RESEARCH PAPER

Optimization and Evaluation of Silver Nanoparticles Synthesized from Ichnocarpus frutescens Using Box–Behnken Design and Their Antimicrobial Activity

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ABSTRACT

The present study aimed to optimize and evaluate the synthesis of silver nanoparticles using plant extract of Ichnocarpus frutescens by employing a Box–Behnken design (BBD). The effect of independent variables such as reaction temperature, incubation time, and plant extract concentration on particle size and entrapment efficiency was systematically investigated. A total of 13 experimental runs were performed, and the optimized formulation was identified based on the desired responses. The results revealed that particle size ranged from 130.21 nm to 225.69 nm, while entrapment efficiency varied from 58.9% to 90.4%. Among all formulations, SNPF5 showed the smallest particle size (130.21 nm) and highest entrapment efficiency (90.4%) under optimized conditions of 80°C temperature, 16 hours incubation time, and 0.2% extract concentration. The optimized formulation also exhibited good stability with a zeta potential of –36.56 mV and uniform particle distribution (PDI = 0.212). The experimental values were found to be in close agreement with predicted values, confirming the validity of the optimization model. Furthermore, antimicrobial studies demonstrated that the optimized silver nanoparticles exhibited significantly enhanced activity compared to the hydroalcoholic extract against Streptococcus mutans, Klebsiella pneumoniae, and Candida albicans. The increased zone of inhibition indicates improved antimicrobial efficacy due to nanoscale size and enhanced surface interaction. In conclusion, the study successfully optimized silver nanoparticle formulation using Ichnocarpus frutescens extract, demonstrating improved physicochemical properties and superior antimicrobial activity, making it a promising candidate for pharmaceutical and biomedical applications.

Keywords: Ichnocarpus frutescens, Silver nanoparticles, Box–behnken design, Particle size, Entrapment efficiency, Zeta potential, Antimicrobial activity, Optimization, Nanotechnology.

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Introduction

Nanotechnology has emerged as a rapidly advancing field with significant applications in medicine, pharmaceuticals, and biotechnology (Shanmuganathan *et al.,* 2019). Among various nanomaterials, silver nanoparticles have gained considerable attention due to their unique physicochemical properties, including high surface area, small particle size, and remarkable antimicrobial activity. These properties make them highly effective against a broad spectrum of microorganisms, including bacteria and fungi, thereby offering promising potential in the development of novel therapeutic agents (Zhang *et al.,* 2016).

Conventional methods for the synthesis of silver nanoparticles often involve physical and chemical approaches, which may be costly, energy-intensive, and associated with the use of toxic chemicals. In contrast, green synthesis using plant extracts has gained increasing importance as an eco-friendly, cost-effective, and sustainable alternative. Plant-mediated synthesis utilizes bioactive phytoconstituents such as flavonoids, phenolics,

alkaloids, and terpenoids, which act as both reducing and stabilizing agents in the formation of nanoparticles (Tsuji *et al.,* 2002).

Ichnocarpus frutescens, a well-known medicinal plant, has been traditionally used for various therapeutic purposes due to its rich phytochemical profile and biological activities (Pandurangan *et al.,* 2010). The presence of bioactive compounds in this plant makes it a suitable candidate for the green synthesis of silver nanoparticles. These phytochemicals not only facilitate nanoparticle formation but also enhance their biological activity, particularly antimicrobial potential (Dash *et al.,* 2007).

Despite the advantages of green synthesis, the properties of nanoparticles are highly influenced by process variables such as reaction temperature, incubation time, and plant extract concentration. Therefore, optimization of these parameters is essential to achieve nanoparticles with desirable characteristics such as smaller particle size, higher stability, and enhanced biological activity (Syed *et al.,* 2013; Xue *et al.,* 2016). Statistical experimental designs, such as the Box–Behnken design (BBD), provide a systematic and efficient approach for optimization by evaluating the interaction effects of multiple variables with a reduced number of experimental runs.

