

Formulation and In Vitro Evaluation of a Biogenic Silver Nanoparticle-Based Hydrogel from Three Congolese Plants

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ABSTRACT

Introduction

Chronic skin wounds, characterized by impaired healing exceeding six weeks, create a significant global socio-economic burden driven by reactive oxygen species (ROS) overproduction, persistent microbial infection, and sustained pro-inflammatory signaling. Although plant-derived biogenic silver nanoparticles (AgNPs) exhibit multifunctional biological properties, hydrogels incorporating AgNPs synthesized simultaneously from multiple Congolese plant species remain largely underinvestigated.

Purpose

This study aimed to formulate, physically characterize, and biologically evaluate a Carbopol 940-based hydrogel incorporating biogenic AgNPs synthesized from *Citrus limon*, *Curcuma longa*, and *Hymenocardia acida* extracts in a 1:1:1 mass ratio for chronic wound management.

Methods

Biogenic AgNPs were synthesized via optimized green protocols and characterized using UV-Vis spectrophotometry (200–800 nm) and dynamic light scattering (DLS) for particle size, polydispersity index (PDI), and zeta potential (ZP). Six Carbopol 940 hydrogel formulations (F1–F6) incorporating varying AgNP concentrations (0–1000 µg/mL per plant) were evaluated for viscosity, pH, thermal gel transition, and 14-day pH stability. Antibacterial activity was assessed via liquid microdilution (ISO 20776-1) against *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) and confirmed by disk diffusion. Anti-inflammatory activity was evaluated using the heat-induced egg albumin denaturation assay (IC₅₀), while free-radical scavenging was quantified using ABTS and DPPH assays. Non-parametric Kruskal-Wallis tests and Dunn's post-hoc comparisons (α = 0.05) were performed.

Results

UV-Vis spectra confirmed AgNP formation with surface plasmon resonance peaks at 422 nm (*C. limon*), 448 nm (*C. longa*), and 426 nm (*H. acida*). DLS showed hydrodynamic diameters of 50 ± 23 nm (PDI = 0.39, ZP = -32.23 mV) for *C. limon*, 47 ± 4.04 nm (PDI = 0.29, ZP = -35.59 mV) for *C. longa*, and 54.66 ± 15.94 nm (PDI = 0.26, ZP = -30.22 mV) for *H. acida*. The optimized hydrogel (1.5% Carbopol 940, 1000 µg/mL AgNPs) exhibited a stable pH (5.83 ± 0.12) and consistent viscosity (197.9 mPa s; 98.9% stability over 14 days). It demonstrated a broad-spectrum minimum inhibitory concentration (MIC) of 125 µg/mL across all strains, with inhibition zones of 20.0 mm (*S. aureus*), 22.0 mm (*E. coli*), and 24.0 mm (*P. aeruginosa*). Significant anti-inflammatory (IC₅₀ = 17.55 µg/mL) and antioxidant activities (ABTS IC₅₀ = 155.50 µg/mL; DPPH IC₅₀ = 126.70 µg/mL) were observed (p < 0.05).

Conclusion

A stable biogenic AgNP-loaded hydrogel combining extracts from three Congolese medicinal plants was successfully developed. The formulation demonstrated potent *in vitro* antibacterial, anti-inflammatory, and radical-scavenging properties, supporting its potential as an advanced topical dressing for chronic wound healing.

INTRODUCTION

Chronic skin wounds present a major global healthcare burden, affecting an estimated 1% to 2% of the global population (Järbrink et al., 2017). These non-healing lesions, which typically fail to progress through the orderly physiological stages of tissue repair within six weeks, cause prolonged hospitalization, elevated risks of nosocomial infection, severe socioeconomic strain, and substantial loss of patient autonomy (Järbrink et al., 2017). The pathophysiology of chronic wound healing impairment involves complex interactions among cellular senescence, persistent microbial bioburden, excessive matrix metalloproteinase production, and sustained oxidative stress (Eming et al., 2007). In senescent and damaged wound tissue, hyper-activated neutrophils and macrophages produce elevated concentrations of reactive oxygen species (ROS) and pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF- α), interleukin-1 beta (IL-1 β), and interleukin-6 (IL-6) (Eming et al., 2007; Zheng et al., 2023). This ongoing inflammatory state degrades extracellular matrix (ECM) components, impairs endothelial and fibroblast proliferation, and prevents normal tissue remodeling (Cox & Erler, 2011; Sarrazin et al., 2011).

Conventional surgical interventions (such as autografts, allografts, and xenografts) are limited by donor site morbidity, graft rejection risks, high failure rates, and potential disease transmission (Shao et al., 2025). Standard topicals and passive dressings often require prolonged treatment regimens that can span months or years, increasing treatment costs and healthcare burden (Rajan et al., 2024; Wright et al., 2024). Consequently, active wound dressings constructed from hydrophilic polymer hydrogels have gained attention as advanced therapeutic matrices (Lapmanee et al., 2025). Polymeric hydrogels maintain a moist physiological wound bed, absorb excess wound exudate and necrotic debris, facilitate gaseous exchange, and reduce immune reaction while enhancing local biocompatibility (Dhir et al., 2024).

Incorporating metallic nanoparticles—particularly silver nanoparticles (AgNPs)—into polymeric hydrogel matrices represents a promising strategy for chronic wound care (Pangli et al., 2021). AgNPs exert broad-spectrum antimicrobial activity against Gram-positive and Gram-

negative bacteria, fungi, and viruses through multiple mechanisms, including sustained silver ion (Ag⁺) release, microbial cell membrane disruption, respiratory chain inhibition, ROS generation within microbial cells, and intracellular DNA damage (Islam et al., 2025). Furthermore, AgNPs exhibit anti-quorum sensing activity, suppressing bacterial cell-to-cell communication and bio-film formation (Samreen et al., 2022). At controlled low concentrations, AgNPs facilitate healing by upregulating endogenous antioxidant enzymes (such as catalase and superoxide dismutase), suppressing nuclear factor-kappa B (NF- κ B) inflammatory signaling, promoting endothelial cell proliferation, and stimulating angiogenesis (Pangli et al., 2021).

While chemically synthesized AgNPs often require toxic reducing and capping agents, green synthesis uses plant secondary metabolites—such as polyphenols, flavonoids, terpenoids, alkaloids, and organic acids—as eco-friendly reducing, capping, and stabilizing agents (Dhir et al., 2024; Islam et al., 2025). Although single-plant AgNP hydrogel systems have been studied, multi-plant biogenic systems derived from medicinal Congolese flora remain unexplored.

In this study, three medicinal plants utilized in traditional Luba phytotherapy (Kasaï, Democratic Republic of the Congo) were chosen based on their complementary pharmacological profiles, sustainability, and regional abundance:

1. ***Citrus limon* (L.) Osbeck (Rutaceae):** The fruit hydrolysate is rich in citric acid, ascorbic acid, and flavonoids, providing an acidic microenvironment that inhibits pathogens while contributing radical-scavenging properties and supporting collagen cross-linking (Fu et al., 2025; Koolaji et al., 2020; Makni et al., 2018).
2. ***Curcuma longa* L. (Zingiberaceae):** The rhizome contains bioactive curcuminoids with well-established anti-inflammatory, antimicrobial, tissue-regenerative, and ECM-modulating activities (Aggarwal et al., 2007; Buhrmann et al., 2011; Jurenka, 2009).
3. ***Hymenocardia acida* Tul. (Phyllanthaceae):** The leaves contain high amounts of astringent

polyphenols, tannins, and flavonoids with anti-inflammatory, antioxidant, and antimicrobial activity (Adedokun et al., 2022a, 2022b).

