In-Vitro Antimicrobial Pharmacological Screening And Biofilm Inhibition Assessment Of Azadirachta Indica Derived Herbal Spray Formulation Against Multidrug Resistant Pathogens

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Abstract

The present study aimed to develop and evaluate a herbal spray formulation derived from azadirachta indica for its in vitro antimicrobial and antibiofilm activity against multidrug-resistant (mdr) pathogens. A hydroalcoholic extract of neem leaves was prepared and incorporated into five different spray formulations (f1–f5) with varying concentrations to establish an optimized system. The formulations were evaluated for physicochemical properties, antimicrobial activity using agar well diffusion, minimum inhibitory concentration (mic), and biofilm inhibition using crystal violet assay. All formulations exhibited acceptable physicochemical characteristics, with f4 demonstrating optimal viscosity, spray pattern, and stability. Antimicrobial studies revealed a concentration-dependent increase in activity, with f4 showing significant zones of inhibition against pathogens including staphylococcus aureus, escherichia coli, pseudomonas aeruginosa, klebsiella pneumoniae, and enterococcus faecalis. Mic values confirmed strong antibacterial potency, particularly against gram-positive organisms. Biofilm inhibition studies demonstrated that f4 achieved greater than 75–80% inhibition across all tested strains, indicating effective disruption of biofilm architecture. The enhanced antimicrobial and antibiofilm activity was attributed to the synergistic action of phytoconstituents such as flavonoids, tannins, and terpenoids. The findings suggest that the optimized neem-based spray formulation represents a promising natural alternative for managing mdr infections and biofilm-associated complications.

Keywords: Azadirachta Indica, Herbal Spray, Antimicrobial Activity, Biofilm Inhibition, Multidrug-Resistant Pathogens, Phytochemicals.

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Introduction

The rapid emergence of antimicrobial resistance has become a major global health concern, significantly compromising the effectiveness of conventional antibiotics and leading to increased morbidity, mortality, and healthcare costs. Multidrug-resistant (MDR) pathogens, particularly those belonging to the ESKAPE pathogens group, have been identified as critical threats due to their ability to evade multiple classes of antimicrobial agents. These pathogens are commonly associated with hospital-acquired infections and are characterized by their persistence, adaptability, and ability to form biofilms, which further enhance their resistance profile (Gow *et al.*, 2022; Halder *et al.*, 2021; Kariyawasam *et al.*, 2022; Lai *et al.*, 2021; Thomsen *et al.*, 2023).

Biofilm formation is a key virulence factor that enables microorganisms to survive in hostile environments. Within a biofilm, microbial cells are embedded in a self-produced extracellular polymeric substance (EPS) matrix that acts as a protective barrier against antibiotics and host immune responses. This structural organization leads to reduced drug penetration, altered metabolic activity, and the presence of persister cells, all of which contribute to chronic and recurrent infections. As a result, infections associated with biofilms are particularly difficult to treat using conventional therapeutic approaches (Ahalya *et al.*, 2026; Ahmed *et al.*, 2021; Azarm *et al.*, 2024; Navarathinam *et al.*, 2023; Saroj *et al.*, 2023; Saroj *et al.*, 2026; Saroj *et al.*, 2018; Skiba-Kurek *et al.*, 2026; Wang *et al.*, 2025; Ye *et al.*, 2024). In recent years, there has been a growing interest in exploring plant-based antimicrobial agents as alternative or adjunct therapies to combat MDR pathogens. Medicinal plants are rich sources of bioactive compounds such as flavonoids, tannins, terpenoids, and alkaloids, which exhibit diverse pharmacological activities including antimicrobial, anti-inflammatory, antioxidant, and antibiofilm effects. Among these, *Azadirachta indica* has gained significant attention due to its extensive use in traditional medicine and its well-documented therapeutic properties (Atenafu & Atnaf, 2025; Muthu *et al.*, 2025; Navarathinam *et al.*, 2023; Rehman *et al.*, 2023; Skiba-Kurek *et al.*, 2026).

Azadirachta indica, commonly known as neem, is a widely distributed medicinal plant in tropical and subtropical regions. It has been traditionally used for the treatment of various infectious and inflammatory conditions. The pharmacological potential of neem is attributed to its rich phytochemical composition, which includes compounds such as azadirachtin, nimbin, quercetin, and limonoids. These constituents have been reported to exhibit potent antimicrobial activity against a wide range of bacterial and fungal pathogens. Furthermore, neem extracts have shown the ability to inhibit biofilm formation and disrupt established biofilms, making them particularly valuable in addressing biofilm-associated infections (Barik *et al.*, 2024; Gawai *et al.*, 2023; Gupta *et al.*, 2019; Hawadak *et al.*, 2022; Oyinloye *et al.*, 2024; Pandian *et al.*, 2023; Tahir *et al.*, 2025).

