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Green Synthesis of Silver Nanoparticles with *Picrasma javanica* Extract Shows Enhanced Wound Healing in Wistar Rats

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Abstract

Silver and various non-antibiotic alternative therapies were not utilized after the discovery of penicillin and other antibiotics. However, recently silver has gained substantial recognition due to the rise of antibiotic-resistant strains and its limited propensity to develop resistance. Studies have suggested that Silver nanoparticles (AgNPs) prepared by green synthesis have superior wound-healing properties compared to conventional AgNPs despite the latter having improved mechanical properties and being widely used as wound-healing agents. In our study, we formulated AgNPs using a green synthesis method using methanolic extract of *Picrasma javanica* (MePJ) leaves and evaluated its wound healing efficacy in Wistar rats. Before formulation, MePJ was evaluated for phytochemical screening, total phenolic and flavonoid content, and antioxidant, antimicrobial, and anti-inflammatory activity. Synthesis of the AgNPs was done by a green synthesis method where 1% MePJ was used as a reducing agent. A UV-visible spectrophotometer was used to characterize the formed AgNPs. Formulated AgNPs were then incorporated in gel and further characterized for pH, porosity, Viscosity, spreadability, extrudability, antimicrobial activity, and wound healing by excision wound model in Wistar rats using 10% w/w povidone iodine ointment as standard. Formulated AgNPs show good physical characteristics, good antimicrobial activity, and enhanced wound closing compared to MeJP gel and povidone iodine ointment. The epithelization period of the wound decreased in AgNPs compared to Povidone iodine ointment. In conclusion, AgNPs have shortened the wound-closing period and may be utilized as an alternative formulation for wound healing.

Keywords: AgNPs, AgNPs-based nanogel, Green silver nanoparticles, *Picrasma javanica*, Wound healing

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Introduction

Silver and alternative non-antibiotic treatments were discontinued after the discovery of penicillin and other antibiotics [1]. However, due to the rise of antibiotic-resistant strains, silver got considerable attention as it has less propensity to develop resistance [2]. Nanoparticles have unique physicochemical properties and therefore managed to attain attention in various fields worldwide including in the field of medicine [3]. Amongst the various metal nanoparticles silver nanoparticles (AgNPs) have been extensively studied for their wound-healing activity due to their broad-spectrum antimicrobial properties [4]. However, the synthesis of AgNPs using chemical methods uses hazardous chemicals that pose a threat to human health as well as the environment. Using herbal extracts as

an alternative method to synthesize green AgNPs avoids the risks of health and environmental hazards. This method involves the reduction of silver ions using phytochemicals present in the herbal extract, which not only eliminates the use of toxic chemicals but also provides a natural capping agent for AgNPs [5]. The green method of synthesis is preferred over chemical methods because of easy techniques, low production cost, environmentally friendly, and rapid production of nanoparticles [6]. In this method, the plant extract is used as a reducing agent usually added to the $AgNO_3$ solution where it causes a reduction of silver ions to AgNPs. Changes in color to colloidal brown from pale yellow indicate the formation of AgNPs [7].

Picrasma javanica (*P. javanica*), an averaged size tree from the Simaroubaceae family, is widely distributed in tropical regions of Asia, such as Myanmar, Indonesia, and India. This plant possesses various pharmacological properties, including anti-tumor, antimalarial, and antiviral activities, and is particularly known for its secondary metabolites, namely quassinoids [8, 9]. Traditional medicine practices in Myanmar, Thailand, and Indonesia have used *P. javanica* for treating different diseases, which has piqued the interest of researchers to initiate further studies on its potential therapeutic activities [10]. In this study, we have formulated AgNPs using the extract of *P. javanica* and the formulated AgNPs were tested for enhancement in wound healing.

Materials and Methods

Collection of plant material and identification

The plant sample was collected from the Serchhip district of Mizoram, India, and was authenticated by the Botanical Survey of India (BSI), Shillong with Reference No. BSI/ERC/Tech/2020-2021/1452 dated 03/02/2021.

Preparation of methanol extract

The collected fresh leaves of *P. javanica* were cleaned, washed, and dried under shade. The shade-dried plant leaves were crushed and ground with a mixer grinder (Tandem, Model: TMG7843A, India) to powder. The powder was cold-macerated using methanol for 72 hours. The extract was then filtered and concentrated with the help of a rotary vacuum evaporator (IKA, RC2 basic, Germany).

Animal housing and treatments

Either sex Wistar rats, aged 6-8 weeks, were obtained from the Departmental Laboratory Animal House Facility at RIPANS in Aizawl, Mizoram, India. The rats were housed individually and subjected to general captivity conditions, with simulated atmospheric settings (temperature: 25±2 °C; relative humidity: 70±5%) and a 12-hour light-dark cycle. A period of 7 days was allotted for the acclimatization of the animals before experimentation commenced. Before experimentation, approval was taken from the Institutional Animal Ethics Committee (IAEC) of the Department of Pharmacy, RIPANS, Aizawl, Mizoram, India. (Registration no. 149/GO/a/11/CPCSEA vide approval no. IAEC/RIPANS/67).

Qualitative phytochemical analysis

The extract obtained from *P. javanica* leaves was examined to determine the presence of different phytoconstituents including alkaloids, phenols, tannins, flavonoids, steroids, etc. using standard protocol [11].

Quantitative phytochemical analysis

Determination of total phenolic contents

The quantification of the total phenolic content in plant extract was performed by the Folin-Ciocalteu Reagent (FCR) method according to the protocol provided by Gutfinger, 1986 with slight modification [12]. Briefly, 1 ml of *P. javanica* extract was combined with 5 ml of FCR. After 3 minutes, 4 mL of 0.7 M sodium carbonate solution was added to the mixture which was then allowed to stand for 1 hr at room temperature. Absorbance readings were taken at 765 nm using a UV Vis Spectrophotometer (Evaluation 201, Thermo Fisher Scientific, USA). Total phenolic content was determined with the help of a calibration curve which was prepared previously with different concentrations of Gallic acid (10, 20, 40, 60, 80, and 100 µg/mL). The results were expressed as mg GAE (Gallic Acid Equivalent) per dried extract.

