pvt. Ltd., Mumbai), chitosan (Sigma Aldrich, USA), Mueller–Hinton agar (M/s Himedia Laboratories, Mumbai), nutrient broth (M/s Himedia Laboratories, Mumbai), acetic acid, sodium hydroxide, hydrochloric acid, and other analytical-grade chemicals were procured from Merck, India. Fresh *Azadirachta indica* (Neem) leaves were collected locally and used for the green synthesis of silver nanoparticles. The marketed silver gel formulation served as the reference standard. Standard bacterial strains, including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853), were used for antimicrobial studies. Adult male and female Wistar albino rats (180–200 g) were used for the *in vivo* wound healing study. Distilled water was used throughout the study, and all chemicals and reagents were of analytical grade.

Methods

Optimized Preparation of Green-Synthesized Silver Nanoparticles (AgNPs)

Green-synthesized silver nanoparticles (AgNPs) were prepared using *Azadirachta indica* (Neem) leaf extract as an eco-friendly reducing and stabilizing agent. Prior to formulation development, a Quality by Design (QbD) approach was employed to systematically optimize the synthesis process. Based on preliminary screening experiments and risk assessment, silver nitrate concentration (X_1), neem extract concentration (X_2), and reaction pH (X_3) were identified as the critical material attributes (CMAs), whereas particle size (Y_1), polydispersity index (Y_2), and zeta potential (Y_3) were selected as the critical quality attributes (CQAs). A three-factor, three-level Box–Behnken Design (BBD) was applied to evaluate the individual and interactive effects of the selected variables and to establish the optimum synthesis conditions.

Numerical optimization generated an optimized formulation consisting of 0.5336% (w/v) silver nitrate, 1.35% (w/v) neem leaf extract, and reaction pH 7.04, with an overall desirability value of 0.937. These optimized conditions were selected for nanoparticle preparation and experimental validation.

For the synthesis, 0.5336% (w/v) aqueous silver nitrate solution was prepared using freshly distilled water. Separately, 1.35% (w/v) neem leaf extract was prepared and filtered through Whatman No. 1 filter paper to remove insoluble plant residues. The silver nitrate solution was stirred continuously on a magnetic stirrer at room temperature, and the filtered neem extract was added gradually in a dropwise manner. The pH of the reaction mixture was adjusted to 7.04 using dilute sodium hydroxide or hydrochloric acid solution and maintained throughout the reaction. Continuous stirring was carried out until the solution changed from pale yellow to dark brown, indicating the bioreduction of silver ions (Ag^+) into silver nanoparticles.

The reaction mixture was further stirred to ensure complete reduction and stabilization of the nanoparticles. Preliminary confirmation of nanoparticle formation was obtained from the characteristic brown colour of the reaction mixture, while successful synthesis was further verified by UV–Visible spectrophotometric analysis through the appearance of the characteristic surface plasmon resonance (SPR) absorption peak.^{24,25}

The synthesized AgNPs were isolated by centrifugation at 15,000 rpm for 20 minutes. The collected nanoparticle pellet was washed three times with distilled water to eliminate residual silver ions and unbound phytoconstituents. The purified nanoparticles were vacuum dried and stored in

airtight containers until further physicochemical characterization.

Validation of the optimized formulation demonstrated excellent agreement between the predicted and experimental responses. The prepared AgNPs exhibited an average particle size of 36.8 nm, a polydispersity index (PDI) of 0.262, and a zeta potential of -10.40 mV, with percentage deviations of less than 10% from the predicted values. These findings confirmed the adequacy of the Box–Behnken optimization model and demonstrated that the optimized formulation produced uniformly distributed and physically stable green-synthesized silver nanoparticles suitable for subsequent incorporation into the chitosan hydrogel formulation.²⁶

Preparation of Optimized Silver Nanoparticle-Loaded Chitosan Hydrogel

The optimized green-synthesized silver nanoparticles were incorporated into a chitosan-based hydrogel using a simple dispersion technique. Chitosan was accurately weighed and dissolved in 1% (v/v) acetic acid under continuous magnetic stirring until a clear and homogeneous polymer solution was obtained.

The optimized silver nanoparticles were dispersed in distilled water and sonicated for 10–15 minutes to eliminate particle agglomeration and ensure uniform dispersion. The nanoparticle suspension was then added gradually to the chitosan solution under continuous stirring to achieve homogeneous distribution throughout the polymer matrix.

Gel formation was induced by the gradual addition of sodium tripolyphosphate (TPP) as a cross-linking agent under constant stirring. Stirring was continued until a smooth, uniform, and stable hydrogel of suitable consistency was obtained. The prepared hydrogel was allowed to stand undisturbed to remove entrapped air bubbles before being transferred into sterile, airtight containers and stored at room temperature for further studies.