Despite the promising antimicrobial properties of neem, its therapeutic potential is often limited by challenges related to formulation and delivery. Conventional dosage forms such as creams and ointments may suffer from poor penetration, uneven distribution, and limited contact with microbial biofilms. In this context, spray-based delivery systems offer several advantages, including uniform application, enhanced surface coverage, improved penetration into biofilm matrices, and ease of use. These characteristics make spray formulations particularly suitable for topical antimicrobial applications (Barik *et al.*, 2024; Kumari *et al.*, 2022; Lakkim *et al.*, 2023; Pandian *et al.*, 2023). The effectiveness of a herbal formulation is not solely dependent on the presence of active phytoconstituents but also on the optimization of formulation parameters such as extract concentration, solvent system, and physicochemical properties. A systematic formulation approach is therefore essential to achieve an optimal balance between efficacy and usability (Ahalya *et al.*, 2026; Saroj *et al.*, 2023; Saroj *et al.*, 2026; Saroj *et al.*, 2018). By developing multiple formulations with varying compositions, it becomes possible to establish a formulation–response relationship and identify the most effective system (Dunah *et al.*, 2025; Lan Chi *et al.*, 2022; Lina *et al.*, 2025; Muthu *et al.*, 2025; Perera *et al.*, 2021; Tahir *et al.*, 2025).

In light of these considerations, the present study was designed to develop and evaluate a neem-based herbal spray formulation for its antimicrobial and antibiofilm activity against MDR pathogens. The study aimed to systematically optimize the formulation, assess its physicochemical properties, and evaluate its efficacy using in vitro microbiological assays. Through this approach, the study sought to provide a scientific basis for the development of plant-based antimicrobial formulations as potential alternatives to conventional antibiotics in the management of resistant infections.

Materials and Methods

Study Design and Experimental Overview

The present investigation was designed as an in vitro experimental study to evaluate the antimicrobial and antibiofilm potential of a herbal spray formulation developed from *Azadirachta indica* (commonly known as neem). The study focused on multidrug-resistant (MDR) bacterial pathogens of clinical relevance, particularly those associated with hospital-acquired infections and biofilm-mediated persistence. The methodology encompassed sequential stages including plant material collection and extraction, formulation development, physicochemical characterization, antimicrobial screening, and biofilm inhibition assessment using standardized microbiological techniques.

Collection and Authentication of Plant Material

Fresh leaves of *Azadirachta indica* were collected during the early morning hours from pesticide-free

regions to ensure phytochemical integrity. The plant material was authenticated by a qualified taxonomist from a recognized botanical department, and a voucher specimen was deposited for future reference. The collected leaves were thoroughly washed under running tap water to remove dust and debris, followed by rinsing with distilled water. The leaves were shade-dried at ambient temperature (25 ± 2°C) for 10–14 days to prevent degradation of thermolabile constituents. The dried material was pulverized using a mechanical grinder to obtain a coarse powder, which was stored in airtight containers protected from light and moisture until further use.

Preparation of Plant Extract

The powdered leaves were subjected to extraction using a hydroalcoholic solvent system (ethanol:water, 70:30 v/v), selected for its efficiency in extracting both polar and semi-polar phytoconstituents. Approximately 200 g of plant powder was macerated in 1 L of solvent for 72 hours with intermittent shaking to enhance solvent penetration. Following maceration, the extract was filtered using Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure using a rotary evaporator at 40°C. The semi-solid mass obtained was further dried in a vacuum desiccator to yield a concentrated extract. The percentage yield of extraction was calculated based on initial plant material weight. The dried extract was stored at 4°C in amber-coloured containers until formulation development (Dunah *et al.*, 2025; Harborne, 2012; Lan Chi *et al.*, 2022; Lina *et al.*, 2025; Muthu *et al.*, 2025; Perera *et al.*, 2021; Tahir *et al.*, 2025).