In the present study, an attempt was made to synthesize silver nanoparticles using the hydroalcoholic extract of *Ichnocarpus frutescens* through a green synthesis approach. The formulation variables were optimized using Box–Behnken design to achieve minimum particle size and maximum entrapment efficiency. Furthermore, the synthesized nanoparticles were characterized using various physicochemical parameters and evaluated for their antimicrobial activity against selected microbial strains. This study aims to provide an optimized, eco-friendly nanoparticle system with enhanced antimicrobial efficacy for potential pharmaceutical applications.

Materials and Methods

Materials

The plant material *Ichnocarpus frutescens* was collected and used for the preparation of hydroalcoholic extract. Silver nitrate ($AgNO_3$) was used as the metal precursor for the synthesis of silver nanoparticles. Ethanol and distilled water were used for extraction and preparation of solutions. Nutrient agar medium and nutrient broth were used for microbial culture studies. The test microorganisms used included *Streptococcus mutans*, *Klebsiella pneumoniae*, and *Candida albicans*. Magnesium stearate and other general laboratory reagents were of analytical grade and used as received. All glassware and instruments were properly cleaned and sterilized before use, and all chemicals and reagents employed in the study were of analytical grade.

Methods

Formulation of *Ichnocarpus frutescens* leaf extract Loaded silver nanoparticles (AgNPs)

Preparation of extract loaded silver nanoparticles

The silver nanoparticles (AgNPs) were synthesized using a green synthesis approach with slight modifications to previously reported methods, and the process variables were systematically optimized using a Box–Behnken Design (BBD) under the Design of Experiments (DoE) framework. In this study, three independent variables were selected: reaction temperature (A: 40°C, 60°C, and 80°C), incubation time (B: 8 h, 16 h, and 24 h), and plant extract concentration (C: 0.05%, 0.1%, and 0.2%) (Puja and Singh, 2025).

For each experimental run, a measured volume of *Ichnocarpus frutescens* extract was mixed with 2 mM silver nitrate ($AgNO_3$) solution in predetermined ratios according to the design matrix. The reaction mixture was subjected to continuous magnetic stirring and maintained at the specified temperature. Formation of AgNPs was preliminarily indicated by a visible color change from colorless to brown due to surface plasmon resonance. The reaction mixtures were then incubated for the designated time periods as defined by the BBD model.

The synthesis process was monitored using UV–visible spectroscopy to confirm nanoparticle formation and determine the characteristic surface plasmon resonance peak. Following synthesis, the colloidal suspensions were centrifuged at 12,000 rpm for 20 minutes to separate the nanoparticles. The supernatant was discarded, and the obtained pellets were washed repeatedly with deionized water to remove unreacted silver ions and residual phytoconstituents. The purified AgNPs were then dried and stored at 4°C for further characterization and evaluation.

Independent variables (factors) and their levels in Box–Behnken Design

Table 1: Independent Variables (Factors) and Their Levels in Box–Behnken Design

Factor Code	Independent Variable (Factor)	Low Level (–1)	Medium Level (0)	High Level (+1)
A	Reaction Temperature (°C)	40	60	80
B	Incubation Time (h)	8	16	24
C	Plant Extract Concentration (%)	0.05	0.10	0.20

Table 2: Dependent Variables (Responses)

Response Code	Response Parameter	Objective/Goal
Y_1	Particle Size (nm)	Minimize
Y_2	Entrapment Efficiency (%)	Maximize

Table 3: Experimental Design Matrix Showing Process Variables for SNPF1–SNPF13 (Box–Behnken Design)

F. Code	Std	Run	Factor 1	Factor 2	Factor 3
			A:Reaction Temperature	B:Incubation Time	C:Plant Extract Concentration
			(°C)	(h)	(%)
SNP F1	14	1	60	16	0.125
SNP F2	10	2	60	24	0.050
SNP F3	11	3	60	8	0.200
SNP F4	6	4	80	16	0.050
SNP F5	8	5	80	16	0.200
SNP F6	1	6	40	8	0.125
SNP F7	12	7	60	24	0.200
SNP F8	9	8	60	8	0.050
SNP F9	7	9	40	16	0.200
SNP F10	4	10	80	24	0.125
SNP F11	5	11	40	16	0.050
SNP F12	3	12	40	24	0.125
SNP F13	2	13	80	8	0.125

Final Equation in Terms of Coded Factors

Particle Size (Y_1) = $155.684 - 17.14875A - 7.30125B - 30.98C + 5.0725AB - 3.16AC + 0.43BC + 19.64175A^2 + 12.65175B^2 + 5.78425C^2$.