We hypothesized that a Carbopol 940-based topical hydrogel incorporating a tri-plant biogenic AgNP composite (*C. limon*, *C. longa*, and *H. acida* in a 1:1:1 ratio) would exert synergistic antibacterial, anti-inflammatory, and free-radical scavenging activities. The specific objectives were to:

1. Synthesize biogenic AgNPs using optimized plant-mediated protocols and systematically characterize their physicochemical properties using UV-Vis spectrophotometry and Dynamic Light Scattering (DLS);
2. Formulate Carbopol 940-based hydrogel matrices incorporating the biogenic AgNPs and evaluate their organoleptic, rheological, pH, and short-term stability parameters;
3. Evaluate the *in vitro* antibacterial performance against representative chronic wound pathogens (*S. aureus*, *E. coli*, *P. aeruginosa*) using liquid microdilution (ISO 20776-1) and agar disk diffusion assays;
4. Quantify the *in vitro* anti-inflammatory capacity (albumin denaturation inhibition) and free-radical scavenging capacity (ABTS and DPPH assays); and
5. Perform rigorous non-parametric statistical analyses to evaluate formulation performance relative to monoplant controls and standard reference drugs.

METHODS

Materials, Chemicals, and Reagents

Anhydrous silver nitrate (AgNO_3 , $\geq 99.0\%$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, $\geq 98\%$), and oleic acid were purchased from Fisher Scientific (Leicestershire, UK). Polysorbate 80 (Tween 80), 2,2-diphenyl-1-picrylhydrazyl (DPPH, $\geq 95\%$), sodium hydroxide (NaOH), ciprofloxacin ($\geq 98\%$), amikacin ($\geq 98\%$), diclofenac sodium ($\geq 98\%$), and quercetin ($\geq 95\%$) were acquired from Sigma-Aldrich (St. Louis, MO, USA). Carbopol 940 (polyacrylic acid polymer), triethanolamine

(TEA), resazurin sodium salt, glycerol, propylene glycol, isopropyl alcohol, and ethanol (96.5% v/v) were of pharmaceutical grade. Fresh hen egg white albumin, sodium citrate, and demineralized water were prepared by the Pharmacy Department of the University Clinics of Kinshasa.

Plant Material Collection and Extraction

Fresh fruits of *Citrus limon* and rhizomes of *Curcuma longa* were purchased at the Somba Zikida central market in Kinshasa, Democratic Republic of the Congo (DRC) in September 2025. Leaves of *Hymenocardia acida* were collected from the Ngaza plateau (Ngaza commune, Kananga, Kasai-Central Province, DRC). Botanical identification and authentication were performed by Mr. Nlandu Lukebiako, head botanist at the National Institute for Agronomic Studies and Research (INERA), Faculty of Sciences, University of Kinshasa.

1. ***Citrus limon* Hydrolysate:** Whole fresh *C. limon* fruits (2948 g) were washed with demineralized water, mechanically homogenized, blended, and diluted with demineralized water to a final volume of 3000 mL. The macerate was stored at 4°C for 60 days to allow complete autolytic hydrolysis, then filtered through Whatman No. 11 filter paper to yield 2700 mL of clear hydrolysate (extraction yield: 37.14%), which was stored at 4°C.
2. ***Curcuma longa* Extract:** Air-dried rhizomes were ground into a powder and sieved (2.0 mm mesh, ISO 3310-1). Powdered rhizome (190 g) was macerated in 2000 mL of an 80:20 (v/v) ethanol-water mixture for 48 hours at room temperature (25°C) under periodic agitation. The filtrate was collected using Whatman No. 11 filter paper (1700 mL; extraction yield: 23.16%).
3. ***Hymenocardia acida* Extract:** Air-dried leaves were pulverized and sieved (2.0 mm mesh). Powdered leaves (1239 g) were macerated in 10,000 mL of 80:20 (v/v) ethanol-water for 48 hours at 25°C. Filtration yielded 8000 mL of extract (extraction yield: 3.30%).

Table 1 summarizes the extraction operating conditions.

Table 1:
Operating Conditions for Extraction of Plant Material

Plant Species	Starting Mass (g)	Pomace Mass (g)	Total Liquid Volume (mL)	Filtrate Volume (mL)	Maceration Duration	Temperature (°C)	Extraction Yield (%)
<i>Citrus limon</i> (fruits)	2948	1853	3000	2700	60 days	4	37.14
<i>Curcuma longa</i> (rhizomes)	190	146	2000	1700	48 hours	25	23.16
<i>Hymenocardia acida</i> (leaves)	1239	1198	10000	8000	48 hours	25	3.30

Green Synthesis of Biogenic Silver Nanoparticles (AgNPs)

Synthesis conditions were optimized using a preliminary factor-screening experiment comprising 82 trials (*C. limon*: 4 biological x 3 technical replicates; *C. longa*: 12 biological x 3 technical replicates; *H. acida*: 12 biological x 3 technical replicates). Target specifications were: hydrodynamic particle diameter ≤50 nm, polydispersity index (PDI) < 0.40, and zeta potential (ZP) ≤ -30 mV. Nine independent validation batches were synthesized for each plant species under optimized conditions:

1. **C. limon AgNPs:** 100 mL of 98.2% (v/v) *C. limon* hydrolysate was mixed with 900 mL of aqueous AgNO₃ (5 mM) and stirred at 1500 rpm at 37°C for 20 minutes. Then, 200 mL of trisodium citrate solution (0.94% w/v) was added as a secondary stabilizer. The pH was adjusted to 9.20 using 0.1 N NaOH, and the reaction mixture was incubated in the dark at 25°C for 24 hours.
2. **C. longa AgNPs:** 100 mL of 3% (w/v) *C. longa* extract was added to 900 mL of 3 mM AgNO₃ solution. The mixture was stirred at 1500 rpm and 65°C for 20 minutes at pH 7.01, followed by incubation in the dark for 48 hours.
3. **H. acida AgNPs:** 400 mL of 1% (w/v) *H. acida* leaf extract was mixed with 600 mL of 1 mM AgNO₃ solution. The reaction mixture was stirred at 1500 rpm and 75°C for 20 minutes, adjusted to pH 9.20, and incubated in the dark for 72 hours.

Following incubation, each colloidal suspension was centrifuged at 4000 rpm (2683 x g) for 20 minutes. The resulting nanoparticle pellets were washed three times with demineralized water and dried in an oven at 37 ± 2°C for 72 hours. Yield (Y) was calculated as:

$$Y (\%) = [(W_2 - W_1) / W_{\text{sample}}] \times 100$$

where W₁ is the mass of the empty container, W₂ is the mass of the container with dried AgNPs, and W_{sample} is the initial dry mass of extract or powder.