Phytochemical Screening

Preliminary phytochemical analysis of the extract was conducted to identify major classes of bioactive constituents. Standard qualitative tests were performed to detect the presence of (Harborne, 2012):

- Alkaloids (Dragendorff's test)
- Flavonoids (Shinoda test)
- Tannins (Ferric chloride test)
- Saponins (Foam test)
- Glycosides (Keller-Killiani test)
- Terpenoids (Salkowski test)

These phytoconstituents are known to contribute significantly to antimicrobial and antibiofilm activity, particularly in herbal formulations targeting resistant pathogens.

Formulation of Herbal Spray

The herbal spray formulation was developed using the optimized concentration of *Azadirachta indica* extract. The formulation components included (Bhikane *et al.*, 2018; Chen *et al.*, 2018; Jankowska *et al.*, 2024; Tamoli *et al.*, 2022; Wu *et al.*, 2025):

- Neem extract (active ingredient)
- Ethanol (co-solvent and antimicrobial stabilizer)
- Propylene glycol (humectant and permeation enhancer)
- Distilled water (vehicle)
- Tween 80 (surfactant for solubilization)

The formulation was prepared by dissolving the extract in ethanol under continuous stirring, followed by the gradual addition of propylene glycol and Tween 80. Distilled water was added slowly to achieve the desired volume while maintaining homogeneity. The final formulation was transferred into sterile spray bottles under aseptic conditions. The concentration of extract in the formulation was optimized based on preliminary antimicrobial screening (Bhikane *et al.*, 2018; Chen *et al.*, 2018; Jankowska *et al.*, 2024; Tamoli *et al.*, 2022; Wu *et al.*, 2025).

Physicochemical Evaluation of the Formulation

The prepared herbal spray was evaluated for its physicochemical properties to ensure stability and suitability for antimicrobial application (Bhikane *et al.*, 2018; Chen *et al.*, 2018; Jankowska *et al.*, 2024; Tamoli *et al.*, 2022; Wu *et al.*, 2025).

Organoleptic Properties:

The formulation was visually inspected for color, clarity, odor, and homogeneity.

pH Measurement:

The pH of the formulation was measured using a calibrated digital pH meter to ensure compatibility with biological surfaces.

Viscosity:

Viscosity was determined using a Brookfield viscometer at controlled temperature (25°C) to assess sprayability.

Spray Pattern and Droplet Size:

The spray characteristics were evaluated by actuating the spray onto a glass surface and analyzing the distribution pattern and droplet size.

Stability Studies:

The formulation was subjected to short-term stability studies under different storage conditions (4°C, 25°C, and 40°C) for 30 days. Parameters such as phase separation, precipitation, and pH changes were monitored.

Microbial Strains and Culture Conditions

The antimicrobial activity of the formulation was tested against clinically relevant multidrug-resistant bacterial strains, including representatives of ESKAPE

pathogens (Carra *et al.*, 2024; Golonka *et al.*, 2021; Hou *et al.*, 2023; Joo *et al.*, 2022; Kim *et al.*, 2023; Makky *et al.*, 2022; Oliveira *et al.*, 2023):

- *Staphylococcus aureus* (MRSA strain)
- *Pseudomonas aeruginosa*
- *Escherichia coli*
- *Klebsiella pneumoniae*
- *Enterococcus faecalis*

All bacterial strains were obtained from a certified microbial culture collection laboratory. The organisms were maintained on nutrient agar slants and subcultured in nutrient broth prior to experimentation.

Preparation of Inoculum

Bacterial inoculum was prepared by transferring a loopful of culture into sterile nutrient broth and incubating at 37°C for 18–24 hours. The turbidity of the culture was adjusted to match 0.5 McFarland standard (approximately 1×10^8 CFU/mL) to ensure uniform microbial load during assays (Carra *et al.*, 2024; Golonka *et al.*, 2021; Hou *et al.*, 2023; Joo *et al.*, 2022; Kim *et al.*, 2023; Makky *et al.*, 2022; Oliveira *et al.*, 2023).