Viscosity analysis

The viscosity of the silver nanoparticle-loaded hydrogel was evaluated using a Brookfield viscometer equipped with an appropriate spindle. The measurements were performed at a controlled temperature of $25 \pm 1^\circ\text{C}$ and at different rotational speeds to assess the rheological behavior of the formulation. Viscosity plays a critical role in determining the consistency, spreadability, residence time, and stability of the hydrogel. An optimum viscosity is required to ensure easy

application while preventing runoff from the application site.

pH Measurement

The pH of the formulated hydrogel was determined using a calibrated digital pH meter. Approximately 1 g of hydrogel was dispersed in 10 mL of distilled water and allowed to equilibrate for 30 minutes. The electrode of the pH meter was immersed directly into the sample, and measurements were recorded in triplicate at room temperature. Determination of pH is an important parameter for topical formulations, as it influences skin compatibility, drug stability, and patient acceptability. The pH of the hydrogel was maintained within the physiological skin pH range to minimize irritation and ensure safe application.

Spreadability

Spreadability of the hydrogel was determined using the glass slide method. A known quantity of hydrogel was placed between two clean glass slides, and a specified weight was applied on the upper slide to form a uniform film. The time required for the upper slide to move a predetermined distance under the influence of an applied weight was recorded. Spreadability was calculated using the following equation:

Spreadability (S) = (M × L) / T equation (1)

Where:

- S = Spreadability (g·cm/s)
- M = Weight tied to the upper slide (g)
- L = Length moved by the glass slide (cm)
- T = Time taken to separate the slides (s)

Good spreadability indicates ease of application and uniform distribution of the formulation over the skin surface, which contributes to improved therapeutic efficacy.

Occlusion Test

The occlusive properties of the silver nanoparticle-loaded chitosan hydrogel were evaluated using a modified method described by Rai *et al.* Three 100 mL beakers were filled with 50 mL of distilled water and covered with cellulose acetate filter paper (90 mm diameter, pore size 4–7 µm). An accurately weighed amount of hydrogel (200 mg) was uniformly spread over the filter paper surface (19.63 cm²) using a stainless-steel spatula, producing a thin film corresponding to an applied amount of 10.19 mg/cm². A control beaker covered only with filter paper was used as the reference.

The beakers were maintained at 32 ± 1°C and 50–55% relative humidity (RH) for 48 h. The loss of water due to evaporation was determined by weighing the beakers at 6, 24, and 48 h. All measurements were performed in triplicate (n = 3). The occlusion factor (F) was calculated using the following equation:

F = ((A - B) / A) × 100 equation (2)

where:

- A = Amount of water lost from the reference beaker (without hydrogel)

- B = Amount of water lost from the beaker covered with hydrogel

An occlusion factor of 0 indicates no occlusive effect, whereas a value of 100 represents maximum occlusivity. Higher occlusion factor values indicate a greater ability of the hydrogel to prevent water evaporation and maintain skin hydration.

Determination of Silver Nanoparticle Content by UV-Visible Spectrophotometry

The silver nanoparticle (AgNP) content of the optimized chitosan hydrogel was determined using a UV-Visible spectrophotometric method. A standard stock solution of green-synthesized AgNPs (1000 µg/mL) was prepared in distilled water, and serial dilutions were made to obtain concentrations of 10, 20, 40, 60, 80, and 100 µg/mL. The absorbance of each standard solution was measured at 420 nm, corresponding to the characteristic surface plasmon resonance (SPR) peak of silver nanoparticles, using a UV-Visible spectrophotometer with distilled water as the blank. A calibration curve was constructed by plotting absorbance against AgNP concentration, and linear regression analysis was performed.

For estimation of silver nanoparticle content, 1.0 g of the optimized AgNP-loaded chitosan hydrogel was accurately weighed and transferred to a 100 mL volumetric flask. Approximately 50 mL of distilled water was added, and the mixture was stirred followed by sonication for 15 min to ensure complete dispersion of the hydrogel and release of the entrapped silver nanoparticles. The volume was then adjusted to 100 mL with distilled water and filtered through a 0.45 µm membrane filter. An appropriate aliquot of the filtrate was further diluted, when necessary, and its absorbance was measured at 420 nm.

The concentration of silver nanoparticles in the sample was calculated from the calibration curve, and the silver nanoparticle content was determined using the following equation:

Silver nanoparticle content (%) =

$$\frac{\text{Amount of AgNPs estimated}}{\text{Theoretical amount of AgNPs}} \times 100 \quad \dots \text{equation (3)}$$

where the amount of AgNPs estimated was obtained from the calibration curve, and the theoretical amount of AgNPs represented the quantity incorporated during formulation. All analyses were carried out in triplicate, and the results were expressed as mean ± standard deviation (SD).

Antimicrobial Activity

The antibacterial activity of the optimized green-synthesized silver nanoparticle (AgNP)-loaded chitosan hydrogel was evaluated using the agar well diffusion method against three common wound-associated bacterial strains: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853).

Fresh bacterial cultures were grown in nutrient broth and adjusted to 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/mL). Sterile Mueller–Hinton agar (MHA) plates were prepared, and each bacterial suspension was uniformly spread over the agar surface using sterile cotton swabs to obtain a confluent bacterial lawn. Wells of 6 mm diameter were aseptically punched into the agar using a sterile cork borer.

Three formulations were evaluated:

- G1: Plain chitosan hydrogel (Control)
- G2: Marketed silver-based wound healing gel (Standard)
- G3: Optimized green-synthesized AgNP-loaded chitosan hydrogel (Test)

Approximately 100 mg of each formulation was carefully introduced into the respective wells. The inoculated plates were allowed to stand at room temperature for 30 min to facilitate diffusion of the formulations into the agar medium and were subsequently incubated at $37 \pm 1^\circ\text{C}$ for 24 h.