Determination of total flavonoid contents

The quantification of the flavonoid content in plant extract was performed by the aluminum chloride method according to the method described by Chang *et al.* with slight modifications [13]. Briefly, in a concise manner, 1 ml of the extract was combined with 2 ml of distilled water. After a 5-minute interval, 3 ml of 5 % sodium nitrite (NaNO2) and 0.3 ml of 10% aluminum chloride were added. After an interval of another 6 minutes, 2mL of 1M NaOH was introduced and the volume was adjusted to 10 ml with distilled water. After 1 hour, the absorbance reading was recorded at 510 nm. A standard curve was generated using Quercetin at different concentrations (10, 20, 40, 60, 80, and 100 µg/mL). The quantification of total flavonoid content was done using the prepared standard curve. The total flavonoid content was expressed as milligrams of Quercetin equivalents (QE/g) of dried extract.

Anti-oxidant activity

Determination of 2, 2 –diphenyl – 1- picrylhydrazyl (DPPH) scavenging activity

The DPPH method was used to determine the antioxidant activity based on the scavenging effect of the stable DPPH free radical. It follows the protocol originally described by Blois in 1958 with minor adjustments [14]. As a reference standard, Ascorbic acid was used. 0.5 mL of 0.1 mM DPPH solution in methanol was mixed with 3 mL of the extract, as well as 3 mL of a standard prepared in various concentrations (1, 5, 10, 15, and 20µg/mL) respectively. Both extracts and standards were incubated for 30 minutes at 37°C. The Absorbance was then recorded at 517 nm using the UV-Vis spectrophotometer (Evaluation 201, Thermo Fisher Scientific, USA). The same procedure was followed for control and absorbance was recorded accordingly. The scavenging effect of DPPH free radical was calculated using the following equations.

%DPPH radical scavenging= (Absorbance of control-Absorbance of extract)/Absorbance of control ×100 (1)

Determination of reducing power

The method for determining the reducing power of the extract can be performed using Ascorbic acid as a standard as described by Sun *et al*., 2012 with slight modifications [15]. For experimenting, 1 mL of the extract and 1 mL of the standard with various concentrations (20, 40, 60, 80, and 100 µg/mL) were combined with 2.5 mL of phosphate buffer (6.6 pH) and 2.5mL of 1% potassium ferricyanide. The resulting mixture was incubated at 50°C for 30 mins. To stop the reaction 2.5 mL of 10% TCA was added to the mixture followed by centrifugation at 3000 rpm for 10 mins. Then, 2.5 mL of the supernatant was combined with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride solution. The absorbance of the mixture was recorded at 700 nm using the UV-Vis spectrophotometer (Evaluation 201, Thermo Fisher Scientific, USA). Higher absorbance of the reaction mixture indicates higher reducing power.

Anti-microbial activity

Preparation of plant extract disc

A circular paper disc with a diameter of 5 mm was created from Whatman filter paper no.3. The paper discs underwent sterilization before the application of the extracts. Then, 10µL of the plant extracts were added to the paper discs followed by air drying.

Evaluation of the anti-microbial activity of plant extract

An antimicrobial study was conducted as per the method described by Roy *et al.* with minor modifications [16]. Mueller Hinton agar plates were prepared and were inoculated with the test microorganisms adjusted to 0.5 McFarland standard by pour plate method using a sterilized micropipette. Both gram-negative and gram-positive namely *Escherichia coli* (ATCC–10536), *Pseudomonas aeruginosa* (ATCC–10145), *Klebsiella pneumonia* (ATCC–10031), and *Bacillus subtilis* (ATCC–11774) were used for the antimicrobial assay. Bacterial strains were added using a micropipette in the Petri plates and nutrient agar was carefully added to the Petri plate from the conical flask. To ensure and maintain the uniformity of the culture and medium, the dish was gently rotated or moved in a back-and-forth motion. After solidification, sterile forceps were used to place the paper disc containing plant extract and an antibiotic disc containing standard (Ceftriaxone, etc.) onto the agar plate. The plates were left overnight at 37°C and subsequently observed for zones of inhibition.

In-vitro anti-inflammatory activity

Bovine serum albumin (BSA) denaturation

The experiment was performed following the methods described by Gunathilake *et al*. with minor modifications [17]. Briefly, a test solution was prepared consisting of 0.45 ml of 5% w/v BSA aqueous solution and 0.05 ml of test solution (100, 200, 300, 400, and 500 µg/ml). The control solution consisted of 0.45 ml of 5% w/v BSA aqueous solution and 0.05 ml of phosphate buffer. The standard solution contains 0.45 ml of 5% w/v BSA aqueous solution and 0.05 ml of diclofenac sodium (100, 200, 300, 400, and 500 µg/ml). pH of the above solutions was adjusted to pH 6.3 using 1N Hydrochloric acid. Subsequently, the samples were incubated at 37 ºC for 20 minutes followed by a temperature increase to 57 ºC for 3 minutes. After cooling, 2.5 ml of phosphate buffer saline was added to each solution. The turbidity was measured at 660 nm using a UV visible Spectrophotometer (Evaluation 201, Thermo Fisher Scientific, USA).

The percentage inhibition of protein denaturation was calculated by,

I%=[Acontrol–Asample/Acontrol] ×100 (2)

Where Acontrol is Absorbance of control and Asample is Absorbance of the sample.

Synthesis of AgNPs using P. javanica extract

AgNPs were prepared according to the methods described by Nagaich *et al*. with slight modifications [18]. Briefly, 10 mM Silver Nitrate solution was prepared by dissolving 0.17 g of $AgNO_3$ in 100 ml distilled water stirred for 20 minutes. The solution was then kept at room temperature under dark conditions. 10 ml of 1% *P. javanica* extract was added (dropwise) to the AgNO3 solution and stirred for 60 minutes. The resulting mixture was then incubated at room

temperature in the dark for 24 hours. A color transformation from light green to brown indicated the synthesis of AgNPs.

Detection and characterization of AgNPs

Visual observation

Visual observation was employed as the primary method of detection. The transformation in color from light green to reddish-brown indicated the synthesis of AgNPs [19]. On the other hand, in control where it lacked extract but contained only 10 mM AgNO3 retained its light green even after being exposed to sunlight for 10 mins.

Ultraviolet-visible (UV–vis) spectroscopy

A UV-visible spectrophotometer is used to observe the formation of AgNPs by detecting color change [20]. The formulated AgNPs were scanned by a UV–vis spectrophotometer (Evaluation 201, Thermo Fisher Scientific, USA) at the range of wavelengths of 250–800 nm.