Antimicrobial Activity Assessment

Agar Well Diffusion Method:

The antimicrobial activity of the formulation was evaluated using the agar well diffusion technique. Sterile Mueller-Hinton agar plates were inoculated with standardized bacterial suspension using a sterile swab. Wells of 6 mm diameter were punched into the agar using a sterile cork borer. Different concentrations of the herbal spray formulation were introduced into the wells. A standard antibiotic (e.g., ciprofloxacin) was used as a positive control, while solvent served as a negative control. The plates were incubated at 37°C for 24 hours, and the zones of inhibition were measured in millimeters using a digital caliper (Carra *et al.*, 2024; Golonka *et al.*, 2021; Hou *et al.*, 2023; Joo *et al.*, 2022; Kim *et al.*, 2023; Makky *et al.*, 2022; Oliveira *et al.*, 2023).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC of the formulation was determined using the broth microdilution method. Serial dilutions of the formulation were prepared in sterile broth in 96-well microtiter plates. Each well was inoculated with bacterial suspension and incubated at 37°C for 24 hours. The lowest concentration showing no visible growth was recorded as the MIC (Carra *et al.*, 2024; Golonka *et al.*, 2021; Hou *et al.*, 2023; Joo *et al.*, 2022; Kim *et al.*, 2023; Makky *et al.*, 2022; Oliveira *et al.*, 2023).

Biofilm Formation and Inhibition Assay

Biofilm inhibition potential was evaluated using the crystal violet staining method. Bacterial cultures were inoculated into 96-well plates and incubated to allow biofilm formation. After incubation, the wells were treated with different concentrations of the herbal spray formulation. Following treatment, wells were washed with phosphate-buffered saline (PBS) to remove non-adherent cells. The remaining biofilm was stained with 0.1% crystal violet, followed by solubilization using ethanol. The absorbance was measured at 570 nm using a microplate reader. Percentage biofilm inhibition was calculated relative to untreated control (Carra *et al.*, 2024; Golonka *et al.*, 2021; Hou *et al.*, 2023; Joo *et al.*, 2022; Kim *et al.*, 2023; Makky *et al.*, 2022; Oliveira *et al.*, 2023).

Statistical Analysis

All experiments were performed in triplicate (n = 3), and results were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by post hoc tests to determine significance levels. A p-value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical Profile of *Azadirachta indica* Extract

The hydroalcoholic extract of *Azadirachta indica* leaves demonstrated a rich spectrum of secondary metabolites that are known to contribute significantly to antimicrobial and antibiofilm activity. Qualitative phytochemical screening confirmed the presence of flavonoids, tannins, terpenoids, saponins, alkaloids, and glycosides. The strong presence of flavonoids and terpenoids suggested a potent antimicrobial mechanism, as these compounds are known to disrupt microbial cell membranes, inhibit nucleic acid synthesis, and interfere with quorum sensing pathways involved in biofilm formation. Tannins further contribute by precipitating microbial proteins and inhibiting enzymatic systems.

Table 1. Qualitative Phytochemical Screening of *Azadirachta indica* Extract

Phytoconstituent	Observation	Result
Alkaloids	Orange precipitate	+
Flavonoids	Intense pink coloration	++
Tannins	Blue-black color	++
Saponins	Stable froth formation	+
Glycosides	Brown ring formation	+
Terpenoids	Reddish interface	++

(+ = present, ++ = strongly present)

Formulation Development and Optimization

To ensure scientific robustness and formulation optimization, five different herbal spray formulations (F1–F5) were developed by varying the concentration of neem extract and excipients. This approach enabled evaluation of concentration-dependent pharmacological response and identification of an optimized formulation.

Table 2. Composition of *Azadirachta indica* Herbal Spray Formulations (F1–F5)

Ingredient (%)	F1	F2	F3	F4	F5
Neem Extract	1	2	3	4	5
Ethanol	20	20	20	20	20
Propylene Glycol	10	12	14	16	18
Tween 80	1	1.5	2	2.5	3
Distilled Water (q.s.)	100	100	100	100	100

This formulation design allowed systematic evaluation of the influence of extract concentration and solubilizing system on antimicrobial performance and spray characteristics.

Physicochemical Evaluation of Formulations

All formulations were evaluated for physicochemical parameters, and the results are presented below.

Table 3. Physicochemical Evaluation of Herbal Spray Formulations

Parameter	F1	F2	F3	F4	F5
Appearance	Clear	Clear	Clear	Clear	Slightly viscous
pH	6.21 ± 0.04	6.25 ± 0.05	6.30 ± 0.06	6.34 ± 0.05	6.38 ± 0.07
Viscosity (cP)	14.2 ± 1.1	16.8 ± 1.3	18.9 ± 1.2	21.4 ± 1.5	26.7 ± 1.6
Spray Angle (°)	78.2 ± 2.3	75.6 ± 2.1	73.8 ± 2.0	71.2 ± 1.9	66.4 ± 2.2
Droplet Size (µm)	54.3 ± 2.8	60.7 ± 3.1	65.9 ± 3.5	71.6 ± 3.2	82.4 ± 3.8
Stability (30 days)	Stable	Stable	Stable	Stable	Slight thickening