Following incubation, the antibacterial activity was determined by measuring the diameter of the clear zone of inhibition (mm) surrounding each well using a digital Vernier caliper. All experiments were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD).

Representative agar plates showing the antibacterial activity of the control, marketed, and optimized formulations against *S. aureus*, *E. coli*, and *P. aeruginosa* are presented in Figure 2 (A–C). The corresponding inhibition zone diameters are summarized in Table 6.

Wound Healing Activity

All animal experiments were reviewed and approved by the Institutional Animal Ethics Committee (IAEC), Santhiram College of Pharmacy (Autonomous), Nandyal, Andhra Pradesh, India, under Approval No. 1519/PO/Re/S/11/CPCSEA/2025/002, and were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA),

Government of India. Adult male Wistar albino rats weighing 180–200 g were used for the in vivo wound healing study. Prior to the experiment, the animals were acclimatized for one week under standard laboratory conditions ($25 \pm 2^\circ\text{C}$, $55 \pm 5\%$ relative humidity, and a 12 h light/dark cycle) with free access to a standard pellet diet and water ad libitum.

Excision Wound Model

The wound healing potential of the developed formulations was assessed using the excision wound model. Prior to surgery, the animals were anesthetized by intraperitoneal administration of ketamine hydrochloride (60 mg/kg body weight). The dorsal thoracic region of each rat was shaved using an electric clipper and disinfected with 70% ethanol.

A circular full-thickness excision wound measuring approximately 200 mm² was created on the dorsal surface using sterile surgical scissors and a scalpel. Care was taken to remove the entire thickness of the skin without damaging the underlying muscle tissue. The wound was left uncovered throughout the experimental period to facilitate topical treatment.

Twenty-four rats were randomly divided into four groups, each containing six animals ($n = 6$).

- Group I (G1): Control – Received no treatment.
- Group II (G2): Standard – Treated with marketed silver nitrate gel formulation.
- Group III (G3): Test – Treated with green-synthesized silver nanoparticle hydrogel formulation.
- Group IV (G4): Test – Treated with neem extract gel formulation.

The assigned formulations were applied topically over the wound surface twice daily from the day of wound creation until complete healing was observed. The wounds were monitored for 21 days, and the healing process was evaluated by measuring wound contraction.

Determination of Wound Contraction

Wound healing progression was assessed by measuring the reduction in wound area at predetermined intervals (Days 0, 3, 6, 9, 12, 15, 18, and 21). The wound margins were traced onto transparent acetate sheets, and the wound area was determined using graph paper or digital planimetric analysis. The percentage of wound contraction was calculated using the following equation:

Percentage Wound Contraction = $\frac{A - B}{A} \times 100$
 ... equation (4)
 where:

- A = Initial wound area (Day 0)
- B = Wound area on the respective observation day

A progressive decrease in wound area was considered indicative of effective wound healing.

Histopathological Evaluation

At the end of the experimental period, healed skin tissues from representative animals of each group were excised for histological examination. The tissue samples were fixed in 10% neutral buffered formalin, dehydrated through ascending grades of ethanol, cleared in xylene, and embedded in paraffin wax. Sections of approximately 5 µm thickness were prepared using a rotary microtome and stained with hematoxylin and eosin (H&E).

The stained sections were examined under a light microscope to evaluate the quality of tissue regeneration. Histological parameters including re-epithelialization, collagen deposition, fibroblast proliferation, neovascularization, inflammatory cell infiltration, and restoration of normal skin architecture were assessed and compared among the treatment groups.

Table 1. Experimental Design for Excision Wound Healing Study

Group	Treatment Code	Treatment Description	Route of Administration	Treatment Frequency	No. of Animals (n)
G1	Control	No treatment	—	—	6
G2	S1	Market silver nitrate gel formulation	Topical	Twice daily	6
G3	S2	Green-synthesized silver nanoparticle hydrogel	Topical	Twice daily	6

		formulation			
G4	S3	Neem extract gel formulation	Topical	Twice daily	6

Accelerated Stability Study

The accelerated stability study of the optimized green-synthesized silver nanoparticle (AgNP)-loaded chitosan hydrogel was performed according to the International Council for Harmonisation (ICH) guidelines. The optimized hydrogel was filled into laminated aluminum collapsible tubes, sealed properly, and stored in a stability chamber maintained at $40 \pm 2^\circ\text{C}$ and $75 \pm 5\%$ relative humidity (RH) for a period of three months.

Samples were withdrawn at 0 (initial), 1, 2, and 3 months and evaluated for changes in physicochemical and functional properties. The parameters investigated included appearance, color, homogeneity, pH, viscosity, spreadability and silver nanoparticle content. All analyses were performed in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD). The formulation was considered stable if no significant changes in the evaluated parameters were observed throughout the storage period.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation (SD) (n = 6). Statistical comparisons among groups at each observation period were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

pH of Silver Nanoparticle-Loaded Chitosan Hydrogel

The pH of the formulated silver nanoparticle-loaded chitosan hydrogel was found to be 5.87 ± 0.35 (n = 3). The obtained pH falls within the normal skin pH range (4.5–6.5), indicating its suitability for topical application and minimizing the risk of skin irritation.