Preparation of AgNPs-based gel

Formulated AgNPs were incorporated with gel bases prepared according to the method described by Jadhav *et al*. with slight modifications [21]. Briefly, in 100ml of AgNPs solution, the required quantity of Carbopol 934 was added by mechanical stirring at room temperature for 1 hour to attain proper swelling of the polymer. 2ml of Glycerol and 3-5 drops of Triethanolamine were added as viscosity enhancers. The pH of the gel was neutralized by using 1M NaOH. The prepared gel was stored in an amber color container, in a cool and dry place. Different combinations of AgNPs gel have been given in **Table 1a**.

Characterization and optimization of AgNPs nanogels

pH, appearance, and homogeneity

The physical appearance and uniformity of the prepared AgNP nanogels were assessed through visual observation. pH of the gel was determined using a digital pH meter (Eutech instruments, pH 700, Netherlands). The measurement of pH was carried out three times and the mean of the three readings was recorded.

Porosity measurement

The porosity of the AgNPs nanogel was measured as per the method recommended by Gupta *et al*. with minor changes [22]. The solvent replacement technique was employed for porosity determination. Briefly, 1 gm of AgNPs gel was placed in absolute ethanol and kept overnight by soaking. Then, an excess amount of ethanol from the hydrogel's surface was removed by blotting, and the gel was reweighed. The formula for calculating porosity is as follows.

$$\text{Porosity} = ((M2-M1))/pV \quad (3)$$

Here, M1 and M2 are the mass of hydrogel before and after the immersion in absolute ethanol, respectively; p is the density of absolute ethanol and V is the volume of the hydrogel.

Viscosity determination

Brookfield viscometer (DV-E Viscometer, Spindle 63) was used to measure the Viscosity of AgNPs nanogel at 25 °C. 30 g of gel was placed inside a beaker and kept undisturbed for 10 mins before studying the viscosity. The effect of shear on viscosity was recorded at 6, 20, 40, 60, 80, and 100 rpm. The change in viscosity was recorded in centipoise (Cp) and the viscosity shear-rate profile of the gel was plotted in graphs.

Spreadability

The spreadability of the formulated gel was measured as per the methods described by Roy *et al*. with slight modifications [23]. Two sets of glass slides with standard dimensions were used in this experiment. One of the slides had the AgNPs gel placed over it, while another slide was placed above the gel, sandwiching an area measuring 7.5 cm between the two slides. 100 g weight was then placed on the top of the upper slide, applying uniform pressure on the gel to form a thin layer, and the excess gel was scraped off from the slides. The weight was then removed. Afterward, two slides were attached to stand securely in a manner that allowed only the upper slide to slide off effortlessly when subjected to a force by a weight attached to it. A 50 g weight was then carefully fastened to the upper slide. The duration required by the upper slide to cover a distance of 7.5 cm due to the gravitational pull of the weight attached to it was recorded three times and the average time was calculated.

Spreadability was calculated by using the following formula: -

$$S = M \times L / T \quad (4)$$

Where, S= spreadability, M-weight tied to upper slides (50 g), L – Length of the glass slide (7.5 cm), T-time taken in sec.

Extrudability

A collapsible tube, which was closed and contained approximately 15 grams of gel, was firmly pressed at the crimped end. To prevent any rollback, a clamp was applied. After removing the cap, the gel was extruded, and the amount of gel that was extruded within 10 seconds was

collected and weighed. The extruded gel percentage was computed.

Grittiness

The formulation was evaluated microscopically and using hand for the presence of gritty particles if any.

Evaluation of the anti-microbial activity of AgNPs gel

The antimicrobial efficacy of the formulated AgNPs gel was evaluated as per the method described by Roy *et al.* with minor adjustments [16]. Briefly, Mueller Hinton agar plates were prepared and were inoculated with the test microorganisms adjusted to 0.5 McFarland standard by the pour plate method. Bacterial strains both gram-negative and gram-positive namely *Escherichia coli* (ATCC – 10536), *Pseudomonas aeruginosa* (ATCC–10145), *Klebsiella pneumonia* (ATCC–10031), and *Bacillus subtilis* (ATCC–11774) were used for the antimicrobial assay. Bacterial strains were added using a micropipette in the Petri plates and nutrient agar was carefully added to the Petri plate from the conical flask. To ensure and maintain the uniformity of the culture and medium, the dish was gently rotated or moved in a back-and-forth motion. After solidification, sterile forceps were used to place the paper disc containing AgNPs gel, 10 mM $AgNO_3$, gel with MePJ, and an antibiotic disc containing standard (Ceftriaxone, etc.) onto the agar plate. The plates were left overnight at 37 °C and subsequently observed for zones of inhibition.

Ex-vivo skin irritation study

Wistar rats were used for the study and the study was done as per the standard protocol. The animals are kept under standard testing conditions and are fed a standard chow diet with unrestricted access to water. Animals were kept in simulated atmospheric conditions (temperature: 25±2 °C; relative humidity: 70±5%; 12 hours of light-dark alternative cycles). For the preparation of the test, 24 hours before hair from the back and flanks were shaved, exposing an area of approximately 6 cm2 of skin. Both the control gel and AgNPs gel were evenly applied to a 4 cm^2 area of shaved skin. The presence of any skin reactions at the applied site was assessed and scored once daily at 1, 24, 48, 72 hours, and 7 days (post-test observation period) accordingly.

Wound healing activity testing

The wound healing study was conducted by excision wound model as per the method described by Demilew *et al*. with minor adjustments [24]. Healthy adult Wistar rats (100-250 gm, 6–8 weeks) were used and grouped into three major groups viz. Control (Gel with MePJ extract), AgNPs gel, and standard (povidone-iodine 10% w/w), and each group contained 6 animals. All the experiments were conducted according to CPCSEA guidelines for laboratory animal use and care.

Excision wound model

On a wounding day, all rats were weighed before the experiment. The rats were anesthetized by administering ketamine (0.5 mg/kg b. w. i.p.). After the dorsal area of the animal was cleaned with 70% alcohol and shaved. A wound area was made by cutting through its entire thickness. The injury day was considered day 0. Wound hemostasis was achieved by utilizing a cotton swab drenched with normal saline. The wound was opened and treated once daily with topical application of the gel (i.e. Gel with MePJ, AgNPs gel, and Standard povidone iodine ointment (10% w/w) till they were completely healed. The wound was monitored and the wound area was measured on 0 till the 12^{th} post-wounding day. The duration of epithelization was considered the number of days needed to completely fall off dead tissue from the wound.