Final Equation in Terms of Actual Factors

Particle Size (Y_1) = 532.8845 − 6.99388(Reaction Temperature) − 9.23030(Incubation Time) − 555.21111(Plant Extract Concentration) + 0.031703(Reaction Temperature × Incubation Time) − 2.10667(Reaction Temperature × Plant Extract Concentration) + 0.71667(Incubation Time × Plant Extract Concentration) + 0.049104(Reaction Temperature²) + 0.197684(Incubation Time²) + 1028.31111(Plant Extract Concentration²).

Final Equation in Terms of Coded Factors

Entrapment Efficiency = $83.2 + 5.375A + 2.8B + 10.425C - 2.3AB + 0.7AC + 0.25BC - 6.725A^2 - 4.675B^2 - 2.525C^2$.

Final Equation in Terms of Actual Factors

Entrapment Efficiency = −51.6056 + 2.4579 (Reaction Temperature) + 3.4979 (Incubation Time) + 216.5556 (Plant Extract Concentration) − 0.01438 (Reaction Temperature × Incubation Time) + 0.46667 (Reaction Temperature × Plant Extract Concentration) + 0.41667 (Incubation Time × Plant Extract Concentration) − 0.01681 (Reaction Temperature²) − 0.07305 (Incubation Time²) − 448.8889 (Plant Extract Concentration²).

Evaluation of silver nanoparticles

Determination of Particle Size (nm)

The particle size of the synthesized nanoparticles was determined using Dynamic Light Scattering (DLS) technique (Kim *et al.,* 2019). An accurately weighed quantity of the optimized nanoparticle formulation was dispersed in distilled water and sonicated for 5–10 minutes to obtain a uniform suspension and to prevent aggregation.

The prepared sample was transferred into a disposable polystyrene cuvette and analyzed using a

particle size analyzer operating at a fixed scattering angle of 90°. All measurements were performed at room temperature (25 ± 2°C). The average particle size (Z-average mean diameter) and polydispersity index (PDI) were recorded. Each sample was analyzed in triplicate, and the mean ± standard deviation was calculated to ensure reproducibility and reliability of the results.

Entrapment efficiency (EE%)

Entrapment efficiency (EE%) was determined by separating the free flavonoids (unentrapped) drug from the nanoparticle dispersion using centrifugation. A known volume of the nanoparticle suspension was centrifuged at 12,000 rpm for 20–30 minutes at 4°C. After centrifugation, the supernatant containing the unentrapped drug was carefully collected and analyzed spectrophotometrically at the predetermined λ_{max} 420.0 nm using a validated calibration curve (Khatami *et al.,* 2016). The amount of drug present in the supernatant was subtracted from the total amount of drug initially added during formulation to calculate the amount of drug entrapped within the nanoparticles. The entrapment efficiency was calculated using the following formula:

$$\text{Entrapment Efficiency (EE\%)} = \frac{\text{Total Drug Added} - \text{Free (Unentrapped) Drug}}{\text{Total Drug Added}} \times 100$$

UV–Visible Spectroscopy Analysis

UV–Visible spectroscopic analysis was performed to confirm the formation of silver nanoparticles. After completion of the biosynthesis process, a small aliquot of the reaction mixture was collected and appropriately diluted with distilled water to avoid saturation of absorbance (Liaqat *et al.,* 2022). The absorbance spectrum was recorded using a UV–Visible spectrophotometer over a wavelength range of 300–700 nm, with distilled water serving as the blank. The spectrum was scanned at room temperature, and the characteristic surface plasmon resonance (SPR) peak was monitored. The appearance of a distinct absorption band in the range of 400–450 nm confirmed the formation of silver nanoparticles.

