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FORMULATION AND EVALUATION OF POLYHERBAL GEL FOR ANTIMICROBIAL ACTIVITY

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ABSTRACT: The present study was designed to develop, optimise, and comprehensively evaluate a polyherbal antimicrobial gel made up from *Azadirachta indica*, *Curcuma longa*, *Curcuma longa*, *Aloe vera*, and *Centella asiatica*. Dry powder extracts were subjected to preliminary phytochemical screening, quantitative estimation of total phenolic content (TPC) and total flavonoid content (TFC), and validated HPTL.C In vitro antimicrobial activity of individual and combined extracts was evaluated by disc diffusion, broth microdilution minimum inhibitory concentration (MIC) determination, and checkerboard synergy assay (Fractional Inhibitory Concentration Index, FICI). Three polyherbal gel formulations (F1, F2, F3) were prepared using Carbopol 940 at 0.5%, 1.0%, and 1.5% w/w respectively. Physicochemical evaluation, in vitro antimicrobial activity by agar well diffusion, in vitro drug release, accelerated stability studies, and primary skin irritation by Draize patch test were performed. All five plant extracts demonstrated broad-spectrum antimicrobial activity. Formulation F2 (Carbopol 940, 1.0% w/w) was identified as optimal: pH 6.1 ± 0.1, viscosity 6,180 ± 204 cP, spreadability 8.2 ± 0.4 g.cm/s, and drug content within 95–105% for all markers. F2 produced a zone of inhibition of 20.4 ± 0.8 mm against clinical MRSA statistically superior to mupirocin 2% cream (16.8 ± 0.6 mm, $p < 0.05$). Drug release followed Korsmeyer-Peppas kinetics (R^2 = 0.9922, n = 0.482) with 78.4% cumulative release at 24 h. The Primary Irritation Index was 0.14 (non-irritant). The developed polyherbal gel is a stable, dermally safe, and pharmacologically potent topical formulation that demonstrated superior anti-MRSA activity compared to the established topical antibiotic mupirocin.

INTRODUCTION: The skin constitutes the largest organ of the human body, serving as the primary physical and immunological barrier against environmental microorganisms. When this barrier is compromised by trauma, systemic disease, immunosuppression, or environmental stress, pathogenic microorganisms can establish infection in the skin and subcutaneous tissues, giving rise to a wide clinical spectrum of skin and soft tissue infections (SSTIs).

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Skin and soft tissue infections represent one of the most prevalent categories of infectious disease worldwide, affecting an estimated 111 million people annually and accounting for approximately 10–15% of all primary care consultations globally [1].

Pharmaceutical formulation science plays a critical mediating role in translating the intrinsic antimicrobial potency of active agents whether synthetic antibiotics or plant derived phytochemicals into clinically effective topical therapy. The formulation vehicle determines the rate and extent of drug release from the dosage form, the penetration depth into different skin layers, the residence time on the skin surface, the stability of active ingredients, the aesthetic

properties that govern patient compliance, and the compatibility of the formulation with the pH and enzymatic environment of the skin surface [2]. Gel formulations offer a superior vehicle platform for plant extract delivery compared to conventional ointments and creams. The hydrophilic matrix of carbopol gels provides exceptional compatibility with the hydroalcoholic extracts used to prepare most plant antimicrobial preparations, enabling high drug loading without phase separation or incompatibility. The pseudoplastic flow behaviour of carbopol gels provides the dual advantages of easy spreading during application and sustained adhesion to the skin surface post-application, extending the effective contact time between active phytochemicals and the target pathogen [3].

The present research has selected five medicinal plants — *Azadirachta indica*, *Curcuma longa*, *Curcuma longa*, *Aloe vera*, and *Centella asiatica* — based on their complementary and synergistic antimicrobial activities, wound-healing properties, and extensive documentation in Ayurvedic and scientific literature. The fundamental pharmacological rationale for combining the five selected plant extracts in a single polyherbal formulation is the principle of synergistic antimicrobial interaction [4].

The novelty of the present work lies in several distinct aspects. First, this is the first study to systematically evaluate the synergistic antimicrobial interaction between all five selected plants using quantitative FICI analysis, providing the pharmacodynamic evidence base for the specific combination employed. Second, the HPTLC-based simultaneous quantification of three marker compounds (curcumin, nimbolide, eugenol) in the final polyherbal gel formulation provides an analytical quality assurance framework that goes beyond existing literature, which has typically quantified only curcumin. Third, the comprehensive antimicrobial evaluation against six clinically relevant pathogens including MRSA, which represents the most clinically challenging target [5]. The aim is to develop, optimise, and comprehensively evaluate a polyherbal antimicrobial gel that meets all pharmacopoeial quality standards, demonstrates superior antimicrobial activity against clinically relevant pathogens including MDR organisms, and provides a validated analytical framework for quality assurance — contributing scientifically sound data towards the development of an effective plant-based topical antimicrobial product for clinical application [6].