Preparation of Biogenic AgNP Hydrogel Formulations

Six hydrogel formulations (F1–F6) were prepared using Carbopol 940 as the gelling matrix. Equal parts (1:1:1 w/w/w) of dried biogenic AgNPs from *C. limon*, *C. longa*, and *H. acida* were combined to form the composite active ingredient:

- **F1:** 0.5% (w/v) Carbopol 940 + AgNPs (1000 µg/mL per plant species; 3000 µg/mL total AgNPs)
- **F2:** 1.5% (w/v) Carbopol 940 + AgNPs (1000 µg/mL} per plant species; 3000 µg/mL total AgNPs)
- **F3:** 1.5% (w/v) Carbopol 940 + AgNPs (500 µg/mL} per plant species; 1500 µg/mL total AgNPs)
- **F4:** 1.5% (w/v) Carbopol 940 + AgNPs (250 µg/mL} per plant species; 750 µg/mL total AgNPs)
- **F5:** 1.5% (w/v) Carbopol 940 + AgNPs (125 µg/mL per plant species; 375 µg/mL total AgNPs)
- **F6:** 1.5% (w/v) Carbopol 940 Base (0 µg/mL AgNPs; vehicle control)

Formulation Procedure:

Carbopol 940 (0.5 g or 1.5 g) was dispersed in 30 mL of preheated demineralized water (70 ± 2°C) under continuous mechanical agitation until a uniform gel was formed. In a separate vessel, 1.8 mL of triethanolamine, 6.0 mL of glycerol, 2.0 mL of propylene glycol, and 5.0 mL of demineralized water were blended and incorporated into the aqueous gel phase. The specified mass of 1:1:1 biogenic AgNPs (0.30 g for F1/F2, 0.15 g for F3, 0.075 g for F4, 0.0375 g for F5, and 0 g for F6) was pre-dispersed in a hydrophobic vehicle containing 1.5 mL of oleic acid and 3.0 mL of Tween 80. This dispersion was added to an alcoholic solution (10 mL isopropyl alcohol and 20 mL ethanol 96.5% v/v), then homogenized into the main gel matrix. The final volume was adjusted to 100 mL with demineralized water. Hydrogels were homogenized over three 45-minute mixing cycles separated by 15-minute rest

intervals, then packaged into light-resistant 15-gram aluminum tubes.

Physicochemical Characterization

Spectrophotometric Characterization

Optical surface plasmon resonance (SPR) spectra of biogenic AgNPs and crude plant extracts were recorded using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) across a wavelength range of 200–800 nm. Briefly, 1.0 mg of dried AgNPs was dispersed in 10 mL of demineralized water, sonicated for 15 minutes, and evaluated against a demineralized water blank.

Particle Size, Polydispersity Index, and Zeta Potential

Hydrodynamic diameter (Φ), polydispersity index (PDI), and zeta potential (ZP) were determined by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) using a Zetasizer Ultra (Malvern Panalytical, Malvern, UK) at $25.0 \pm 0.1^\circ\text{C}$. Samples were diluted 1:50 (v/v) in demineralized water prior to measurement. Particle size distributions were analyzed using both intensity and number-weighted algorithms.

Gelation Parameters, Viscosity, and pH Stability

Gelation time was determined using the inverted tube method. Thermal gel-solution transition temperatures were measured using a calibrated water bath. Rheological measurements were performed using a rotational viscometer (Brookfield Type) at 26.1°C and 30 rpm. pH was recorded using a calibrated digital pH meter (Universal pH Meter, Thermo Scientific). Physicochemical stability (pH, visual phase separation, and viscosity) of hydrogel F2 (1000 $\mu\text{g/mL}$) was evaluated on days 1, 7, and 14 under ambient storage conditions ($25 \pm 2^\circ\text{C}$).

In Vitro Biological Evaluations

Antibacterial Activity

Antibacterial performance was evaluated against reference strains: *Staphylococcus aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853).

1. **Microplate Liquid Dilution Assay (ISO 20776-1):** Minimum inhibitory concentrations (MICs) were determined according to ISO 20776-1 standards. Test samples (hydrogel, single-plant AgNPs,

crude extracts, AgNO_3 , and reference antibiotics) were dissolved in dimethyl sulfoxide (DMSO, 250 μL) and diluted with Mueller-Hinton broth (9.75 mL) to yield 1.0 mg/mL stock solutions. Serial two-fold dilutions were prepared in 96-well microplates (7.83 to 2000 $\mu\text{g/mL}$). Bacterial suspensions were standardized to 5×10^5 CFU/mL. Positive control wells contained Mueller-Hinton broth (8 μL) and bacterial inoculum (2 μL); negative control wells contained sample solution (20 μL) and broth (100 μL). Plates were incubated at $37 \pm 0.5^\circ\text{C}$ for 24 hours. After incubation, 3 μL of 1% resazurin solution was added to each well, incubated for 4 hours, and assessed visually (a color change from blue to pink indicated microbial growth). Assays were conducted with 10 biological replicates ($n=10$) and 3 technical triplicates.

2. **Agar Disk Diffusion Assay:** To confirm microdilution findings, inhibition zone diameters (mm) were measured on Mueller-Hinton agar plates inoculated with standardized bacterial suspensions. Sterile paper disks (6.0 mm diameter) were impregnated with test solutions or controls (ciprofloxacin and amikacin), placed on agar surfaces, and incubated at 37°C for 24 hours. Zones of inhibition were measured using a digital vernier caliper.

Anti-Inflammatory Activity (Inhibition of Albumin Denaturation)

Anti-inflammatory activity was evaluated using the heat-induced egg albumin denaturation assay (Anokwah et al., 2022; Hasan et al., 2025). Reaction mixtures contained 0.5 mL of 10% (v/v) fresh egg albumin solution (in normal saline) and 1.0 mL of test sample or diclofenac sodium standard at concentrations of 10, 25, 50, and 100 $\mu\text{g/mL}$. Reaction tubes were homogenized, incubated at 37°C for 15 minutes, then heated in a water bath at $70 \pm 5^\circ\text{C}$ for 5 minutes to induce protein denaturation. After cooling to 25°C , absorbance was measured at 660 nm using a UV-Vis spectrophotometer. Percentage inhibition of albumin denaturation was calculated as:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Half-maximal inhibitory concentrations (IC₅₀) were calculated using non-linear regression analysis (log[inhibitor] vs. response).

Antioxidant Activity (ABTS and DPPH Assays)

Free-radical scavenging capacity was assessed using ABTS and DPPH radical assays (Dudonné et al., 2009).

1. **ABTS Assay:** ABTS radical cation (ABTS•⁺) was generated by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate in the dark at 25°C for 16 hours. The reagent was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm. 20µL of sample (10, 50, 100, 250, 500, and 1000 µg/mL) was added to 1980 µL of diluted ABTS solution, incubated in the dark for 30 minutes, and read at 734 nm. Quercetin served as the positive control.
2. **DPPH Assay:** 20 µL of test sample across the same concentration gradient was mixed with 1980 µL of 0.2 mM ethanolic DPPH solution. Tubes were incubated in the dark at 25°C for 30 minutes, and absorbance was recorded at 517 nm. Quercetin served as the positive control.