In vitro* antimicrobial activity of hydroalcoholic extract and optimized silver nanoparticles of *Ichnocarpus frutescens

Nutrient agar medium (NAM) was prepared by dissolving 13 g of powder in 1000 mL of distilled water and sterilized in an autoclave at 121°C and 15 psi for 25 minutes. The sterile medium was poured into petri plates (about 20 mL per plate) and allowed to solidify. The agar plates were then swabbed with fresh bacterial culture using sterile cotton swabs. Wells of 6 mm diameter were made using a sterile cork borer. Different concentrations of hydroalcoholic extract and optimized silver nanoparticles (25, 50, and 100 mg/mL) were prepared, and 100 µL of each was added into the respective wells using a micropipette (Crisan *et al.,* 2024). The plates were kept at room temperature for 2 hours to allow diffusion and then incubated at 37°C for 24 hours. After incubation, the zones of inhibition were measured in millimeters, and the experiment was performed in triplicate to obtain average values.

Results and Discussion

The present study employed a Box–Behnken design (BBD) to optimize the formulation variables influencing the synthesis of nanoparticles. The independent variables selected were reaction temperature (A), incubation time (B), and plant extract concentration (C), as shown in Table 1**,** while particle size (Y_1) and entrapment efficiency (Y_2) were considered as dependent responses (Table 2). The experimental design matrix (Table 3) included 13 runs, enabling a systematic evaluation of the effects of formulation variables and their interactions.

The results presented in Table 4 indicate that particle size ranged from 130.21 nm to 225.69 nm, while entrapment efficiency varied between 58.9% and 90.4%. It was observed that higher reaction temperature and increased plant extract concentration significantly reduced particle size and enhanced entrapment efficiency. Formulation SNPF5 exhibited the smallest particle size (130.21 nm) along with the highest entrapment efficiency (90.4%), suggesting the synergistic effect of optimal process parameters. In contrast, formulations prepared at lower temperature and extract concentration, such as SNPF6 and SNPF11, showed larger particle size and lower entrapment efficiency, indicating insufficient stabilization and reduced nanoparticle formation efficiency.

The statistical analysis and optimization criteria (Table 5) confirmed that the optimized conditions were reaction temperature of 80°C, incubation time of 16 hours, and plant extract concentration of 0.2%. These optimized parameters correspond to formulation SNPF5, as shown in Table 6, indicating that higher temperature and adequate incubation time

facilitate better reduction and stabilization of nanoparticles.

The validation of the optimized formulation (Table 7) demonstrated close agreement between experimental and predicted values, with minimal residual error for both particle size and entrapment efficiency. The optimized formulation SNPF5 showed a particle size of 130.21 nm (predicted: 129.82 nm) and entrapment efficiency of 90.40% (predicted: 90.45%), confirming the reliability of the model. Additionally, the zeta potential value of –36.56 mV indicates good stability of the nanoparticles due to sufficient electrostatic repulsion, while the low polydispersity index (PDI = 0.212) suggests uniform particle size distribution.

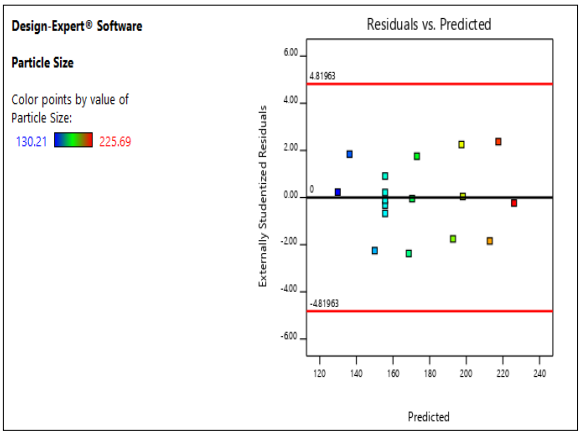
The graphical representations further support these findings. The normal probability plots (Figure 1 and Figure 2) showed that the residuals were normally distributed, indicating the adequacy of the model. Residual vs. predicted plots confirmed the absence of significant deviation, suggesting good model fitting. The contour and 3D response surface plots illustrated the interactive effects of formulation variables, revealing that increasing temperature and extract concentration leads to smaller particle size and improved entrapment efficiency.