MATERIALS AND METHODS:

Plant Material Collection and Authentication: Dried plant materials of *Azadirachta indica* (leaves), *Curcuma longa* (rhizome), and *Curcuma longa* (leaves), along with *Centella asiatica* (whole plant), were procured from authenticated herbal suppliers in Pune, Maharashtra. Each plant material was authenticated by a qualified botanist at ASPM College of Pharmacy, Sangulwadi, and herbarium specimen was deposited in the Department of Pharmacognosy. *Aloe vera* (inner leaf gel) was obtained from fresh plants grown on the college premises. All plant materials were dried under shade at ambient temperature, reduced to a coarse powder, and stored in airtight amber glass containers until use [7]. Percentage moisture content of each dried plant material was determined by the loss-on-drying method. Foreign organic matter content was determined in accordance with WHO guidelines for quality control of herbal materials. Ash values (total ash, acid-insoluble ash, water-soluble ash) and extractive values (alcohol-soluble extractive, water-soluble extractive) were determined for each plant material as per standard pharmacopoeial methods [8].

Preparation of Standardised Plant Extracts: Hydroalcoholic extracts of all five plants were prepared by Soxhlet extraction method. Coarse powder of each plant material (500 g) was loaded into the Soxhlet extractor thimble and extracted with 70% ethanol v/v as solvent (1,500 mL) for 18 hours (approximately 16–18 cycles) maintaining the temperature at 60°C. The extract was filtered and concentrated under reduced pressure using a rotary evaporator (Buchi, Switzerland) at 45°C to obtain the semi-solid extract. The semi-solid extract was further lyophilised (Heto Power Dry LL3000 freeze-dryer) at −50°C under vacuum (50 mTorr) for 48 hours to obtain the dry powder extract. Percentage yield was calculated on dry weight basis [9]. *Aloe vera* inner leaf gel was freshly obtained by filleting mature *Aloe vera* leaves (3–4 years old), removing outer leaf tissue and removing

the anthraquinone-containing yellow latex layer with thorough washing.

The collected inner gel was lyophilised immediately (−50°C, 72 hours) to obtain a dry powder extract for formulation incorporation, preserving the acemannan and anthraquinone content without enzymatic degradation [10].

Phytochemical Screening: Preliminary phytochemical screening of each plant extract was performed using standard chemical tests to detect the presence or absence of major phytochemical groups, in accordance with Harborne (1973) and Evans (2002) standard procedures. The following tests were performed: Mayer's test and Dragendorff's test for alkaloids; Molisch's test and Fehling's test for carbohydrates; Foam test and Hemolysis test for saponins; Ferric chloride test and Lead acetate test for tannins; Shinoda test for flavonoids; Salkowski test and Liebermann–Burchard test for sterols and terpenoids; Borntrager's test for anthraquinones; Legal's test for cardiac glycosides. Results were recorded as present (+) or absent (−) for each extract [11].

Quantitative Phytochemical Estimation:

Total Phenolic Content (TPC): was determined by the Folin-Ciocalteu spectrophotometric method. Plant extract (1 mg/mL in methanol) was reacted with Folin-Ciocalteu reagent (diluted 1:10 in water) and 20% sodium carbonate solution. Absorbance was measured at 760 nm after 30 minutes incubation at ambient temperature. TPC was expressed as milligrams gallic acid equivalents per gram of dry extract (mg GAE/g DE) using a calibration curve constructed with gallic acid (20–100 µg/mL, $R^2 = 0.9976$).

Total Flavonoid Content (TFC) was determined by the $AlCl_3$ colorimetric method. Plant extract (1 mg/mL in methanol) was reacted with $AlCl_3$ (2% in ethanol) and potassium acetate solution (1 M). Absorbance was measured at 415 nm after 30 minutes. TFC was expressed as milligrams quercetin equivalents per gram of dry extract (mg QE/g DE) using quercetin calibration curve (10–50 µg/mL, $R^2 = 0.9981$) [12].

HPTLC Fingerprinting and Marker Compound Quantification: HPTLC analysis was performed using pre-coated silica gel 60 F254 aluminium plates (20 × 10 cm, Merck). Samples and standards were applied as 8-mm bands (10 µL each) using an automatic TLC applicator (CAMAG Linomat 5). Chromatographic development was carried out in a twin-trough chamber (CAMAG, pre-saturated with mobile phase vapour for 20 minutes). Densitometric scanning was performed using CAMAG TLC Scanner 3 at appropriate wavelengths for each marker compound.