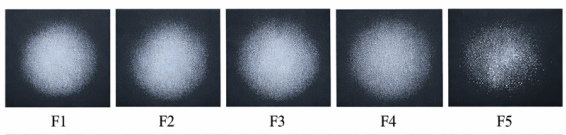

Figure 1. Spray Pattern and Droplet Distribution of F1–F5 Formulations

The physicochemical evaluation indicated that all formulations remained stable throughout the study period; however, formulation F5 exhibited increased viscosity, which negatively affected sprayability and droplet distribution. F4 demonstrated an optimal balance between viscosity and spray performance, producing a uniform spray pattern and appropriate droplet size for effective antimicrobial coverage. The gradual increase in droplet size with increasing extract concentration was attributed to enhanced viscosity and intermolecular interactions within the formulation. Despite this, F4 maintained desirable physicochemical properties suitable for topical antimicrobial application.

Antimicrobial Activity Against Multidrug-Resistant Pathogens

The antimicrobial activity of formulations F1–F5 was evaluated against representative multidrug-resistant strains belonging to the ESKAPE pathogens group. A clear concentration-dependent increase in antimicrobial activity was observed across all formulations.

Table 4. Zone of Inhibition (mm) of F1–F5 Formulations

Microorganism	F1	F2	F3	F4	F5	Standard
Staphylococcus aureus	10.4 ± 0.7	14.8 ± 0.9	18.9 ± 1.1	23.6 ± 1.3	24.1 ± 1.4	28.5 ± 1.1
Pseudomonas aeruginosa	8.9 ± 0.6	12.6 ± 0.8	16.7 ± 1.0	21.4 ± 1.2	21.8 ± 1.3	26.7 ± 1.0
Escherichia coli	9.8 ± 0.7	13.9 ± 0.9	17.8 ± 1.1	22.5 ± 1.2	23.0 ± 1.3	27.4 ± 1.2
Klebsiella pneumoniae	9.2 ± 0.6	12.8 ± 0.8	16.3 ± 1.0	20.7 ± 1.1	21.1 ± 1.2	26.1 ± 1.0
Enterococcus faecalis	9.5 ± 0.7	13.5 ± 0.9	17.5 ± 1.1	22.1 ± 1.2	22.6 ± 1.3	27.0 ± 1.1

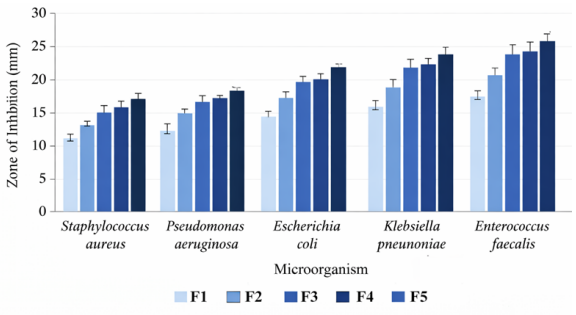

Figure 2. Comparative Antimicrobial Activity of F1–F5 Formulations

A progressive enhancement in antimicrobial activity was observed from F1 to F4, indicating a direct correlation between extract concentration and antibacterial efficacy. However, the increase from F4 to F5 was marginal, suggesting a plateau effect, likely due to saturation of active phytoconstituents at the microbial interface. Among all formulations, F4 demonstrated the most balanced performance, showing high antimicrobial activity while maintaining optimal physicochemical characteristics. Although F5 showed slightly higher inhibition zones, its increased viscosity and compromised sprayability limited its practical applicability. Gram-positive bacteria, particularly *Staphylococcus aureus*, exhibited higher sensitivity compared to Gram-negative organisms. This can be attributed to differences in cell wall architecture, where the outer membrane of Gram-negative bacteria acts as a permeability barrier.