Percentage scavenging was calculated using the equation provided in Section 2.6.2, and IC₅₀ values were derived from concentration-response curves.

Quality Control and Analytical Instrument Calibration

Quality assurance procedures were enforced according to United States Pharmacopeia (USP Chapters <41> and <1251>) and European Pharmacopoeia (Ph. Eur. 2.2.3) standards:

1. **Analytical Balance:** An OHAUS electronic balance (capacity: 100 g, sensitivity: 0.01 mg) was tested for linearity, accuracy, and eccentric loading using standard weights (5 g, 10 g, 20 g, 50 g, and 100 g) in ascending and descending series. Standard criteria: absolute bias < 1.0 mg and coefficient of variation (CV) < 1.0%.

2. **pH Meter:** A Universal digital pH meter was calibrated using certified standard buffer solutions (pH 4.01, 7.00, and 9.01; Ph. Eur. 2.2.3) at 25 ± 1°C. Calibration curves exhibited linear slopes within 95 – 105% of theoretical Nernstian behavior (R² ≥ 0.999).
3. **Micropipettes:** A 1000µL micropipette was calibrated gravimetrically at 25 ± 1°C using demineralized water (n=6 per volume setting). Precision (CV) and accuracy (relative bias) were both < 3.0%.

Statistical Analysis

Data were analyzed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and R software version 4.5.0 (R Foundation for Statistical Computing, Vienna, Austria). Distribution normality was evaluated using the Shapiro-Wilk test. Homogeneity of variances was evaluated using Levene’s and Bartlett’s tests. Because data sets exhibited unequal variances across treatment groups, non-parametric statistics were applied. Group differences were evaluated using the Kruskal-Wallis test, followed by Dunn’s post-hoc pairwise multiple comparison test with Bonferroni correction. Statistical significance was set at p < 0.05. Results are presented as mean ± standard deviation (SD) for independent replicates (n=3 to n=10).

RESULTS

Physicochemical Characterization of Biogenic AgNPs

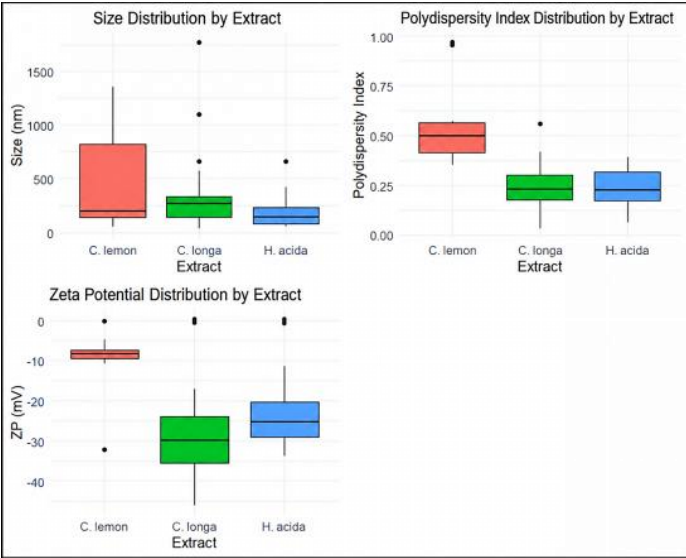
Optical Characterization by UV-Vis Spectroscopy

Synthesis of biogenic AgNPs was visibly confirmed by a rapid color transition of reaction mixtures from pale yellow to dark brown within 15 to 45 minutes. Spectrophotometric analysis demonstrated distinct surface plasmon resonance (SPR) absorption peaks characteristic of spherical silver nanoparticles (Table 2, Figure 1).

Table 2:
UV-Vis Spectrophotometric Characteristics of Plant Extracts and Synthesized Biogenic AgNPs

Plant Source	Crude Extract Spectral Range (nm)	Crude Extract Peak λ _{max} (nm)	Crude Extract Absorbance	AgNP Spectral Range (nm)	AgNP SPR Peak λ _{max} (nm)	AgNP Absorbance
<i>Citrus limon</i>	100–360	224	3.186	390–800	422	0.590
<i>Curcuma longa</i>	244–324	250	1.450	360–800	448	0.381
<i>Hymenocardia acida</i>	212–278	232	3.197	360–800	426	0.391

Figure 1:
The metric characteristics of biogenic AgNPs from *C. lemon*, *C. longa* and *H. acida* during formulation trials



Metric Characteristics by Dynamic Light Scattering (DLS)
DLS measurements confirmed the synthesis of stable, nanometer-scale particles (Table 3). Adjusting the reaction pH to alkaline conditions (9.20) and adding trisodium citrate as a co-stabilizer significantly improved particle size uniformity and surface c

Table 3:
Hydrodynamic Particle Diameter, Polydispersity Index (PDI), and Zeta Potential (ZP) of Optimized Biogenic AgNPs

Nanoformulation System	Extract Concentration (w/v)	Component Ratios (Extract : AgNO ₃ : Citrate)	Reaction pH	Particle Size Distribution by Number (Φ, nm)	Polydispersity Index (PDI)	Zeta Potential (ZP, mV)
1. <i>C. limon</i> AgNPs	98.2% hydrolysate	1 : 9 : 2 (5 mM AgNO ₃ , 0.94% citrate)	9.20	50.00 ± 23.00	0.39 ± 0.01	-32.23 ± 0.73
2. <i>C. longa</i> AgNPs	3.0% extract	1 : 9 : 0 (3 mM AgNO ₃)	7.01	47.00 ± 4.04	0.29 ± 0.00	-35.59 ± 0.19
3. <i>H. acida</i> AgNPs	1.0% extract	4 : 6 : 0 (5 mM AgNO ₃)	9.20	54.66 ± 15.94	0.26 ± 0.01	-30.22 ± 2.54

Note. Data presented as mean ± SD (n=3).

Yields of Extraction and Nanoformulation

The mass conversion yields for plant extraction and subsequent biogenic nanoparticle synthesis are presented in Table 4.

Table 4:
Extraction Yields and Biogenic Nanoparticle Conversion Yields

Plant Species	Extract Mass Yield (%)	AgNP Synthesis Yield (%)
<i>Citrus limon</i>	37.14	0.16
<i>Curcuma longa</i>	23.16	5.75
<i>Hymenocardia acida</i>	3.30	6.31

Physicochemical Characterization of Hydrogel Formulations

Six hydrogel formulations (F1–F6) were systematically evaluated for gelation kinetics, viscosity, pH, thermal

stability, and 14-day storage integrity (Table 5 and Table 6).