Table 2. pH of Optimized Hydrogel

S. No.	pH
1	5.9
2	6.2
3	5.5
Mean $\pm$ SD	5.87 ± 0.35

The results indicate good formulation uniformity and compatibility with the skin, making the hydrogel suitable for wound healing applications.

Viscosity of Silver Nanoparticle-Loaded Chitosan Hydrogel

The viscosity of the silver nanoparticle-loaded chitosan hydrogel was found to be 4208 ± 101.01 cPs. The relatively high viscosity indicates the formation of a stable hydrogel network and provides good consistency for topical application. The low variation among measurements demonstrates formulation uniformity and reproducibility. This viscosity is suitable for wound dressing applications, ensuring adequate retention at the application site and sustained release of silver nanoparticles.

Table 3: Viscosity of Silver Nanoparticle-Loaded Chitosan Hydrogel

S. No.	Viscosity (cPs)
1	4206
2	4310
3	4108
Mean $\pm$ SD	4208 ± 101.01

Rheological Characterization: Spreadability of AgNP-Chitosan Hydrogel

The spreadability of the silver nanoparticle-loaded chitosan hydrogel was found to be 12.11 ± 3.11 g·cm/s. The formulation exhibited good spreadability, indicating ease of application and uniform distribution over the skin or wound surface. The obtained value suggests an optimum balance between viscosity and flow characteristics, enabling the hydrogel to remain at the site of application while allowing convenient spreading. These results indicate that the developed hydrogel possesses suitable rheological properties for topical wound-healing applications.

Table 4. Spreadability of Green-Synthesized Silver Nanoparticle-Loaded Chitosan Hydrogel

S. No.	Time (s)	Weight (g)	Length (cm)	Spreadability (g ² cm/s)
1	12	20	7.5	12.50
2	10	20	7.5	15.00
3	17	20	7.5	8.82
Mean $\pm$ SD	—	—	—	12.11 ± 3.11

Occlusive effect of the Hydrogel

The occlusion factor of the silver nanoparticle-loaded chitosan hydrogel was found to be $76.69 \pm 5.20\%$. The high occlusion value indicates that the hydrogel effectively reduced water evaporation, thereby enhancing moisture retention. This occlusive property is beneficial for wound-healing applications, as it helps maintain a moist environment that promotes cell migration, tissue regeneration, and faster wound closure. The results suggest that the developed hydrogel possesses good barrier-forming ability and is suitable for topical wound management.

Table 05. Occlusion factor of the silver nanoparticle-loaded chitosan hydrogel

S. No.	Water Loss from Reference	Water Loss from Hydrogel-	Occlusion Factor, F (%)
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	Beaker, A (g)	Treated Beaker, B (g)	
1	12.0	3.5	70.83
2	15.0	2.9	80.67
3	14.0	3.0	78.57
Mean $\pm$ SD	—	—	76.69 ± 5.18

Silver Nanoparticle Content

The UV-Visible spectrophotometric method was successfully employed to quantify the silver nanoparticle (AgNP) content in the optimized chitosan hydrogel. The calibration curve constructed using AgNP standard solutions over the concentration range of 10–100 $\mu\text{g/mL}$ exhibited excellent linearity, confirming compliance with the Beer-Lambert law. The regression equation obtained was $y = 0.0111x + 0.0018$, with a correlation coefficient (R^2) of 0.9992, demonstrating a strong linear relationship between absorbance and AgNP concentration. The high correlation coefficient indicates the reliability, accuracy, and suitability of the analytical method for quantitative estimation of silver nanoparticles within the selected concentration range.

The optimized AgNP-loaded chitosan hydrogel exhibited a silver nanoparticle content of $98.64 \pm 0.71\%$, indicating efficient incorporation of nanoparticles into the hydrogel matrix with minimal processing losses. The high recovery value suggests that the preparation method ensured homogeneous dispersion of AgNPs throughout the polymeric network, which is essential for achieving uniform therapeutic performance and reproducible antimicrobial and wound healing activity.

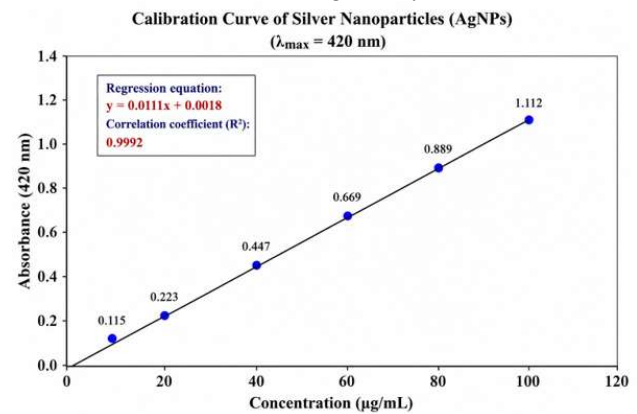


Figure 1. Calibration curve of green-synthesized silver nanoparticles determined by UV-Visible spectrophotometry at 420 nm.