Statistical analysis

Data represented in the manuscripts are given as Mean±SD (Standard Deviation). Statistical tests were done using GraphPad InStat statistical software. Differences between the groups were done using the Dunnetts multiple comparison test. Differences between the two groups were analyzed using a two-way ANOVA unpaired t-test taking P values less than 0.05 considered as significant.

Results and Discussion

Extractive yield and preliminary phytochemical screening

Extractive yield is a measure of the amount of plant material that can be extracted using a particular solvent. For the preparation of the extract Cold maceration method was used and the extractive yield of *P. javanica* leaves was found to be 19.7±4.5%. **Table 1b** summarizes the extractive value, density, and pH of the extract.

Phytochemicals are natural compounds found in plants that have various biological activities. The preliminary phytochemical screening is a qualitative test that determines the presence of various classes of phytochemicals such as alkaloids, flavonoids, tannins, saponins, phenolic compounds, etc. The phytochemical investigation of the extract revealed the presence of alkaloids, phenolic compounds, cardiac glycosides, flavonoids, tannins, and quinones **(Table 1c)**. The presence of these compounds indicates that the extract is bioactive and may have biological activities [8, 9].

Table 1. (a) AgNPs formulation composition (b) the Extractive yield, density, and pH for methanol extract of ***P. javanica*** leaves (c) Presence of phytochemicals in methanolic extract of *P. javanica* leaves.

Sl No	(a) AgNPs formulation composition			
	Ingredients	Formulations		
		F1	F2	F3
1	AgNPs **(**ml**)**	100	100	100
2	Carbopol **934**	2 %	4%	6%
3#	Glycerine	2ml	2ml	2ml
4	Triethanolamine	1 ml	1ml	1ml
	(b) Methanolic Extract of P. *javanica* leaves			
5		Test		Result (Mean±SD)
6		**%**Yield		19**.**7 ±4.5%
7		Density		0.80±0.14g/cm3
8		pH		6.04±0.52
	(c) Presence of phytochemicals in methanolic extract of *P. javanica* leaves.			
9		Test		Absent/Present
10		Alkaloids		Present
11		Phenolic Compounds		Present
12		Carbohydrates		Absent
13		Cardiac Glycosides		Present
14		Proteins and Amino Acids		Absent
15		Flavonoids		Present
16		Tannins		Present
17		Quinones		Present

Total phenolic and flavonoid content

The preliminary phytochemical screening revealed the presence of phenolic and flavonoids in the plant extract. The total phenolic content of the extract was determined by the FCR method. The total phenolic content was determined with the help of the standard curve **Figure 1a** and the content was found to be 239±0.58 mg Gallic acid equivalent/gram (GAE/gm). The total phenolic content of MePJ suggests that it has potential wound-healing properties. Phenolic compounds have been known for their antioxidant, anti-inflammatory, and antimicrobial properties, which are essential in wound healing processes [25]. Reactive oxygen species (ROS) delay wound healing by causing cellular damage. Antioxidants help in wound healing by neutralizing the harmful effects of ROS [26]. Moreover, the Phenolic compound scavenges ROS and protects cells from oxidative stress thereby facilitating the wound healing process. Phenolic compounds are also reported to have anti-inflammatory and antimicrobial properties which reduce inflammation and infection thereby helping faster wound healing process [25, 26]. Similarly, for quantification of total flavonoid content aluminum chloride method was used AND with the help of standard curve **Figure 1b**, and the total flavonoid was estimated to be 283±0.78 mg quercetin equivalent /gram (QE/g). The result indicates that the MePJ is a rich source of flavonoids. Flavonoids a plant-derived phytoconstituents with wide ranges of bioactivities including antioxidant, anti-inflammatory, and wound healing activities [27, 28]. The process of wound healing is intricate and comprises of series of steps including inflammation, proliferation, and remodeling [29]. Flavonoids have been reported to possess wound-healing properties by promoting the different stages of wound healing [30].

Antioxidant activity

The presence of phenolic and flavonoid compounds in *P. javanica* leaves is believed to be a contributor to antioxidant activity.

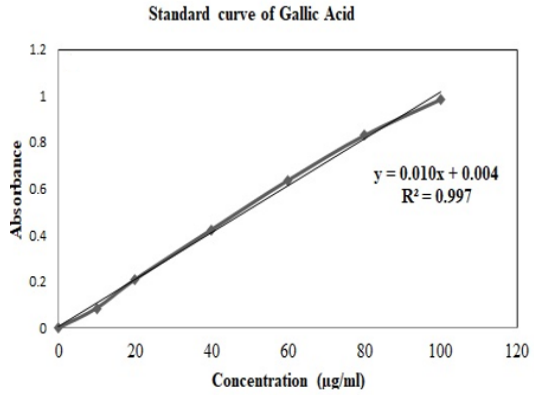
2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

A comparison of DPPH scavenging activity between MePJ and Ascorbic acid has been depicted in **Figure 1c**. The IC_{50} value of the MePJ was 12.141 µg/ml, while ascorbic acid had an IC_{50} value of 8.083 µg/ml, indicating that ascorbic acid exhibits a higher DPPH scavenging activity. Antioxidant activity increases with decreased IC_{50} value.

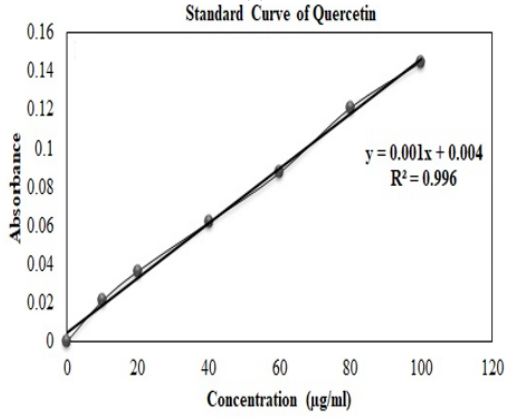
Wound healing is a complex process that involves several biological pathways, including inflammation, cell proliferation, and tissue remodeling [29]. ROS generated during the inflammatory phase can impair or delay the wound-healing process due to higher oxidative stress [30]. Despite having a higher IC_{50} compared to ascorbic acid, MePJ may still protect the cellular damage and help in wound healing progress.