Table 5:
Physicochemical Properties and Gelation Characteristics of Hydrogel Formulations F1–F6

Formulation	Carbopol 940 Polymer (% w/v)	AgNP Concentration (µg/mL per plant)	Gelation Temp. (°C)	Gelation Time (min)	Apparent Viscosity at 30 rpm (mPa s)	pH at 25°C	Gel–Solution Transition Temp. (°C)
F1	0.5	1000	70 ± 2	< 1.0	Liquid (< 50)	5.86 ± 0.02	N/A
F2	1.5	1000	70 ± 2	< 1.0	197.90 ± 0.00	5.83 ± 0.12	37.0 ± 0.5
F3	1.5	500	70 ± 2	< 1.0	198.30 ± 0.00	5.87 ± 0.09	38.0 ± 0.1
F4	1.5	250	70 ± 2	< 1.0	198.60 ± 0.00	5.99 ± 0.05	38.0 ± 0.5
F5	1.5	125	70 ± 2	< 1.0	198.70 ± 0.00	6.43 ± 0.03	39.0 ± 0.3
F6 (Base)	1.5	0	70 ± 2	< 1.0	198.90 ± 0.00	8.10 ± 0.50	39.0 ± 0.1

Note. N/A = Not applicable due to incomplete polymer crosslinking at 0.5% Carbopol. Data presented as mean ± SD (n=3).

Formulation F2 (1.5% Carbopol 940, 1000 µg/mL AgNPs per plant) demonstrated high physical stability over 14 days under room temperature storage conditions (25 ± 2°C) (Table 6)

Table 6:
Fourteen-Day Stability Assessment of Hydrogel Formulation F2

Evaluation Parameter	Day 1	Day 7	Day 14	Target Specification
Homogeneity & Suspended Phase	Uniform grey, soft gel	Uniform, no precipitation	Uniform, no phase separation	Homogeneous single phase
pH	5.83 ± 0.12	5.75 ± 0.06	5.65 ± 0.00	3.00 – 8.00
Viscosity (mPa s)	198.70 ± 0.00	198.50 ± 0.00	197.90 ± 0.00 (98.9% ret.)	approx 200.00text{ mPa s}

In Vitro Biological Evaluations

Antibacterial Performance

The optimized biogenic AgNP hydrogel (F2) demonstrated superior broad-spectrum antibacterial activity compared to individual biogenic AgNPs and crude plant extracts (Table 7, Figure 2).

Table 7:
Minimum Inhibitory Concentrations (MIC) and Zone of Inhibition Diameters for Hydrogel F2, Individual AgNPs, Plant Extracts, and Reference Standards

Test Material / Control	<i>S. aureus</i> (ATCC 29213) MIC (µg/mL)	<i>S. aureus</i> Inhibition Zone (mm)	<i>E. coli</i> (ATCC 25922) MIC (µg/mL)	<i>E. coli</i> Inhibition Zone (mm)	<i>P. aeruginosa</i> (ATCC 27853) MIC (µg/mL)	<i>P. aeruginosa</i> Inhibition Zone (mm)
Ciprofloxacin	1.95 ± 0.00	16.0 ± 0.0	7.85 ± 0.00	35.0 ± 0.0	0.46 ± 0.00	33.0 ± 0.0
Amikacin	—	24.0 ± 0.0	—	26.0 ± 0.0	—	27.0 ± 0.0
Biogenic Hydrogel F2	125.00 ± 0.00	20.0 ± 0.0	125.00 ± 0.00	22.0 ± 0.0	125.00 ± 0.00	24.0 ± 0.0
AgNPs (<i>C. limon</i>)	2000.00 ± 0.00	15.5 ± 2.12	1666.66 ± 577.35	17.0 ± 0.0	2000.00 ± 0.00	18.0 ± 0.0
AgNPs (<i>C. longa</i>)	2000.00 ± 0.00	No zone	2000.00 ± 0.00	No zone	2000.00 ± 0.00	No zone
AgNPs (<i>H. acida</i>)	> 2000.00	No zone	> 2000.00	No zone	> 2000.00	No zone
Hydrolysate (<i>C. limon</i>)	> 2000.00	No zone	250.00 ± 0.00	No zone	> 2000.00	No zone
Extract (<i>C. longa</i>)	> 2000.00	No zone	1000.00 ± 0.00	No zone	> 2000.00	No zone
Extract (<i>H. acida</i>)	> 2000.00	No zone	> 2000.00	No zone	> 2000.00	No zone
text{AgNO}_3 Precursor	531.24 ± 0.00	No zone	> 250.00	No zone	> 250.00	No zone

Note. Data presented as mean ± SD (n=10 for MIC; n=3 for disk diffusion). "No zone" indicates absence of a clear inhibition zone at the tested concentration. — = Not determined.

Figure 2:
Antibacterial properties of the biogenic hydrogel analysed using the agar diffusion method

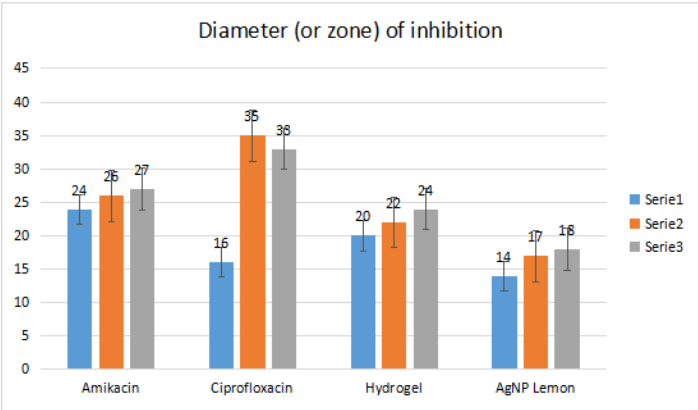


Table 8:
Anti-Inflammatory Activity (IC₅₀) and Descriptive Statistics for Protein Denaturation Inhibition

Treatment Group	Minimum (µg/mL)	Median (µg/mL)	Maximum (µg/mL)	Mean ± SD (µg/mL)	Standard Error (SEM)	95% Confidence Interval (µg/mL)
Diclofenac Sodium	27.00	27.23	27.52	27.25 ± 0.26	0.15	26.60 – 27.89
Biogenic Hydrogel + AgNPs	16.96	17.34	18.34	17.55 ± 0.71	0.41	15.78 – 19.31

Anti-Inflammatory Activity (Albumin Denaturation Inhibition)
Evaluation of heat-induced protein denaturation revealed that the composite biogenic AgNP hydrogel exhibited potent anti-inflammatory activity, comparable to the reference non-steroidal anti-inflammatory drug diclofenac sodium (Table 8, Figures 3-4).

AgNPs (<i>C. limon</i>)	21.54	21.64	22.48	21.89 ± 0.52	0.29	20.60 – 23.17
AgNPs (<i>C. longa</i>)	1.08	1.13	1.16	1.12 ± 0.04	0.02	1.03 – 1.22
AgNPs (<i>H. acida</i>)	1.06	1.06	1.08	1.07 ± 0.01	0.01	1.04 – 1.09
Hydrolysate (<i>C. limon</i>)	1.12	1.14	1.14	1.13 ± 0.01	0.01	1.10 – 1.16
Extract (<i>C. longa</i>)	0.37	0.40	0.46	0.41 ± 0.04	0.03	0.30 – 0.52
Extract (<i>H. acida</i>)	0.40	11.37	17.10	9.62 ± 8.49	4.89	-11.45 – 30.70
Hydrogel Base (F6)	0.00	0.00	0.46	0.15 ± 0.27	0.15	-0.50 – 0.81

Note. Lower IC₅₀ values indicate higher anti-inflammatory potency. Data represent n=3 technical triplicates across 9 biological sets.