Antimicrobial activity

The antibacterial activity of the optimized green-synthesized AgNP-loaded chitosan hydrogel was evaluated against three clinically relevant wound pathogens, namely *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. As presented in Figure 2(A–C) and Table 6, the optimized formulation (G3) produced the largest

zones of inhibition against all tested microorganisms compared with the marketed silver-based gel (G2) and the plain chitosan hydrogel (G1), demonstrating its superior broad-spectrum antibacterial activity. In Figure 2A, the agar well diffusion plate against *Staphylococcus aureus* (ATCC 25923) shows that the optimized AgNP-loaded chitosan hydrogel (G3) produced the largest inhibition zone, followed by the marketed silver gel (G2), whereas the plain chitosan hydrogel (G1) exhibited only a small inhibition zone. This finding indicates that the incorporation of green-synthesized silver nanoparticles markedly enhanced antibacterial activity against Gram-positive bacteria. Similarly, Figure 2B illustrates the antibacterial activity against *Escherichia coli* (ATCC 25922). The optimized formulation (G3) exhibited a noticeably larger inhibition zone than both the marketed formulation (G2) and the control hydrogel (G1), confirming its improved effectiveness against Gram-negative bacteria. The antibacterial activity against *Pseudomonas aeruginosa* (ATCC 27853) is presented in Figure 2C. Although *P. aeruginosa* is recognized for its intrinsic resistance to many antimicrobial agents, the optimized AgNP-loaded chitosan hydrogel (G3) produced the widest inhibition zone among all formulations, exceeding that of the marketed silver gel. The control formulation showed only minimal antibacterial activity.

Table 6. Zone of Inhibition of Different Formulations Against Wound Pathogens

Formulation		<i>S. aureus</i> (mm)	<i>E. coli</i> (mm)	<i>P. aeruginosa</i> (mm)
Plain chitosan hydrogel	G1	10.8 ± 0.6	9.7 ± 0.5	8.9 ± 0.4
Marketed silver gel	G2	21.6 ± 0.8	20.8 ± 0.7	19.7 ± 0.6
Optimized AgNP-loaded chitosan hydrogel	G3	24.8 ± 0.7	23.5 ± 0.8	22.6 ± 0.5

Values are expressed as Mean ± SD (n = 3).

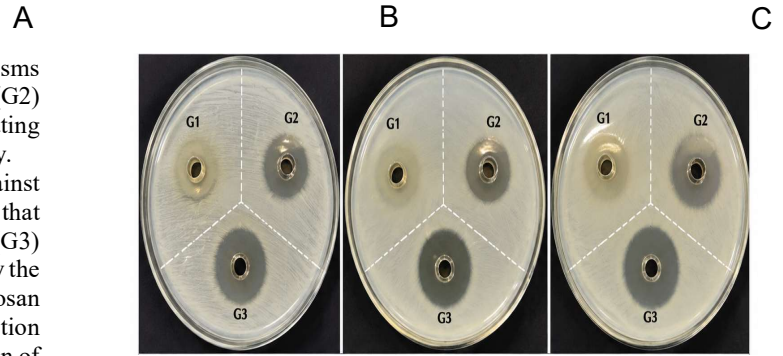


Figure 2. Representative agar well diffusion plates showing the antibacterial activity of different hydrogel formulations against wound-associated bacterial pathogens.

(A) *Staphylococcus aureus* (ATCC 25923): The optimized AgNP-loaded chitosan hydrogel (G3) exhibited the largest zone of inhibition, followed by the marketed silver gel (G2), whereas the plain chitosan hydrogel (G1) produced only a small inhibition zone, indicating comparatively lower antibacterial activity.

(B) *Escherichia coli* (ATCC 25922): The optimized AgNP-loaded chitosan hydrogel (G3) demonstrated superior antibacterial efficacy with the widest inhibition zone, followed by the marketed silver gel (G2). The control hydrogel (G1) showed minimal inhibition.

(C) *Pseudomonas aeruginosa* (ATCC 27853): The optimized AgNP-loaded chitosan hydrogel (G3) produced the greatest inhibition zone against *P. aeruginosa*, exceeding that of the marketed silver gel (G2), while the plain chitosan hydrogel (G1) exhibited the smallest zone of inhibition.

Abbreviations: G1 – Plain chitosan hydrogel (Control); G2 – Marketed silver-based wound healing gel (Standard); G3 – Optimized green-synthesized AgNP-loaded chitosan hydrogel (Test).

The enhanced antibacterial performance of G3 can be attributed primarily to the small particle size (36.8 nm) and uniform distribution of green-synthesized silver nanoparticles within the chitosan matrix. The nanoscale dimensions provide a high surface area that facilitates intimate interaction with bacterial cell membranes. Silver nanoparticles exert their antibacterial action through multiple mechanisms, including disruption of membrane integrity, generation of reactive oxygen species (ROS), interaction with sulfhydryl-containing proteins, and inhibition of DNA replication, ultimately leading to bacterial cell death. The simultaneous operation of these mechanisms reduces the possibility of bacterial resistance.