Reducing power assay

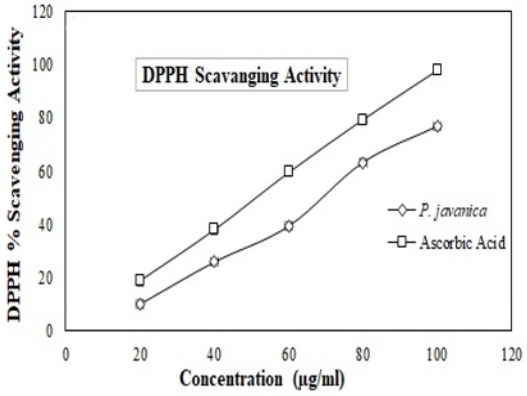
In this experiment, we evaluated the reducing ability of MePJ by monitoring the alteration of Fe3+ to Fe2+ and detecting a color shift from yellow to dark green in reaction. As the concentration of MePj increases, the absorbance value also increases indicating good reductive potential. The reducing power of the standard ascorbic acid and MePJ leaves is given in **Figure 1d**.



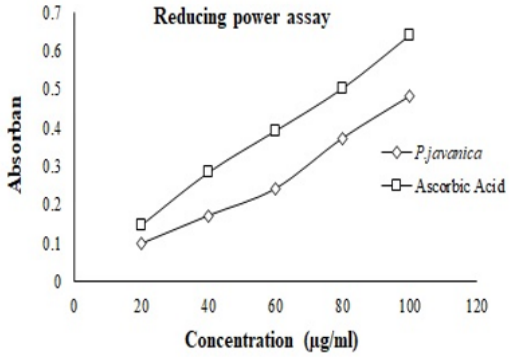
(a)



(b)



(c)



(d)

Figure 1. (a, b) Total phenolic and flavonoid content determination (c, d). Antioxidant activities of MePJ (a) Standard curve of Gallic acid, (b) Standard curve of Quercetin, (c) DPPH scavenging activity- The IC50 value of MePJ is 12.141 µg/ml and the IC50 value of ascorbic acid is 8.083 µg/ml, (d) Reducing power of MePJ.

Anti-microbial activity

The zone of inhibitions shown by the MePJ and standard antibiotic disc ceftriaxone were calculated by using a transparent scale in triplicate. The zone of inhibition produced by the MePJ and ceftriaxone against four different species of ATCC-grade microorganisms has been shown in **Figure 2I**. The zone of inhibition (Mean±SD) shown by the MePJ and ceftriaxone disc has been given in **Table 2a**. The zone of inhibition is a measure of the effectiveness of antibiotics against bacterial growth. MePJ and Ceftriaxone discs were tested against *Escherichia coli* (ATCC–10536), *Pseudomonas aeruginosa* (ATCC–10145), *Klebsiella pneumonia*e (ATCC–10031), *Bacillus subtilis* (ATCC–11774) and the zone of inhibition values were 9.33±0.58, 23±2; 10±0.57, 25.33±1.52; 13±1, 27.66±1.15 and 9±1, 26±1 respectively. *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*e, and *Bacillus subtilis* are common bacterial pathogens that can cause various infections,

including wound infections. Infections can delay the healing process by causing inflammation, increasing the risk of further tissue damage, and impairing the ability of the body to repair the wound [25]. MePJ has inhibited the growth of the bacterial strains under study means MePJ may be utilized to prevent wound infections and promote faster wound healing.

In vitro anti-inflammatory activity

Denaturation of a protein refers to the alteration or disruption of the natural structure and functions of the protein molecules. Denaturation or disruption of the protein molecules leads to the loss of their biological properties and this process has been associated with the development of inflammation. BSA is often used to measure the amount of other proteins by comparing an unknown protein quantity to the known quantities of BSA. The BSA denaturation method is one of the techniques that is used to study anti-inflammatory activity. Percentage inhibition of MePJ at different concentrations and Diclofenac (standard) has been given in **Table 2b**. Based on the results of the protein denaturation anti-inflammatory testing, it appears that MePJ is more effective at inhibiting the inflammatory process than diclofenac. The IC_{50} value for MePJ (40.95 µg/ml) is significantly lower than the IC_{50} value for diclofenac (98.1 µg/ml), indicating that a lower concentration of MePJ is required to achieve the same level of inhibition as a higher concentration of diclofenac. This suggests that MePJ may be a more potent anti-inflammatory agent than diclofenac.

Table 2. (a) Summary of Zone of inhibition shown by the MePJ and Ceftriaxone against the different microbial species, (b) inhibition of bovine serum albumin denaturation of Diclofenac sodium and MePJ

(a) Summary of Zone of inhibition shown by the MePJ and Ceftriaxone against the different microbial species

SL No	Test Organisms	Zone of Inhibition (mm)	
		P. javanica extract (mm) (Mean±SD)	Ceftriaxone (mm) (Mean±SD)
1	*Escherichia coli* (ATCC–10536)	9.33±0.58	23±2
2	*Pseudomonas aeruginosa* (ATCC–10145)	10±0.57	25.33±1.52
3	*Klebsiella pneumoniae* (ATCC–10031)	13±1	27.66±1.15
4	*Bacillus subtilis* (ATCC–11774)	9±1	26±1

All the values are (Mean±SD) of triplicate determinants.

(b) Inhibition of bovine serum albumin denaturation of Diclofenac sodium and MePJ

5	*Concentration (µg/ml)*	% Inhibition		P -value
		Diclofenac sodium (Mean±SEM)	MePJ (Mean±SEM)	
6	100	47.29 ± 3.42	53.13 ± 1.88	0.06
7	200	62.50 ± 2.95	65.63 ± 2.99	0.26
8	300	75.00 ± 2.04	71.88 ± 3.79	0.27
9	400	81.25 ± 4.63	83.25 ± 3.37	0.57
10	500	90.63 ± 5.09	87.50 ± 4.17	0.45

Detection and characterization of formulated AgNPs

Visual observation

The formation of green synthesis of AgNPs using MePJ was confirmed by the color change from colorless to light brown indicating the formation of AgNPs **(Figure 2II-a)**. The utilization of green synthesis presents a viable method for the production of nanoparticles, which is both ecologically sound and eliminates the need for harmful substances [31]. MePJ, as a plant extract, can be an alternative non-toxic biocompatible reducing agent to conventional reducing agents used in nanoparticle synthesis [32]. The color change indicates the reduction of silver ions to form AgNPs.