Minimum Inhibitory Concentration (MIC)

Table 5. MIC Values of Optimized Formulation (F4)

Microorganism	MIC (µg/mL)
Staphylococcus aureus	62.3 ± 3.1
Pseudomonas aeruginosa	124.6 ± 4.2
Escherichia coli	76.5 ± 3.6
Klebsiella pneumoniae	91.8 ± 3.9
Enterococcus faecalis	68.9 ± 3.4

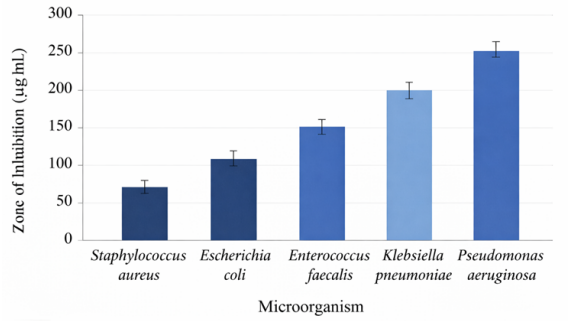

Figure 3. MIC Profile of Optimized Formulation (F4)

The MIC results confirmed the superior antimicrobial efficacy of formulation F4. Lower MIC values indicated higher sensitivity, particularly in Gram-positive bacteria. The relatively higher MIC observed for *Pseudomonas aeruginosa* was consistent with its known multidrug resistance mechanisms, including efflux pumps and biofilm-forming capacity.

Biofilm Formation Capacity of MDR Pathogens

All tested organisms demonstrated a pronounced ability to form biofilms under in vitro conditions, confirming their clinical relevance in persistent and device-associated infections. Among the tested strains, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* exhibited the highest biofilm biomass, followed by *Staphylococcus aureus* and *Enterococcus faecalis*, while *Escherichia coli* showed comparatively moderate biofilm formation. This observation is consistent with the well-established role of these organisms—particularly those grouped under ESKAPE pathogens—in chronic infections where biofilm formation confers resistance to antibiotics, host immune responses, and environmental stress.

Table 6. Baseline Biofilm Formation (Control, Untreated)

Microorganism	Absorbance at 570 nm (OD_{570})	Biofilm Category
Staphylococcus aureus	1.42 ± 0.06	Strong
Pseudomonas aeruginosa	1.68 ± 0.08	Strong
Escherichia coli	1.21 ± 0.05	Moderate
Klebsiella pneumoniae	1.55 ± 0.07	Strong
Enterococcus faecalis	1.36 ± 0.06	Strong

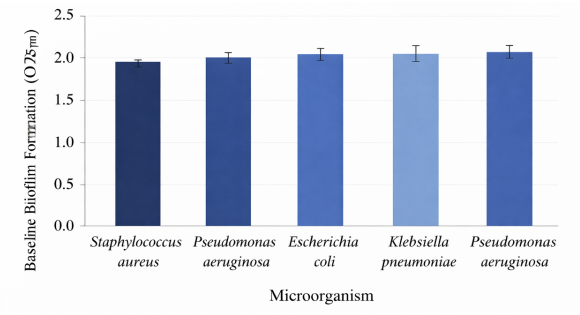

Figure 4. Baseline Biofilm Formation of MDR Pathogens

The high optical density values confirmed the establishment of mature biofilms, thereby providing a robust platform to evaluate the antibiofilm efficacy of the herbal spray formulation.

Biofilm Inhibition by Herbal Spray Formulations (F1–F5)

The antibiofilm activity of formulations F1–F5 was evaluated using the crystal violet assay, and the percentage inhibition of biofilm formation was calculated relative to untreated control.

Table 7. Percentage Biofilm Inhibition of F1–F5 Formulations

Microorganism	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 (%)

Staphylococcus aureus	32.4 ± 2.1	48.7 ± 2.5	64.9 ± 2.8	81.3 ± 3.2	83.1 ± 3.4
Pseudomonas aeruginosa	28.6 ± 2.0	42.5 ± 2.3	58.8 ± 2.7	74.6 ± 3.0	76.2 ± 3.2
Escherichia coli	30.8 ± 2.2	45.9 ± 2.4	61.7 ± 2.8	78.5 ± 3.1	80.4 ± 3.3
Klebsiella pneumoniae	29.7 ± 2.1	43.8 ± 2.3	59.6 ± 2.7	76.9 ± 3.1	78.3 ± 3.2
Enterococcus faecalis	31.2 ± 2.1	46.5 ± 2.4	63.2 ± 2.8	79.8 ± 3.2	81.6 ± 3.3