The superior antibacterial efficacy of the optimized hydrogel may also result from the synergistic contribution of its individual components. Neem (*Azadirachta indica*) phytochemicals, including

flavonoids, terpenoids, tannins, and phenolic compounds, possess inherent antimicrobial and antioxidant properties that complement the activity of AgNPs. In addition, chitosan contributes intrinsic antibacterial activity through electrostatic interaction between its protonated amino groups and negatively charged bacterial cell membranes, leading to increased membrane permeability and leakage of intracellular components.

Among the tested microorganisms, the optimized formulation demonstrated the greatest antibacterial activity against *S. aureus*, followed by *E. coli* and *P. aeruginosa*. This variation may be attributed to structural differences in bacterial cell envelopes. The thick but porous peptidoglycan layer of Gram-positive bacteria permits easier penetration of nanoparticles, whereas the outer membrane of Gram-negative bacteria provides an additional permeability barrier. Nevertheless, the optimized formulation exhibited substantial inhibitory activity against all three pathogens, indicating broad-spectrum antimicrobial efficacy.

The results demonstrate that the optimized green-synthesized AgNP-loaded chitosan hydrogel possesses superior antibacterial activity compared with the marketed formulation. The synergistic combination of green-synthesized silver nanoparticles, neem phytoconstituents, and chitosan offers an effective multifunctional wound dressing capable of preventing wound-associated infections while promoting rapid wound healing.

Wound Contraction Study

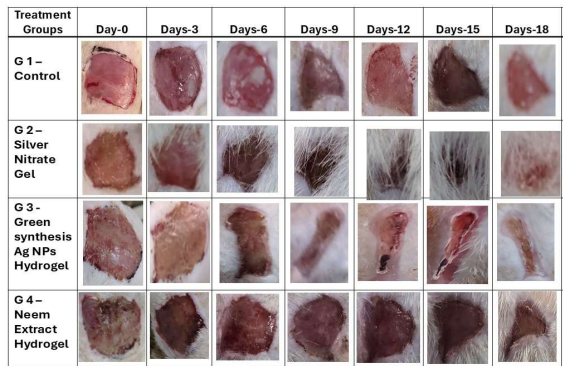


Figure 3. Sequential photographic representation of wound contraction and healing in excision wound rats treated with Control (G1), Silver Nitrate Hydrogel (G2), Green Synthesized Silver Nanoparticle Hydrogel (G3), and Neem Hydrogel (G4) from Day 0 to Day 21.

Representative photographs of the excision wounds revealed a progressive reduction in wound size in all experimental groups over the 21-day treatment period. However, the rate of wound contraction and tissue regeneration differed considerably among the groups. The Control group (G1) showed the slowest healing, with delayed wound contraction and incomplete epithelialization throughout the observation period. In contrast, all treatment groups exhibited improved wound healing compared with the control.

The Green Synthesized Silver Nanoparticle (AgNP) Hydrogel (G3) demonstrated the most rapid and complete wound healing. A marked reduction in wound area was evident from Day 6, followed by accelerated granulation tissue formation and progressive epithelialization. By Day 21, the wounds in this group were almost completely healed, with restoration of normal skin architecture and visible hair regrowth, indicating effective tissue remodeling. The enhanced healing may be attributed to the synergistic action of silver nanoparticles and neem phytochemicals, which possess potent antimicrobial, antioxidant, and anti-inflammatory properties. These properties help reduce microbial load, minimize oxidative stress, stimulate fibroblast proliferation, enhance collagen synthesis, and promote angiogenesis, thereby accelerating wound repair.

The Silver Nitrate Hydrogel (G2) also showed a significant improvement in wound healing compared with the control. Wound contraction progressed steadily with healthy granulation tissue formation and epithelialization; however, the healing response was comparatively slower than that observed with the green synthesized AgNP hydrogel. The antimicrobial activity of silver ions likely prevented wound infection and facilitated tissue regeneration, although the absence of plant-

derived bioactive compounds may have limited its overall therapeutic efficacy.

The Neem Hydrogel (G4) exhibited moderate wound healing activity throughout the study. Wound contraction occurred gradually with continuous epithelialization, but complete wound closure was achieved later than in the AgNP-treated groups. The wound healing effect of neem hydrogel can be attributed to the presence of bioactive phytoconstituents such as flavonoids, tannins, terpenoids, and polyphenols, which possess antimicrobial, antioxidant, and anti-inflammatory activities that support the natural wound healing process.

Overall, the photographic observations clearly indicate that the Green Synthesized AgNP Hydrogel (G3) produced the greatest improvement in wound healing among all treatment groups. The accelerated wound contraction, enhanced epithelialization, and complete tissue remodeling observed in this group demonstrate the therapeutic potential of green synthesized silver nanoparticles as an effective topical wound healing formulation. These findings are consistent with the proposed mechanism of silver nanoparticles in promoting rapid tissue repair while maintaining excellent biocompatibility.²⁷

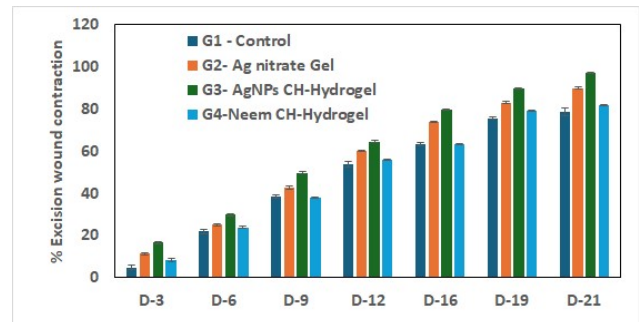


Figure 4. Percentage wound contraction in excision wound rats treated with Control (G1), Silver Nitrate Gel (G2), Green Synthesized AgNPs Chitosan Hydrogel (G3), and Neem Chitosan Hydrogel (G4) during the 21-day treatment period.