Ultraviolet-visible (UV–vis) spectroscopy

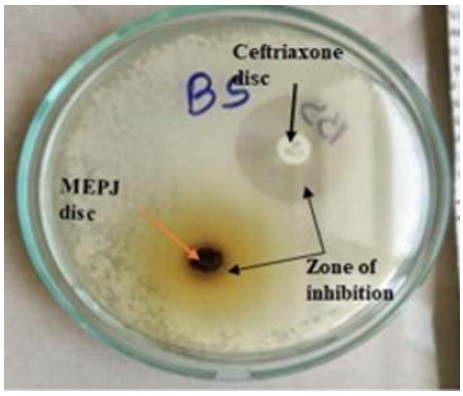
A UV-visible spectrophotometer was used to detect the formulated AgNPs. A sharp peak at 420 nm indicated the formation of AgNPs, whereas 10mM of AgNO3 does not provide any peak **(Figure 2II-b)**. In contrast, when

AgNO3 (silver nitrate) is measured using a UV-visible spectrophotometer, it does not produce any characteristic peak at 420 nm or any other wavelength in the visible range. This is because AgNO3 is a salt and does not form nanoparticles under normal conditions. The formation of AgNPs is typically achieved by reducing silver ions using a reducing agent such as sodium borohydride, which leads to the formation of silver nanoparticles [33]. The presence of a peak at 420 nm indicates that MePJ has reduced the silver ions and formed nanoparticles.

Characterization of AgNPs gel

Physical appearance and pH

Three formulations ((F1, F2, and F3) of hydrogels were developed which exhibited a brownish-red color and had uniform and smooth appearances. There was no evidence of phase separation **(Figure 2II-c)**. The pH values of all formulations prepared were ranged from 6.8 to 7.0.



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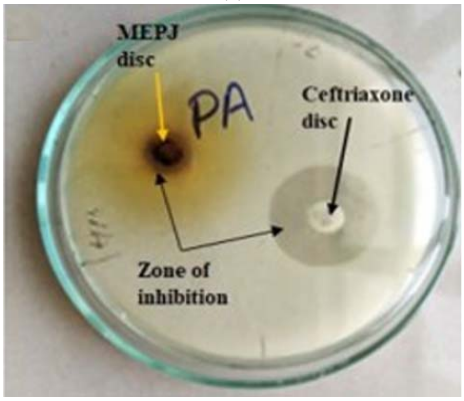

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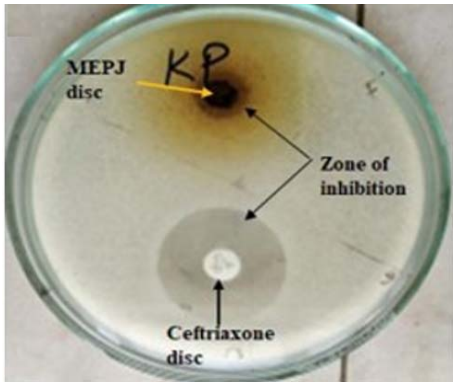

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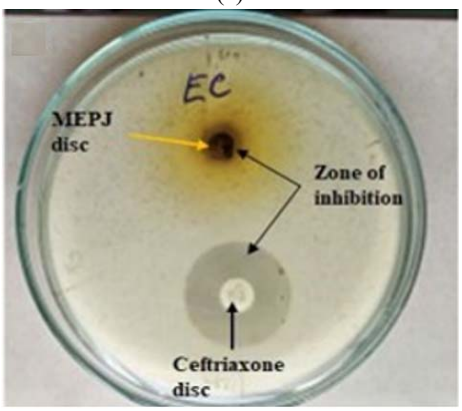

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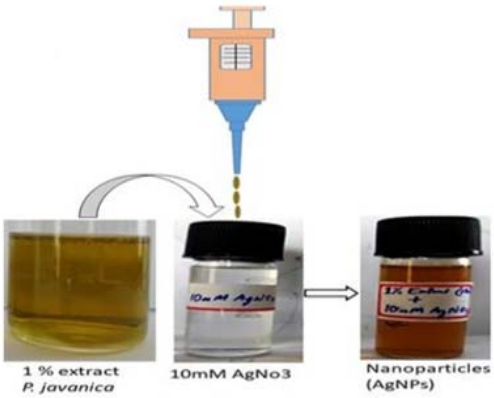

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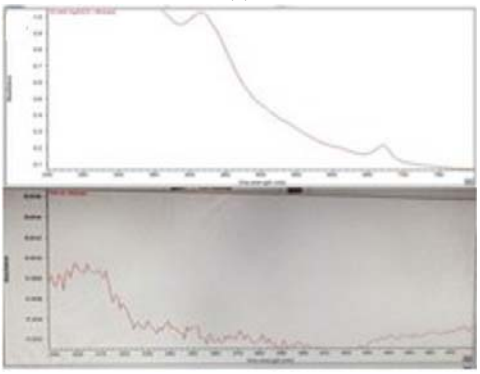

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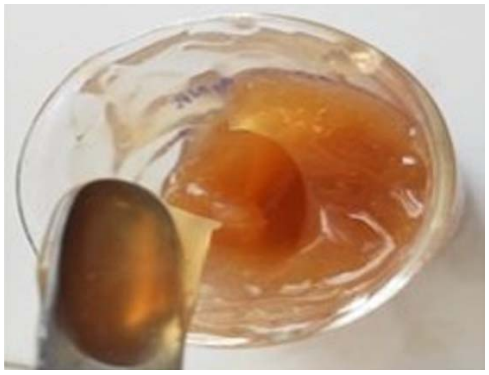


(c)

(II)

Figure 2. I. (a-d) Zone of inhibition produced by the MePJ and ceftriaxone against. a) *Bacillus subtilis* (ATCC–11774), (b) *Klebsiella pneumonia*e (ATCC – 10031), (c) *Pseudomonas aeruginosa* (ATCC – 10145), and (d) *Escherichia coli* (ATCC –10536). II (a-c). Green synthesis of AgNPs using MePJ extract. (a) Formation of AgNPs showing the color change from light green to brown, (b) UV spectra of AgNPs show a peak at 420 nm while 10mM AgNO3 solution does not show any proper peak, (c) Formulated gel containing AgNPs