Figure 3:

Graph showing the results of the anti-inflammatory activity of the hydrogel, AgNPs and extracts from three Congolese plants, analysed using GraphPad Prism Nonlinear Fit of IC₅₀/log (inhibitor vs. response variable) slope, mean, SD, n=3, least squares fit, interpolated curve, 95% confidence interval

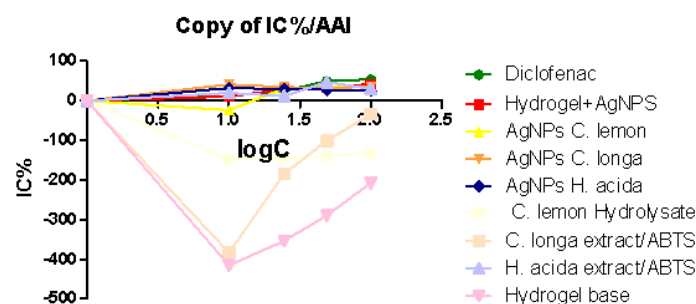
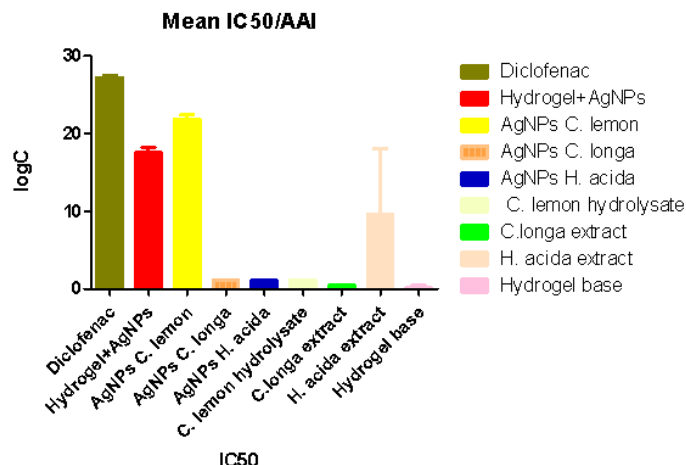


Figure 4:

Graph showing the Characteristics of the anti-inflammatory activity of the hydrogel, AgNPs, and extracts from three Congolese plants, analyzed using GraphPad, including one-way ANOVA, and Turkey's multiple comparison test for IC₅₀ values, mean, SD, n=3, P<0,05



Free-Radical Scavenging Activity (ABTS and DPPH Assays)

The antioxidant capacity of test formulations was evaluated using ABTS and DPPH radical scavenging assays (Table 9 and Table 10, Figures 5-8).

Table 9:

ABTS Free-Radical Scavenging Activity (IC₅₀) and Descriptive Statistics

Treatment Group	Minimum (µg/mL)	Median (µg/mL)	Maximum (µg/mL)	Mean ± SD (µg/mL)	Standard Error (SEM)	95% Confidence Interval (µg/mL)
Quercetin (Standard)	19.58	19.59	19.64	19.60 ± 0.03	0.02	19.52 – 19.68
Biogenic Hydrogel F2	155.00	155.30	156.20	155.50 ± 0.63	0.36	153.95 – 157.05
AgNPs (<i>C. limon</i>)	76.52	76.72	77.05	76.76 ± 0.27	0.16	76.09 – 77.43
AgNPs (<i>C. longa</i>)	6.14	6.14	6.16	6.15 ± 0.01	0.004	6.13 – 6.17
AgNPs (<i>H. acida</i>)	398.80	510.00	512.60	473.80 ± 64.95	37.51	312.42 – 635.18
Hydrolysate (<i>C. limon</i>)	809.30	827.90	852.10	829.77 ± 21.46	12.39	776.46 – 883.08
Extract (<i>C. longa</i>)	51.65	53.77	54.06	53.16 ± 1.32	0.76	49.89 – 56.43
Extract (<i>H. acida</i>)	5.05	5.06	5.07	5.06 ± 0.01	0.01	5.04 – 5.08
Hydrogel Base (F6)	0.00056	0.00079	0.00085	0.00074 ± 0.00015	0.00009	0.00037 – 0.0011

Note. Data represent n=3 technical triplicates across 9 biological sets.

Figure 5:
Graph showing the results of the hydrogel's, AgNPs' and extracts from three Congolese plants' antioxidant activity as measured by the ABTS assay, analysed using GraphPad Prism Nonlinear Fit of IC%,log(log(inhibitor-normalised response)), slope, mean, SD, n=3; coefficient of determination < 0.05

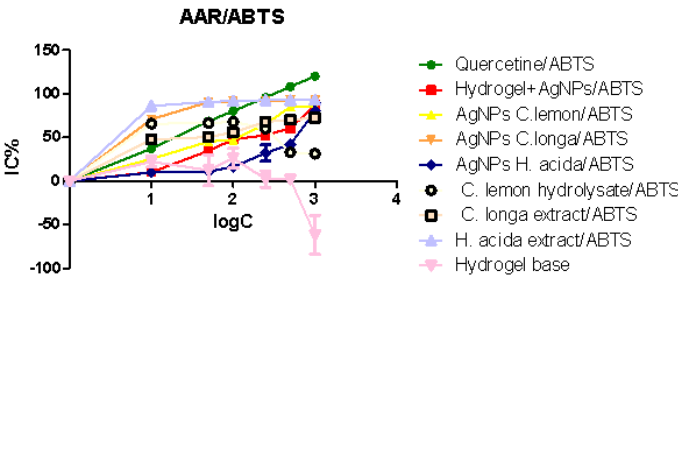


Figure 6:
Graph showing the results of the antioxidant activity of the hydrogel, AgNPs and extracts from three Congolese plants, as determined by the ABTS assay, analysed using GraphPad, including a one-way ANOVA, P-value < 0.05

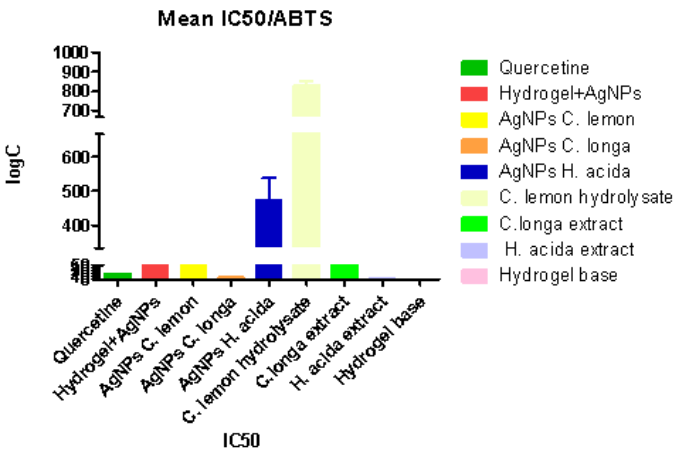


Table 10:
DPPH Free-Radical Scavenging Activity (IC₅₀) and Descriptive Statistics