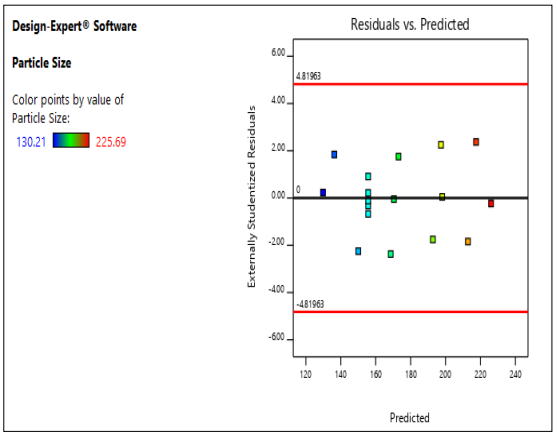
The particle size distribution graph of the optimized formulation (Figure 3) confirmed the nanoscale size range with narrow distribution, while the zeta potential graph (Figure 4) validated the stability of the formulation. Furthermore, UV spectral analysis (Figure 5) showed characteristic peaks confirming the formation of nanoparticles and successful reduction of metal ions by the plant extract, indicating its role as both reducing and stabilizing agent.

In addition to physicochemical characterization, the antimicrobial activity results (Table 8) demonstrated that both the hydroalcoholic extract and the optimized silver nanoparticle formulation (SNPF5) exhibited significant antimicrobial effects against tested microorganisms. The extract showed moderate activity with zones of inhibition increasing in a concentration-dependent manner. However, the optimized SNPF5 formulation exhibited markedly enhanced antimicrobial activity compared to the crude extract at all concentrations. For instance, against *Klebsiella pneumoniae*, the zone of inhibition increased from 14 mm (extract at 100 mg/ml) to 20 mm (SNPF5 at 100 mg/ml). Similarly, for *Streptococcus mutans* and *Candida albicans*, the nanoparticle formulation showed significantly higher inhibition zones. This enhanced activity can be attributed to the nanoscale size, increased surface area, and improved penetration ability of silver nanoparticles, which facilitate better interaction with microbial cell membranes and lead to increased antimicrobial efficacy.

The study demonstrates that process variables significantly influence nanoparticle characteristics, and the Box–Behnken design is an effective tool for optimization. The optimized formulation SNPF5 exhibited desirable physicochemical properties, including small particle size, high entrapment efficiency, good stability, and uniform distribution, along with superior antimicrobial activity. These findings suggest that the developed silver nanoparticle formulation holds strong potential for pharmaceutical and antimicrobial applications.