Curcumin (*Curcuma longa* Marker): Mobile phase: chloroform:methanol:formic acid (9.3:0.5:0.2 v/v/v). Detection: 430 nm (absorption mode). Rf: 0.52 ± 0.02. Calibration range: 100–600 ng/band.

Nimbolide (*Azadirachta indica* Marker): Mobile phase: petroleum ether:ethyl acetate (6:4 v/v). Detection: 254 nm (absorption mode). Rf: 0.64 ± 0.02. Calibration range: 50–300 ng/band.

Eugenol (*Curcuma longa* Marker): Mobile phase: toluene:ethyl acetate (9:1 v/v). Detection: 280 nm (absorption mode). Rf: 0.58 ± 0.02. Calibration range: 200–1,200 ng/band.

HPTLC Method Validation: The HPTLC methods for all three marker compounds were validated in accordance with ICH Q2(R1) guidelines for Linearity, Limits of Detection (LOD) and Quantification (LOQ), Precision (Repeatability), Accuracy (Recovery), Specificity [13].

***In-vitro* Antimicrobial Activity of Plant Extracts:**

Microbial Strains: The following standard and clinical strains were used: *Staphylococcus aureus* ATCC 25923 (Gram-positive), Methicillin-resistant *Staphylococcus aureus* (MRSA, clinical isolate, confirmed by oxacillin disc diffusion and mecA PCR), *Escherichia coli* ATCC 25922 (Gram-negative), *Pseudomonas aeruginosa* ATCC 27853 (Gram-negative), *Candida albicans* ATCC 10231 (yeast), and *Trichophyton rubrum* ATCC 28188 (dermatophyte). All strains were maintained on appropriate media (Mueller-Hinton agar for bacteria, Sabouraud Dextrose Agar for fungi) at 4°C and sub-cultured every 4 weeks [14].

Disc Diffusion Assay: Disc diffusion antimicrobial susceptibility testing was performed in accordance

with CLSI M02 standard procedure. Bacterial inocula were prepared by the direct colony suspension method to achieve turbidity equivalent to 0.5 McFarland standard (1–2 × 10^8 CFU/mL). The inoculum was uniformly swabbed across the surface of Mueller-Hinton agar (MHA) plates. Plant extract solution (10 mg/mL in DMSO:water 1:9) was impregnated onto sterile Whatman No. 1 filter paper discs (6 mm diameter, 10 µL per disc). Discs were placed on inoculated plates using sterile forceps. Positive control discs (mupirocin 5 µg/disc for *S. aureus*/MRSA, ciprofloxacin 5 µg/disc for Gram-negatives, fluconazole 25 µg/disc for C. albicans, terbinafine 30 µg/disc for T. rubrum) and negative control discs (DMSO:water 1:9, 10 µL) were included on each plate. Plates were incubated at 37°C for 24 hours (bacteria) and 28°C for 48 hours (T. rubrum). Zone of inhibition diameters (mm) were measured in triplicate experiments [15].

Broth Microdilution MIC Determination: Minimum Inhibitory Concentration (MIC) determination was performed by broth microdilution method in accordance with CLSI M07 guidelines using 96-well microtiter plates. Two-fold serial dilutions of each plant extract (concentration range: 0.0625–64 mg/mL) were prepared in Mueller-Hinton Broth (MHB) for bacteria and RPMI-1640 (pH 7.0 with MOPS) for *C. albicans*. Inoculum was adjusted to 0.5 McFarland (1–5 × 10^5 CFU/mL for bacteria, 1–5 × 10^3 CFU/mL for *C. albicans*) and added to each well. Positive control (broth + organism, no extract) and negative control (broth + extract, no organism) wells were included on each plate. Plates were incubated at 37°C for 24 hours (bacteria) and 35°C for 48 hours (*C. albicans*) without agitation. MIC was defined as the lowest concentration showing no visible turbidity [16].

Checkerboard Synergy Assay: Synergistic interactions between extract pairs were evaluated by the checkerboard microdilution assay. Serial two-fold dilutions of each extract pair (Extract A: 0.125–8 × MIC_A; Extract B: 0.125–8 × MIC_B) were prepared in MHB in 96-well microtiter plates in a 12 × 8 matrix. Each combination well received both extracts at the specified concentrations with a total volume of 100 µL. Inoculum (100 µL of 2 × 10^5 CFU/mL) was added and plates incubated at 37°C for 24 hours. FICI was calculated as:

FICI = (MIC_A combination / MIC_A alone) + (MIC_B combination / MIC_B alone)

Interpretation: FICI ≤ 0.5 = synergistic; 0.5 < FICI ≤ 4.0 = additive/indifferent; FICI > 4.0 = antagonistic. Experiments were performed in triplicate [17].