Treatment Group	Minimum (µg/mL)	Median (µg/mL)	Maximum (µg/mL)	Mean ± SD (µg/mL)	Standard Error (SEM)	95% Confidence Interval (µg/mL)
Quercetin (Standard)	25.88	25.91	25.94	25.91 ± 0.03	0.02	25.83 – 25.99
Biogenic Hydrogel F2	124.10	126.70	128.80	126.53 ± 2.65	1.53	120.00 – 133.10
AgNPs (C. limon)	157.10	157.80	158.50	157.80 ± 0.70	0.40	156.08 – 159.52
AgNPs (C. longa)	6.00	6.01	6.02	6.01 ± 0.01	0.006	5.98 – 6.04
AgNPs (H. acida)	6.00	6.01	6.02	6.01 ± 0.01	0.006	5.98 – 6.04
Hydrolysate (C. limon)	39.90	39.96	40.02	39.96 ± 0.06	0.03	39.83 – 40.09
Extract (C. longa)	612.00	614.60	617.20	614.60 ± 2.60	1.50	608.10 – 621.10
Extract (H. acida)	10.30	10.32	10.34	10.32 ± 0.02	0.01	10.28 – 10.36
Hydrogel Base (F6)	6340.00	6341.00	6342.00	6341.00 ± 1.00	0.58	6338.50 – 6343.50

Note. Data represent n=3 technical triplicates across 9 biological sets.

Figure 7:
Graph showing the results of the DPPH-based antioxidant activity of the hydrogel, AgNPs and extracts from three Congolese plants, analysed using GraphPad Prism Nonlinear Fit of IC%,log(log(inhibitor-normalised response)), slope, mean, SD, n=3; coefficient of determination < 0.05

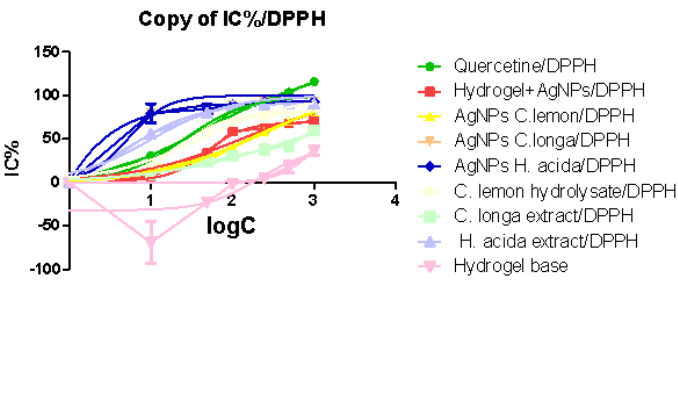
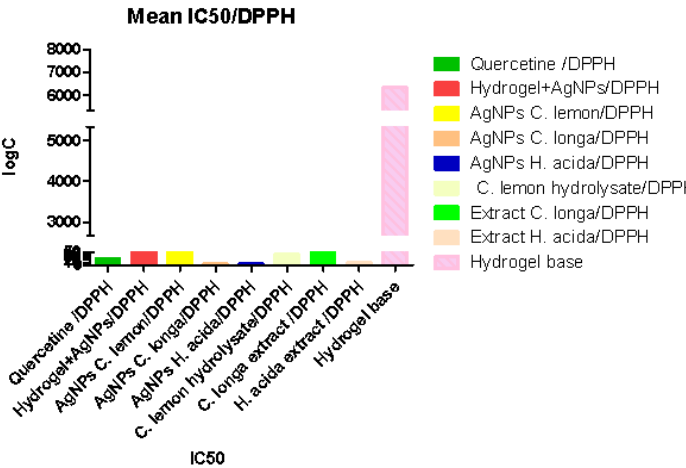


Figure 8:
Graph showing the results of the antioxidant activity of the hydrogel, AgNPs and extracts from three Congolese plants, as determined by the DPPH assay



Non-Parametric Hypothesis Testing and Post-Hoc Comparisons

Normality testing (Shapiro-Wilk) indicated normal distributions within individual parameter sub-groups ($p > 0.05$). However, tests for equal variances revealed severe heteroscedasticity across the combined formulation matrix:

- **Anti-inflammatory Levene’s test:** $F = 23.46$, $p < 0.0001$; Bartlett’s test: $K^2 = 123.46$, $p < 0.0001$.

- **ABTS Radical Assay Levene’s test:** $F = 47.23$, $p < 0.0001$; Bartlett’s test: $K^2 = 734.23$, $p < 0.0001$.
- **DPPH Radical Assay Levene’s test:** $F = 8934.57$, $p < 0.0001$; Bartlett’s test: $K^2 = 2345.68$, $p < 0.0001$.

Consequently, non-parametric Kruskal-Wallis analysis was performed, followed by Dunn’s post-hoc pairwise comparison tests with Bonferroni correction (Table 11).

Table 11: Summary of Non-Parametric Kruskal-Wallis Analysis and Dunn’s Post-Hoc Pairwise Comparisons

Bioassay Parameter	Kruskal-allis Test Statistic	Degrees of Freedom (df)	p-Value	Dunn’s Post-Hoc Pairwise Comparison	Difference in Rank Sum	Adjusted p-Value	Significance Conclusion ($p < 0.05$)
Anti-Inflammatory	$\chi^2 = 24.12$	8	0.0023	Diclofenac vs. <i>C. longa</i> Extract	20.00	0.0008	Significant
				Diclofenac vs. Hydrogel Base (F6)	20.00	0.0012	Significant
				AgNPs (<i>C. limon</i>) vs. <i>C. longa</i> Extract	18.00	0.0034	Significant
				Biogenic Hydrogel F2 vs. <i>C. longa</i> Extract	17.50	0.0078	Significant
				Diclofenac vs. AgNPs (<i>C. limon</i>)	5.10	0.7654	Non-Significant
				Diclofenac vs. Biogenic Hydrogel F2	4.20	0.5432	Non-Significant
ABTS Antioxidant	$\chi^2 = 24.59$	8	0.0018	<i>C. limon</i> Hydrolysate vs. Hydrogel Base	25.71	0.0024	Significant
				AgNPs (<i>H. acida</i>) vs. Hydrogel Base	25.71	0.0089	Significant
				Biogenic Hydrogel F2 vs. Hydrogel Base	22.10	0.0234	Significant
				AgNPs (<i>C. limon</i>) vs. Hydrogel Base	21.00	0.0341	Significant
DPPH Antioxidant	$\chi^2 = 24.88$	8	0.0017	Hydrogel Base vs. AgNPs (<i>C. longa</i>)	-20.00	0.0012	Significant
				Hydrogel Base vs. AgNPs (<i>H. acida</i>)	-20.00	0.0012	Significant
				Hydrogel Base vs. Extract (<i>H. acida</i>)	-18.50	0.0023	Significant
				Extract (<i>C. longa</i>) vs. AgNPs (<i>C. longa</i>)	16.00	0.0156	Significant
				Extract (<i>C. longa</i>) vs. AgNPs (<i>H. acida</i>)	16.00	0.0156	Significant

DISCUSSION

The biogenic synthesis of silver nanoparticles using aqueous extracts of *Citrus limon*, *Curcuma longa*, and *Hymenocardia acida* was confirmed by visual color changes and distinct surface plasmon resonance (SPR) absorption bands between 420 and 450 nm (Table 2). These SPR spectra reflect collective oscillations of free conduction electrons on the metallic nanoparticle surface when excited by electromagnetic radiation, consistent with spherical silver nanostructures (Al Moudani et al., 2024; Alkhulaifi et al., 2020). Synthesis of *C. limon* AgNPs yielded an SPR peak at 422 nm, sharpened by the addition of trisodium citrate as an secondary electrolyte that prevented aggregation (Prathna et al., 2011). *C. longa* and *H. acida* AgNPs exhibited SPR peaks at 448 nm and 426 nm, respectively.