The Silver Nitrate Gel (G2) also significantly accelerated wound healing, achieving approximately 90% wound contraction by Day 21, although its efficacy was lower than that of the green synthesized AgNP hydrogel. The Neem Chitosan Hydrogel (G4) showed moderate wound healing activity, with approximately 82% wound contraction at the end of the study, reflecting the beneficial effects of neem phytoconstituents and the chitosan hydrogel matrix on tissue repair. In contrast, the Control group (G1) demonstrated the slowest healing response, attaining only about 79% wound contraction by Day 21.

Table 7. Percentage wound contraction (%; Mean $\pm$ SD, n = 6)

Group	D-3	D-6	D-9	D-12	D-16	D-19	D-21
G1 Control	4.84 $\pm$ 0.98	21.92 $\pm$ 1.17	38.50 $\pm$ 0.65	53.71 $\pm$ 1.27	63.16 $\pm$ 0.85	75.63 $\pm$ 0.59	78.78 $\pm$ 1.75
G2 Silver Nitrate Gel	11.40 $\pm$ 0.48**	25.26 $\pm$ 0.51**	42.65 $\pm$ 0.77**	60.27 $\pm$ 0.38**	73.95 $\pm$ 0.31**	83.16 $\pm$ 0.61**	90.01 $\pm$ 0.33**
G3 Green AgNP Hydrogel	16.69 $\pm$ 0.61**	29.94 $\pm$ 0.42**	49.84 $\pm$ 0.65**	64.59 $\pm$ 0.38**	79.76 $\pm$ 0.17**	89.61 $\pm$ 0.11**	97.01 $\pm$ 0.17**
G4 Neem Hydrogel	8.32 $\pm$ 0.73*	23.94 $\pm$ 0.40	37.88 $\pm$ 0.38	56.01 $\pm$ 0.45*	63.36 $\pm$ 0.39	79.09 $\pm$ 0.13*	81.92 $\pm$ 0.17*

Values are expressed as Mean $\pm$ SD (n = 6).

* $p < 0.05$, ** $p < 0.001$ versus G1 (Control)

††† $p < 0.001$ versus G2 and G4 (Tukey's multiple comparison test)

The percentage wound contraction increased progressively in all experimental groups throughout the study. One-way ANOVA demonstrated significant differences among the treatment groups at each observation period ($p < 0.001$). Tukey's post hoc analysis revealed that the Green AgNPs hydrogel (G3) exhibited significantly greater wound contraction than the control (G1), silver nitrate gel (G2), and neem hydrogel (G4) from Day 3 onwards ($p < 0.001$). By Day 21, G3 achieved the highest wound contraction ($97.01 \pm 0.17\%$), followed by G2 ($90.01 \pm 0.33\%$), G4 ($81.92 \pm 0.17\%$), and G1 ($78.78 \pm 1.75\%$). The silver nitrate gel also produced significantly greater wound contraction than the control throughout the experimental period ($p < 0.001$). Neem hydrogel showed moderate improvement compared with the control, with statistically significant differences observed mainly during the later stages of wound healing.

Percentage wound contraction increased progressively in all groups throughout the study.

The AgNPs chitosan hydrogel (G3) exhibited the highest wound contraction, increasing from $16.69 \pm 0.61\%$ on Day 3 to $97.01 \pm 0.17\%$ on Day 21. The Ag nitrate gel (G2) showed the second-highest healing response ($90.01 \pm 0.33\%$ on Day 21), followed by the Neem chitosan hydrogel (G4) ($81.92 \pm 0.17\%$) and the control group (G1) ($78.78 \pm 1.75\%$). These findings indicate that incorporation of green synthesized silver nanoparticles into the chitosan hydrogel markedly accelerated wound closure compared with the conventional silver nitrate gel and neem hydrogel.

Histopathological Findings

Histopathological examination performed on Day 21 demonstrated distinct differences among the treatment groups.

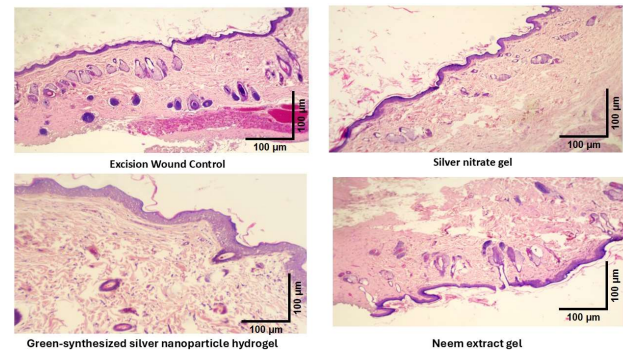


Figure 5. Representative photomicrographs of H&E-stained skin sections showing histopathological changes following topical treatment of excision wounds: (A) G1—excision wound control, (B) G2—marketed silver nitrate gel, (C) G3—green-synthesized silver nanoparticle hydrogel, and (D) G4—neem extract gel.