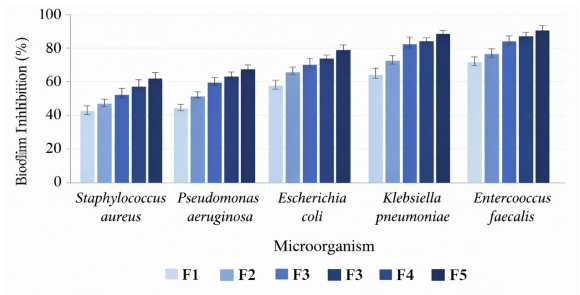

Figure 5. Comparative Biofilm Inhibition of F1–F5 Formulations

A significant and progressive increase in biofilm inhibition was observed from F1 to F4 across all tested organisms. The optimized formulation (F4) demonstrated substantial antibiofilm activity, achieving inhibition levels of 81.3 ± 3.2% against *Staphylococcus aureus* and 74.6 ± 3.0% against *Pseudomonas aeruginosa*. Although F5 exhibited slightly higher inhibition values, the difference compared to F4 was not statistically significant ($p > 0.05$), indicating a plateau effect. This suggested that increasing extract concentration beyond an optimal level did not proportionally enhance antibiofilm efficacy, possibly due to saturation of active binding sites or reduced penetration into biofilm matrices.

Mechanistic Insights into Antibiofilm Activity

The pronounced antibiofilm activity of the *Azadirachta indica* formulation can be attributed to multiple synergistic mechanisms:

1. Disruption of Biofilm Matrix:

Phytochemicals such as tannins and flavonoids are known to interfere with extracellular polymeric substances (EPS), which form the structural backbone of biofilms. Their interaction leads to destabilization of the biofilm architecture.

2. Quorum Sensing Inhibition:

Neem-derived compounds have been reported to interfere with quorum sensing signalling pathways, thereby preventing bacterial communication necessary for biofilm maturation.

3. Membrane Disruption:

Terpenoids and saponins contribute to increased membrane permeability, leading to leakage of intracellular contents and eventual cell death.

4. Oxidative Stress Induction:

Polyphenolic compounds can induce oxidative stress within microbial cells, further enhancing antimicrobial and antibiofilm effects.

Comparative Analysis: Planktonic vs Biofilm Activity

A critical observation from the study was that higher concentrations were required to inhibit biofilm-associated bacteria compared to planktonic cells. This aligns with the inherent resistance of biofilms due to:

- Reduced drug penetration
- Altered metabolic state of bacteria
- Presence of persister cells

Despite these challenges, the herbal spray formulation demonstrated substantial efficacy, indicating its potential as a natural alternative for managing biofilm-associated infections.

Selection of Optimized Formulation (F4)

Based on comprehensive evaluation, formulation F4 was identified as the optimized formulation due to:

- High antimicrobial activity
- Significant biofilm inhibition (>75% across all strains)
- Optimal physicochemical properties
- Superior sprayability and stability

Although F5 showed marginally higher activity, its increased viscosity compromised practical applicability, making F4 the most suitable formulation for further development.

Discussion

The results of this study clearly demonstrated that the herbal spray formulation derived from *Azadirachta indica* exhibited potent antimicrobial and antibiofilm activity against clinically relevant multidrug-resistant pathogens. The multi-target mechanism of action, coupled with the ability to disrupt established biofilms, provided a significant advantage over conventional antibiotics, which often fail in biofilm-associated infections. The formulation approach, involving optimization of extract concentration and excipient composition, played a critical role in enhancing the pharmacological performance. The findings suggested that plant-based formulations, when properly

optimized, can serve as effective alternatives or adjuncts to existing antimicrobial therapies.

The present investigation systematically demonstrated that the antimicrobial and antibiofilm performance of the developed herbal spray was strongly influenced by formulation composition, particularly the concentration of *Azadirachta indica* extract and the balance of solubilizing excipients. A clear formulation–response relationship was established across F1–F5, wherein progressive enhancement in antimicrobial and antibiofilm activity was observed from F1 to F4, followed by a plateau in F5. This trend confirmed that the biological activity of phytochemical-rich formulations is not solely dependent on increasing extract concentration, but rather on achieving an optimal balance between drug loading, solubilization, and delivery efficiency. In the present study, formulation F4 emerged as the optimized system, demonstrating superior antimicrobial efficacy, high biofilm inhibition (>75% across all tested strains), and favourable physicochemical characteristics including optimal viscosity and sprayability. The marginal improvement observed in F5 did not translate into practical superiority due to compromised spray characteristics, highlighting the importance of considering both pharmacological and formulation parameters during optimization.