Viscosity

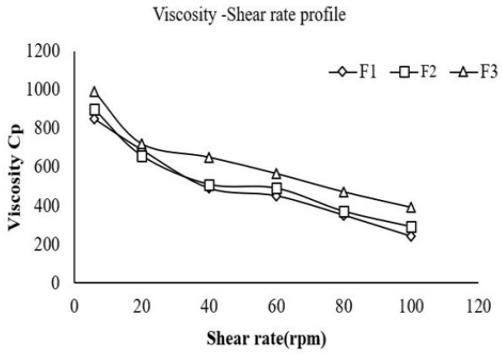
Figure 3I depicts the viscosity-shear rate profile of the formulated gel formulations (F1, F2, and F3) with non-Newtonian flow behavior. Two important characteristics of a gelling system are viscosity and homogeneity. The formulation must have ideal viscosity to facilitate smooth application on the skin. As the shear rate is increased, all three formulations show decreased viscosity.

All the formulations have shown decreased viscosity with increasing shear rate. Increased concentration of the carbopol shows higher viscosity. This could be due to the large polymeric network in the solution with a higher concentration of polymers. As the concentration decreases, the formation of the polymeric network decreases with the concentration. The decreased viscosity with increased shear rate may be due to the polymeric entangled network tending to direct themselves in the direction of flow hence showing shear thinning properties [34].

Since the concentration of polymer plays a considerable role in the diffusion of active drugs from the gel and gel with less concentration of polymer with good physicochemical characteristics is chosen for further evaluation in antimicrobial activity and wound closing efficacy in animal models. Based on the data available in **Table 3a**, F1 was further considered for biological evaluation.

Anti-microbial activity of AgNPs gel

The formulated AgNPs gel, gel with MePJ, 10mM $AgNO_3$, and standard ceftriaxone disc were used for the evaluation of antimicrobial activity against four ATCC grade microorganisms namely *Escherichia coli* (ATCC–10536), *Pseudomonas aeruginosa* (ATCC–10145), *Klebsiella pneumonia* (ATCC–10031), *Bacillus subtilis* (ATCC–11774) **(Figure 3II-a-d)**. The zone of inhibition (Mean±SD) has been shown in **Table 3b**. The MePJ gel exhibited a relatively low inhibitory effect with a zone of inhibition of 4.66±0.57 mm compared to AgNPs gel which showed a significantly larger zone of inhibition of 10±1 mm. The 10 mM $AgNO_3$ solution had a relatively small zone of inhibition of 2.66±0.57 mm. Similarly, the zone of inhibition shown by AgNPs gel is significantly higher compared to both MePJ gel and 10mM $AgNO_3$ solution against other three bacterial species namely *Pseudomonas aeruginosa* (ATCC–10145), *Klebsiella pneumonia* (ATCC–10031), *Bacillus subtilis* (ATCC–11774). The reason for the higher zone of inhibition shown by AgNPs compared to normal MeJP gel and 10mM AgNO3 may be due to AgNPs having a larger surface area to volume ratio compared to bulk silver, making them more effective in interacting with microbial cells. This increased surface area allows for more Ag+ ions to be released, which can bind to and disrupt the cell membranes of microbes [35]. The other reason may be because the plant extracts used in green synthesis contain bioactive compounds that can enhance the antimicrobial activity of AgNPs due to the synergistic effect of AgNPs [36].



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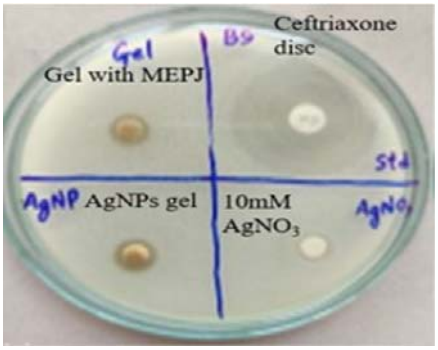
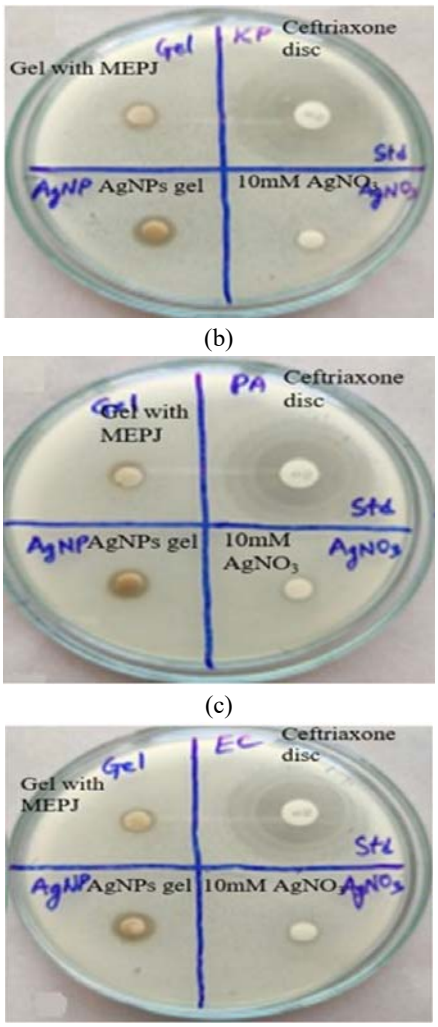


(a)



(II)

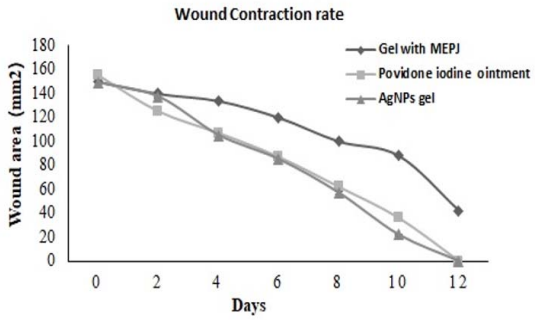
Figure 3. (I) Viscosity-shear rate profile of the formulated gel formulations. With increasing shear rate viscosity decreases. (II) (a-d) Antimicrobial efficacy of MePJ gel, AgNPs gel, 10mM AgNO3, and ceftriaxone against. (a) Bacillus subtilis (ATCC–11774), (b) Klebsiella pneumoniae (ATCC–10031), (c) Pseudomonas aeruginosa (ATCC –10145),) and (d) Escherichia coli (ATCC –10536).