Dynamic light scattering (DLS) measurements showed average particle sizes (Φ) of 50 ± 23 nm for *C. limon*, 47 ± 4.04 nm for *C. longa*, and 54.66 ± 15.94 nm for *H. acida* (Table 3). PDI values were < 0.40 , indicating narrow, monodisperse size distributions. Zeta potential values ($\leq -$

30 mV) confirmed strong electrostatic repulsion among suspended nanoparticles, ensuring long-term colloidal stability (Dhir et al., 2024). Reaction pH strongly influenced particle nucleation kinetics and final size: increasing the pH from acidic to alkaline (9.20) accelerated precursor reduction, reducing reaction completion times from 45 minutes to 15 minutes for *H. acida*. Alkaline conditions promote the deprotonation of plant phenolic hydroxyl groups, increasing their electron-donating capacity for silver ion (Ag^+) reduction (Ibrahim, 2015; Kambale et al., 2020).

Formulation F2 (1.5% Carbopol 940, 1000 $\mu\text{g/mL}$ AgNPs per plant) maintained ideal physical properties over 14 days of storage, with consistent viscosity (197.9 mPa s; 98.9% retention) and stable pH (5.83 ± 0.12) appropriate for topical application to human skin (Table 5 and Table 6). Inclusion of Tween 80 and oleic acid facilitated uniform entrapment of biogenic AgNPs within the hydrophilic Carbopol polymer matrix without phase separation.

The biogenic hydrogel (F2) demonstrated broad-spectrum antibacterial activity, with an MIC of 125 µg/mL against *S. aureus*, *E. coli*, and *P. aeruginosa* (Table 7). In disk diffusion assays, F2 produced clear zones of inhibition measuring 20.0 mm (*S. aureus*), 22.0 mm (*E. coli*), and 24.0 mm (*P. aeruginosa*), approaching the antibacterial activity of ciprofloxacin (16 -- 35 mm) and amikacin (24 -- 27 mm). Notably, the antibacterial potency of the composite hydrogel was higher than that of individual plant-derived AgNPs (MICs ≥ 1666.66 µg/mL) or crude extracts (MICs ≥ 250 µg/mL). This increased potency reflects synergistic interactions between released silver ions (Ag⁺) and bioactive plant secondary metabolites (such as citric acid, curcuminoids, and polyphenols) co-encapsulated within the hydrogel network (Pangli et al., 2021). Silver ions disrupt bacterial cell walls and metabolic enzymes, while plant polyphenols permeabilize outer membranes, facilitating intracellular nanoparticle uptake (Islam et al., 2025; Samreen et al., 2022).

In the anti-inflammatory assay, hydrogel F2 demonstrated robust inhibition of heat-induced protein denaturation (IC₅₀ = 17.55 ± 0.71 µg/mL), significantly outperforming the standard non-steroidal anti-inflammatory drug diclofenac sodium (IC₅₀ = 27.25 ± 0.26 µg/mL; *p* < 0.05) (Table 8). Post-hoc Dunn tests confirmed no statistically significant difference between the biogenic hydrogel and diclofenac (*p* = 0.5432), establishing its strong anti-inflammatory potential (Table 11). Excessive protein denaturation in the wound bed releases pro-inflammatory autoantigens; suppressing denaturation helps mitigate chronic tissue inflammation (Anokwah et al., 2022; Hasan et al., 2025).

Free-radical scavenging assays confirmed strong antioxidant capacity for hydrogel F2 (ABTS IC₅₀ = 155.50 ± 0.63 µg/mL; DPPH IC₅₀ = 126.53 ± 2.65 µg/mL) (Table 9 and Table 10). *C. longa* and *H. acida* AgNPs individually demonstrated potent DPPH radical scavenging (IC₅₀ = 6.01 µg/mL), likely due to surface-bound curcuminoids and leaf tannins acting as hydrogen donors (Aggarwal et al., 2007; Adedokun et al., 2022a). Neutralizing excess ROS within chronic wound beds is essential to prevent oxidative damage to healthy surrounding tissue, inhibit lipid peroxidation, and allow chronic wounds to transition from a stalled inflammatory state to active

tissue proliferation and re-epithelialization (Eming et al., 2007; Lapmanee et al., 2025).

Limitations

This study was focused on *in vitro* formulation, physical characterization, and preliminary biological testing. Further studies are needed to evaluate real-time transdermal drug release kinetics, cellular cytotoxicity against human dermal fibroblasts and keratinocytes, systemic bio-distribution, and *in vivo* wound healing efficacy in animal models.

CONCLUSION

A biogenic silver nanoparticle-loaded hydrogel was successfully formulated using Carbopol 940 and a tri-plant active extract derived from *Citrus limon*, *Curcuma longa*, and *Hymenocardia acida*. The biogenic AgNPs displayed desirable hydrodynamic sizes (47 -- 55 nm), monodisperse particle distributions, and high negative zeta potential (< -30 mV). The optimized hydrogel formulation (F2) demonstrated excellent physical stability, stable pH, and reliable viscosity over 14 days. Bioassays demonstrated broad-spectrum antibacterial activity (MIC = 125 µg/mL against *S. aureus*, *E. coli*, and *P. aeruginosa*), significant anti-inflammatory activity (IC₅₀ = 17.55 µg/mL), and free-radical scavenging capacity. These findings support the potential of this multi-plant biogenic hydrogel as an active topical dressing for chronic wound management and justify further *in vivo* preclinical evaluations.

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Authors' Contributions

- i. **Conceptualization:** NEL, MPB
- ii. **Investigation:** NEL, KEK (This replaces "Experimental Execution")
- iii. **Formal analysis:** NEL, KEK, MPB (This covers the analytic portion of your data step)
- iv. **Visualization:** NEL, KEK, MPB
- v. **Writing - original draft:** NEL, KEK, MGK, MPB
- vi. **Writing - review & editing:** NEL, KEK, MGK, MPB
- vii. **Project administration:** KEK, MGK, MPB
- viii. **Supervision:** KEK, MGK, MPB

Data Availability Statement: The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Ethical Approval: Ethical approval was not required for this study as it did not involve human participants or animal subjects. Plant materials

were collected in compliance with institutional and national regulatory guidelines.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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