Histopathological examination further supported the macroscopic wound-healing findings. The excision wound control group exhibited comparatively disorganized epidermal and dermal architecture, whereas treatment groups showed progressive improvement in tissue regeneration. The marketed silver nitrate gel demonstrated improved epidermal continuity and dermal organization compared with the control. The green-synthesized silver nanoparticle hydrogel exhibited the most favorable histological appearance, characterized by relatively complete re-epithelialization, improved organization of collagen-rich connective tissue, and restoration of dermal structures. The neem extract gel also produced appreciable tissue regeneration, although the overall tissue organization appeared comparatively less uniform than that observed with the AgNP hydrogel. The enhanced histological response to the green-synthesized AgNP hydrogel may be attributed to the combined antimicrobial activity of silver nanoparticles and the antioxidant,

anti-inflammatory, and wound-supportive properties of neem-derived phytoconstituents. Overall, the histological findings indicate that the developed green-synthesized AgNP hydrogel promoted progression of wound healing toward tissue remodeling and restoration of skin architecture.

Analysis of Accelerated Stability Testing Report

The accelerated stability results are presented in Table 8. The optimized AgNP loaded chitosan hydrogel remained physically stable throughout the three-month storage period under accelerated conditions. No evidence of phase separation, syneresis, discoloration, or microbial growth was observed, and the formulation retained its smooth appearance and homogeneity. The absence of phase separation and syneresis indicates that the chitosan polymer network effectively retained the incorporated silver nanoparticles without compromising gel integrity during storage.

Table 8. Accelerated Stability Study of Optimized AgNP-Loaded Chitosan Hydrogel ($40 \pm 2^\circ\text{C}/75 \pm 5\% \text{ RH}$)

Parameter	Initial	1 Month	2 Months	3 Months
Appearance	Smooth, homogeneous	No change	No change	No change
Color	Light Brown	No change	No change	No change
Homogeneity	Homogeneous	Homogeneous	Homogeneous	Homogeneous
pH	5.87 ± 0.35	5.84 ± 0.61	5.80 ± 0.54	5.82 ± 0.29
Viscosity (cP)	4208 ± 101.01	4159 ± 80.22	4120 ± 82.43	4103 ± 90.17
Spreadability ($\text{g}^\circ\text{cm/s}$)	12.11 ± 3.11	14.35 ± 2.25	13.21 ± 3.23	13.08 ± 4.07
Silver nanoparticle content (%)	98.64 ± 0.71	98.32 ± 0.65	98.01 ± 0.68	97.71 ± 0.74

Values are expressed as Mean $\pm$ SD (n = 3).

Only marginal changes were observed in the evaluated physicochemical parameters. The pH decreased slightly from 6.84 ± 0.03 to 6.79 ± 0.04 , remaining within the acceptable range for topical application. Similarly, viscosity and spreadability exhibited only minimal reductions during storage, indicating that the gel maintained its consistency and ease of application.

The silver nanoparticle content remained above 97% throughout the study, demonstrating excellent retention of AgNPs within the hydrogel matrix. The results demonstrate that the formulation possesses

satisfactory storage stability and supports its potential for further pharmaceutical development as a stable and effective topical wound healing system.

Conclusion

The present study successfully demonstrated the development and preclinical evaluation of a green-synthesized silver nanoparticle-loaded chitosan hydrogel as a multifunctional topical wound healing system. The neem-mediated synthesis approach generated stable silver nanoparticles that were efficiently incorporated into the chitosan hydrogel, resulting in a formulation with desirable physicochemical properties, including skin-compatible pH, appropriate viscosity, good spreadability, high occlusivity, and excellent nanoparticle loading.

The optimized hydrogel exhibited superior broad-spectrum antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, demonstrating enhanced antimicrobial efficacy compared with the marketed silver gel. In the excision wound model, the AgNP-loaded hydrogel significantly accelerated wound contraction, achieving approximately 97% wound closure within 21 days. Histopathological findings further supported enhanced tissue regeneration, characterized by improved re-epithelialization, collagen deposition, fibroblast proliferation, and restoration of normal skin architecture. These therapeutic effects can be attributed to the synergistic action of silver nanoparticles, neem-derived phytochemicals, and chitosan, which collectively provide antimicrobial, antioxidant, anti-inflammatory, and tissue-regenerative benefits.

Furthermore, the formulation maintained its physicochemical integrity and silver nanoparticle content throughout the accelerated stability study, confirming its suitability for pharmaceutical development and storage. The findings establish that the green-synthesized AgNP-loaded chitosan hydrogel is a stable, effective, and eco-friendly wound dressing with significant potential for the management of acute and infected wounds. Future investigations involving chronic wound models, mechanistic studies, toxicity evaluation, and clinical trials are warranted to facilitate its translation into clinical practice.

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