Ex vivo skin irritation study

The optimized gel formulation was found to be homogeneous, with good consistency and appearance. The pH of the formulations fell within the neutral range, hence no skin irritation was evidenced by the skin irritation study. To assess the skin irritation potential of the gel, the AgNPs gel was subjected to evaluation in rats, and there was no sign of erythema or edema during the period of study i.e., 7 days. These findings suggest that the prepared AgNPs gel may be safe for topical application.

Wound healing activity

The wound healing action of AgNPs gel was compared with 1% MePJ gel and standard commercial povidone iodine 10% w/w ointment **(Figure 4a)**. The wound closing rate is higher with AgNPs gel followed by povidone iodine ointment. Complete epithelialization is considered at the point when the scab naturally falls without leaving an exposed wound and the duration taken to this process is considered as epithelization period. Epithelialization of the wound for AgNPs, Povidone iodine ointment, and MePJ gel were 12±0.40, 13±0.40 and 18±0.70 respectively days and there were decreased in the Epithelialization period in the AgNPs gel treated group **(Table 3c)**. **Figure 4b** shows there was no residue in the animal wound treated with AgNPs gel compared to other formulations of MePJ gel and Povidone iodine. The AgNPs gel-treated animals healed faster than the other groups. From the result, we can assume that the formulated AgNPs gels activity enhanced when compared with raw extract, hence showing better wound-closing activity.



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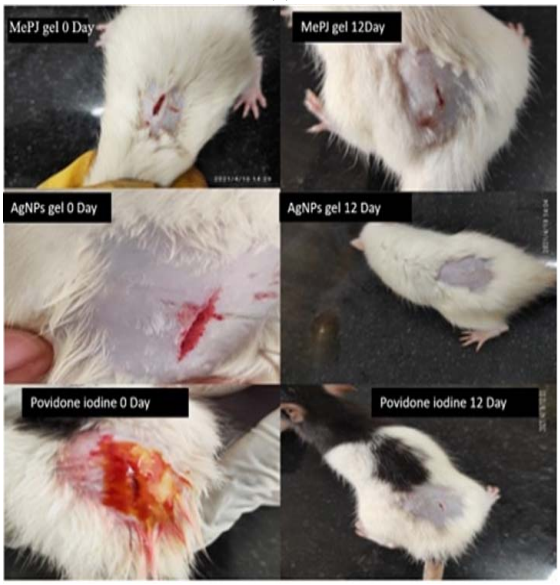

(b)

Figure 4. a) Wound contraction rate of Wistar rats using MePJ gel, AgNPs gel, and povidone-iodine ointment (10%w/w), b) Assessment of wound closing from day 0 to the 12th day

Table 3. (a) Evaluation parameters of AgNPs gels, (b) Antimicrobial evaluation of AgNPs gel, MeJP gel,10 mM AgNO3, and ceftriaxone disc (c) Period of epithelialization in rats

(a) Evaluation parameters of AgNPs gels

SL No	Parameters	F1	F2	F3
1	pH	7.02 ± 0.033	6.92 ± 0.056	6.96 ± 0.041
2	Porosity	63.3 %	58.5 %	55.4 %
3	Homogeneity	Homogenous	Homogenous	Homogenous
4	Physical appearance	Light brown, Smooth, Translucent	Light brown, Smooth, Translucent	Light brown, Smooth, Translucent
5	Spreadability **(**gcm/sec**)**	15.65 ±0.058	12.28 ±0.66	9.4 ±0.49
6	Extrudability	Excellent **(**90.2%)	Good (84.5%)	Good (82.6%)
7	Grittiness	Absent	Absent	Absent

(b) Antimicrobial evaluation of AgNPs gel, MeJP *g*el,10mM AgNO3, and ceftriaxone disc

	Test Organism	Gel with MePJ	AgNPs gel	10mM AgNO3	Ceftriaxone disc
8	*Escherichia coli* **(**ATCC–10536**)**	4.66±0.57	10±1**	2.66±0.57*	24±1**
9	*Pseudomonas aeruginosa* **(**ATCC –10145**)**	6±1	8.66±0.57**	3±0**	25.33±1.15**
10	*Klebsiella pneumoniae* **(**ATCC–10031**)**	6±1	9±1*	3±1*	24.33±1.15**
11	*Bacillus subtilis* **(**ATCC–11774**)**	4±1	8.66±1.53**	2.33±0.57	25.66±1.53**

All the values are **(**Mean±SD**)** of triplicate determinants**.** Significance levels were taken p< 0.05.

(c) Period of epithelialization in rats

12	Treatment groups	Period of epithelialization **(**Days**)** **(**Mean±SD**)**	**%** Decrease in Epithelialization
13	MePJ gel	18±0.707	
14	Povidone iodine ointment (10%w/w)	13±0.408	27.78±0.68
15	AgNPs gel	12±0.408	33.34 ±0.87

Conclusion

The synthesis of green AgNPs and their application in a wound-healing formulation showed promising results in enhancing the healing process in rats. The green synthesis method used in this study is an eco-friendly and cost-effective alternative to conventional methods of AgNP synthesis. The formulated AgNPs gel significantly reduced the wound closure and epithelization period. The antibacterial activity against common pathogenic bacteria indicates that the formulation has the potential ability to prevent wound infection. AgNPs gel was found to be safe in skin irritation tests with no adverse effects observed during the test period. The results of the study of AgNPs gel suggest that the formulation has the potential ability to be used as a novel therapeutic option in wound healing clinical settings. However, further research is warranted to

explore the efficacy and safety of the formulation in humans for optimization for clinical use.

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Conflict of interest: None.

Financial support: None.

Ethics statement: Approval was taken from the Institutional Animal Ethics Committee (IAEC) of the Department of Pharmacy, RIPANS, Aizawl, Mizoram, India. (Registration no. 149/GO/a/11/CPCSEA vide approval no. IAEC/RIPANS/67) for conducting experiments on Animals.

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