Normal Probability Plot of Externally Studentized Residuals for Particle Size

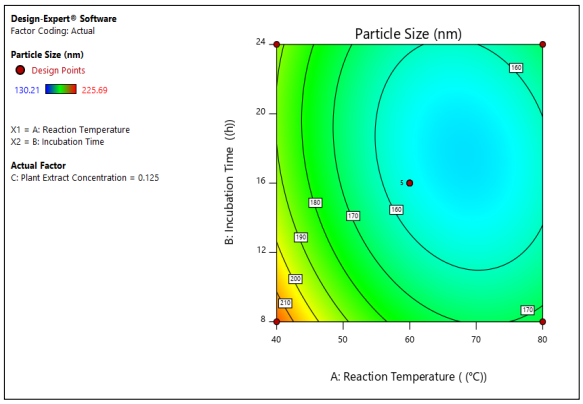


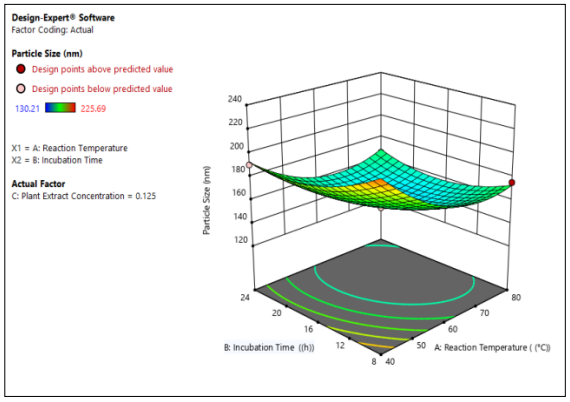


Residuals vs. Predicted Plot for Particle Size

Residuals vs. Predicted Plot for Particle Size

Optimization And Evaluation Of Silver Nanoparticles Synthesized From Ichnocarpus Frutescens Using Box–Behnken Design And Their Antimicrobial Activity

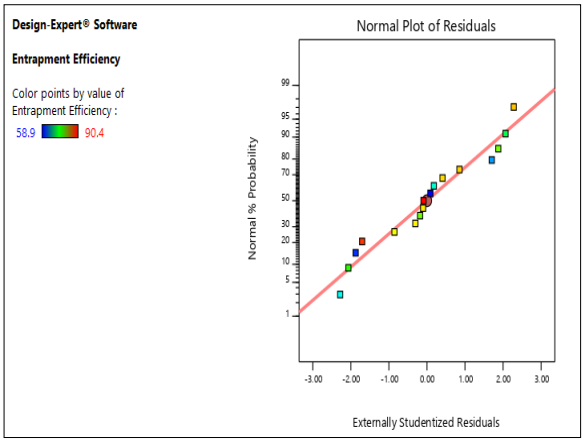


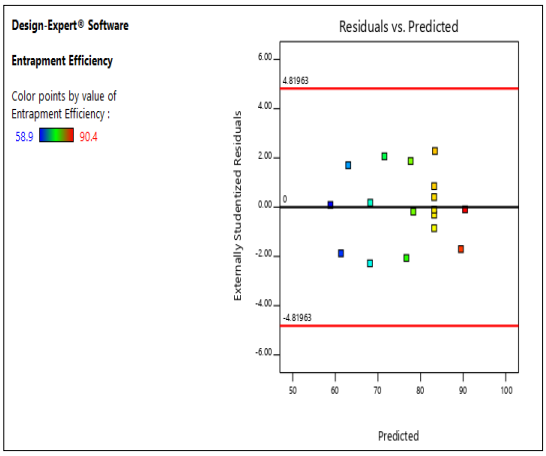


Contour Plot of Reaction Temp. and Incubation Time **3D Plot of Reaction Temp. and Incubation Time**

Figure 1: Normal Probability Plot of Externally Studentized Residuals for Particle Size

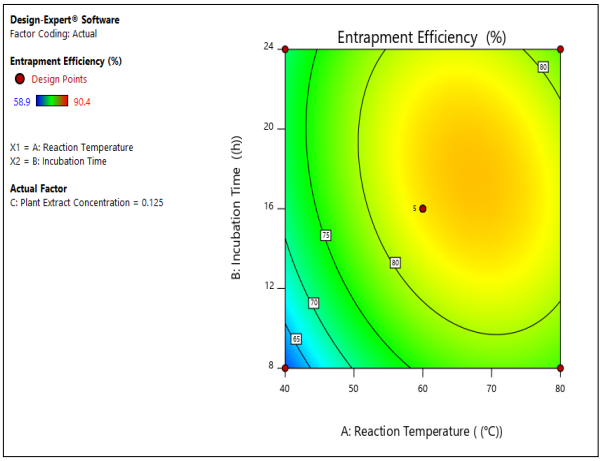
Normal Probability Plot of Externally Studentized Residuals for Entrapment Efficiency