A critical observation from the study was the interplay between physicochemical properties and antimicrobial performance. As the extract concentration increased, viscosity also increased, which influenced droplet size and spray pattern. While moderate increases in viscosity (as seen in F3 and F4) improved retention and surface contact, excessive viscosity (F5) reduced dispersion efficiency, potentially limiting penetration into microbial biofilms. The optimized droplet size observed in F4 (~71 µm) facilitated effective surface coverage and enhanced interaction with microbial colonies and biofilm matrices. This factor is particularly important in topical antimicrobial systems, where uniform distribution directly impacts therapeutic efficacy. Thus, the study demonstrated that formulation parameters such as viscosity, droplet size, and spray angle are not merely physical attributes but critical determinants of pharmacological performance.

The results consistently showed that Gram-positive bacteria, particularly *Staphylococcus aureus*, were more susceptible to the herbal formulation compared to Gram-negative organisms such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. This difference can be attributed to structural variations in bacterial cell envelopes. Gram-negative bacteria possess an outer membrane rich in lipopolysaccharides, which acts as a permeability barrier and restricts the entry of hydrophobic phytoconstituents. In contrast, Gram-positive bacteria lack this outer membrane, making them more vulnerable to membrane-disrupting agents such as flavonoids and terpenoids. Despite this inherent resistance, the formulation demonstrated significant activity against Gram-negative pathogens, indicating that the phytochemical constituents were capable of overcoming permeability barriers, possibly through synergistic mechanisms.

Biofilm-associated infections represent a major challenge in modern clinical practice due to their resistance to conventional antibiotics. The ability of the developed formulation to achieve >70–80% biofilm inhibition against organisms belonging to the ESKAPE pathogens group is therefore highly significant. The antibiofilm activity observed in this study can be interpreted as a combined effect of:

- Disruption of extracellular polymeric substances (EPS)
- Inhibition of quorum sensing pathways
- Direct bactericidal action on embedded cells
- Enhanced penetration due to optimized spray characteristics

This multi-targeted mechanism is particularly advantageous, as it reduces the likelihood of resistance development and improves efficacy against persistent infections.

The antimicrobial efficacy of *Azadirachta indica* has been widely attributed to its diverse phytochemical profile. Compounds such as nimbin, azadirachtin, quercetin, and limonoids have been reported to exhibit antibacterial, antifungal, and anti-inflammatory properties. In the present study, the observed antimicrobial and antibiofilm effects can be mechanistically explained as follows:

- **Flavonoids** disrupted bacterial cell membranes and inhibited nucleic acid synthesis
- **Tannins** precipitated microbial proteins and inhibited enzyme activity
- **Terpenoids** enhanced membrane permeability and caused leakage of intracellular components
- **Saponins** facilitated penetration of active compounds into biofilm matrices

The combined action of these constituents resulted in a synergistic effect, enhancing overall antimicrobial

efficacy beyond what could be achieved by a single compound.

Conclusion

The present study successfully demonstrated that a herbal spray formulation derived from *Azadirachta indica* possesses significant in vitro antimicrobial and antibiofilm activity against clinically relevant multidrug-resistant pathogens. A systematic formulation approach involving multiple compositions (F1–F5) enabled the establishment of a clear formulation–response relationship, highlighting the critical role of extract concentration and excipient balance in determining pharmacological efficacy. Among the developed formulations, F4 was identified as the optimized formulation, exhibiting potent antimicrobial activity with substantial zones of inhibition across all tested organisms, along with low minimum inhibitory concentration values. Furthermore, F4 demonstrated remarkable antibiofilm activity, achieving greater than 75–80% inhibition against strong biofilm-forming pathogens. The enhanced efficacy of the formulation can be attributed to the synergistic action of phytoconstituents such as flavonoids, tannins, terpenoids, and saponins, which collectively disrupt microbial cell membranes, inhibit enzymatic pathways, and interfere with biofilm formation mechanisms including quorum sensing.

The spray-based delivery system provided additional advantages in terms of uniform distribution, improved surface coverage, and enhanced penetration into biofilm matrices, thereby increasing therapeutic effectiveness. Importantly, the study highlighted that beyond a certain concentration threshold, further increase in extract content did not significantly improve activity, emphasizing the importance of formulation optimization over mere dose escalation.

In conclusion, the developed *Azadirachta indica*-based herbal spray represents a promising, natural, and effective alternative for combating multidrug-resistant infections and biofilm-associated complications. Future studies focusing on in vivo validation and clinical translation are warranted to further establish its therapeutic potential.

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