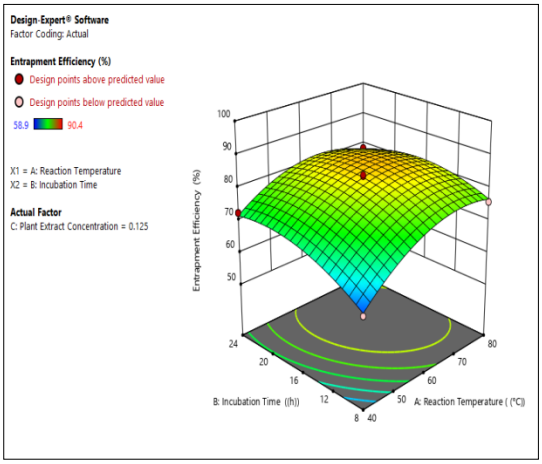




Residuals vs. Predicted Plot for Particle Size

Residuals vs. Predicted Plot for Particle Size





Contour Plot of Reaction Temp. and Incubation Time **3D Plot of Reaction Temp. and Incubation Time**

Figure 2: Normal Probability Plot of Externally Studentized Residuals for Entrapment Efficiency

Table 4: Results of Particle Size and Entrapment Efficiency

F. Code	Response 1	Response 2
	Particle Size	Entrapment Efficiency
	nm	%
SNPF1	158.25	82.4
SNPF2	200.15	67.2
SNPF3	147.23	84.3
SNPF4	198.18	68.3
SNPF5	130.21	90.4
SNPF6	220.36	60.5
SNPF7	138.74	88.7
SNPF8	210.36	63.8
SNPF9	170.36	78.2
SNPF10	165.74	78.5
SNPF11	225.69	58.9
SNPF12	190.36	72.4
SNPF13	175.45	75.8

Table 5: Optimization Criteria and Target Goals for Independent and Dependent Variables

Parameter	Goal	Target Value	Lower Limit	Upper Limit	Importance	Weight	Priority
Reaction Temperature (°C)	Target	80	40	80	1	1	3
Incubation Time (h)	Target	16	8	24	1	1	3
Plant Extract Concentration (%)	Target	0.2	0.05	0.2	1	1	3
Particle Size (nm)	Minimize (Target Achieved)	130.21	130.21	225.69	1	1	3
Entrapment Efficiency (%)	Maximize (Target Achieved)	4	9	4			

Table 6: Process Parameters of Optimized Formulation (SNPF5)

Formulation Code	Std	Run	Reaction Temperature (°C)	Incubation Time (h)	Plant Extract Concentration (%)
SNPF5	8	5	80	16	0.2

Table 7: Optimized Formulation Based on Experimental and Predicted Responses

Formulation Code	Particle Size (nm) Experimental	Particle Size (nm) Predicted	Zeta Potential (mV)	PDI	Residual	Entrapment Efficiency (%) Experimental	Entrapment Efficiency (%) Predicted	Residual
SNPF5	130.21	129.82	-36.56	0.212	0.3888	90.40	90.45	-0.0500

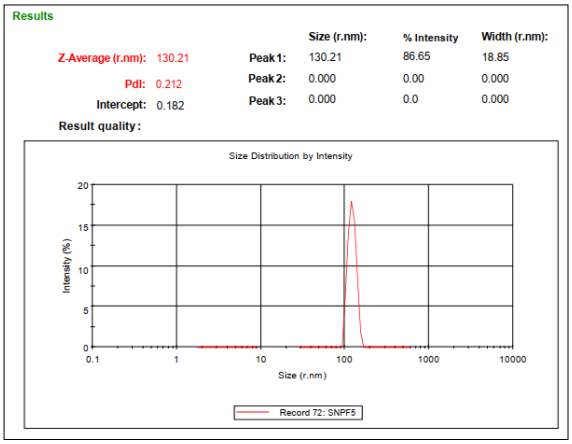


Figure 3: Graph of particle size of optimized formulation SNPF5

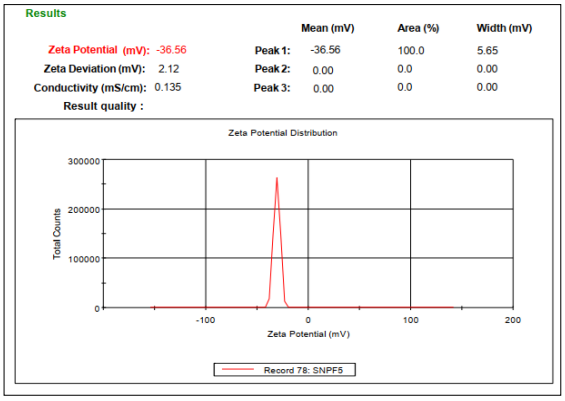
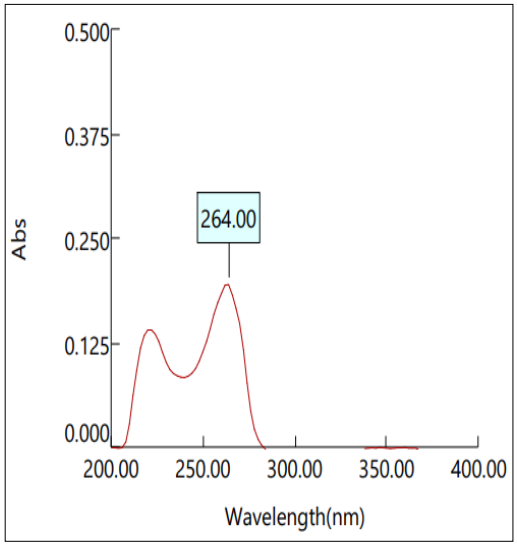
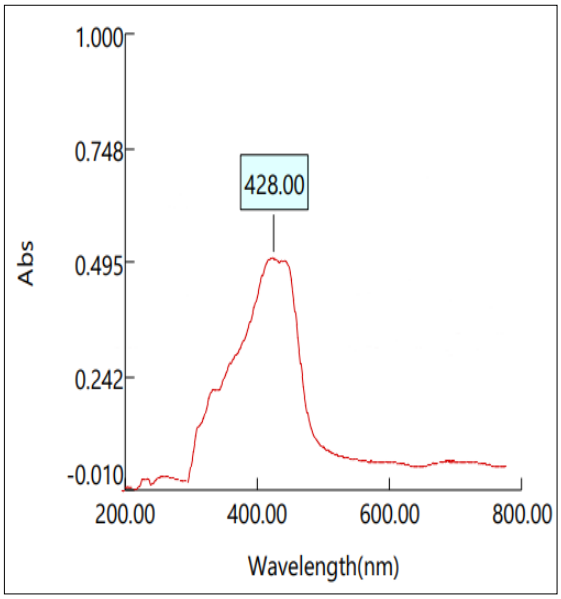


Figure 4: Graph of Zeta Potential of Optimized Formulation SNPF5



UV Spectra of Extract



UV Spectra of silver nanoparticles SNPF5

Figure 5: UV Spectra of extract and silver nanoparticles SNPF5

Table 8: Antimicrobial Activity of Hydroalcoholic Extract and Optimized Silver Nanoparticles (SNPF5) of *Ichnocarpus frutescens*

S. No.	Name of Microbes	Zone of Inhibition (mm) Extract			Zone of Inhibition (mm) Optimized SNPF5		
		25 mg/ml	50 mg/ml	100 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
1.	*Streptococcus mutans*	8 ± 0.5	11 ± 0.94	12 ± 0.57	12 ± 0.62	15 ± 0.78	18 ± 0.65
2.	*Klebsiella pneumoniae*	9 ± 0.74	13 ± 0.86	14 ± 0.5	13 ± 0.70	17 ± 0.82	20 ± 0.73
3.	*Candida albicans*	9 ± 0.47	10 ± 0.5	14 ± 0.86	11 ± 0.58	14 ± 0.69	18 ± 0.80

Conclusion

The present study successfully optimized the synthesis of silver nanoparticles using the plant extract of *Ichnocarpus frutescens* through a Box–Behnken design. The optimized formulation (SNPF5) exhibited desirable physicochemical properties, including minimum particle size, high entrapment efficiency, good stability, and uniform particle distribution. The close agreement between predicted and experimental values confirmed the reliability of the optimization model. Furthermore, the optimized nanoparticles showed significantly enhanced antimicrobial activity compared to the plain extract against selected microbial strains. The study highlights the potential of plant-mediated silver nanoparticles as an effective and promising approach for pharmaceutical and biomedical applications.

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