Preparation of Polyherbal Gel Formulations:

Formulation Composition:

TABLE 1: COMPOSITION OF POLYHERBAL GEL FORMULATIONS (PER 100 G)

Ingredient	F1	F2	F3
Azadirachta indica dry extract	1.0 g	1.0 g	1.0 g
Curcuma longa dry extract	1.0 g	1.0 g	1.0 g
Curcuma longa dry extract	0.5 g	0.5 g	0.5 g
Aloe vera lyophilised gel	2.0 g	2.0 g	2.0 g
Centella asiatica dry extract	0.5 g	0.5 g	0.5 g
Carbopol 940	0.5 g	1.0 g	1.5 g
Glycerol (humectant)	5.0 g	5.0 g	5.0 g
Propylene glycol (co-solvent)	5.0 g	5.0 g	5.0 g
Methylparaben	0.18 g	0.18 g	0.18 g
Propylparaben	0.02 g	0.02 g	0.02 g
Triethanolamine (pH adjustment)	q.s.	q.s.	q.s.
Purified water	q.s. 100 g	q.s. 100 g	q.s. 100 g

Preparation Method: Carbopol 940 was dispersed slowly in freshly prepared purified water (75 mL of total batch) with continuous mechanical stirring (IKA overhead stirrer, 500 rpm) and allowed to hydrate completely for 2 hours at room temperature. In a separate beaker, methylparaben and propylparaben were dissolved completely in propylene glycol with gentle warming (40°C).

Glycerol was measured and added to the propylene glycol-paraben solution. The combined plant extracts (pre-dispersed in the remaining purified water, 15 mL) were added slowly to the Carbopol dispersion under continuous stirring. The preservative-glycerol solution was incorporated. Triethanolamine (10% w/v solution in purified water) was added dropwise with continuous stirring at 500 rpm, monitoring pH continuously, until the target pH of 6.0 ± 0.2 was achieved and the transparent gel formed.

The gel was homogenised at 1,000 rpm for 10 minutes to ensure uniform distribution, then transferred to amber glass jars (50 g) and sealed. Three batches of each formulation (F1, F2, F3) were prepared for evaluation [18].

Evaluation of Polyherbal Gel Formulations:

Appearance and Organoleptic Properties: The prepared gel formulations were examined visually for colour, clarity, homogeneity, and consistency.

pH Measurement: The pH of each gel formulation was measured directly using a calibrated digital pH meter (Eutech Instruments, Thermo Scientific) with a combined glass electrode.

Viscosity: Viscosity of gel formulations was determined using a Brookfield Digital Viscometer (model DV-II+Pro) with spindle no. 6 [19].

Spreadability: Spreadability was determined by the cone and plate method. A quantity of gel (2 g) was placed at the centre of a clean glass plate. A second glass plate was placed on top with a standard weight (100 g) for 5 minutes. The diameter of the gel spread was measured in two perpendicular directions. Spreadability was calculated using: Spreadability (g.cm/s) = m × L / t

Extrudability: Extrudability was determined by filling the gel into a standard aluminium collapsible tube (10 g). The weight required to extrude 0.5 cm of gel from the tube under standardised conditions was measured using a texture analyser. The percentage extrudability relative to a standard reference gel was calculated.

Drug Content (Marker Compound Content): Drug content was determined by HPTLC method. Gel (500 mg) was dissolved in methanol (10 mL), filtered through Whatman 0.45 μm membrane filter, and the filtrate was applied to HPTLC plates. Curcumin, nimbolide, and eugenol contents were quantified by densitometric analysis from the validated calibration curves.

***In-vitro* Antimicrobial Activity of Polyherbal Gel:** Antimicrobial activity of each gel formulation was evaluated by agar well diffusion method. Mueller-Hinton agar plates (bacteria) and Sabouraud Dextrose Agar plates (fungi) were inoculated with standardised suspensions (0.5 McFarland). Wells (8 mm diameter) were bored in agar using a sterile cork borer. Each well received 100 μL of the gel formulation (equivalent to 100 mg gel). Positive control (mupirocin 2% cream equivalent) and negative control (plain Carbopol gel without extracts) were included on each plate. After 24-hour incubation (37°C for bacteria, 28°C for *T. rubrum*), zones of inhibition were measured in millimetres. Each experiment was performed in triplicate.

***In-vitro* Drug Release Study:** Drug release from the optimised gel formulation was evaluated using Franz diffusion cell apparatus with an effective diffusion area of 3.14 cm². Regenerated cellulose acetate membrane (molecular weight cut-off: 12,000–14,000 Da, pore size 0.45 μm) was used as the diffusion barrier, pre-equilibrated for 12 hours in receptor fluid (50% ethanol v/v in phosphate buffer pH 5.5 to simulate skin surface conditions). The gel (500 mg) was placed uniformly on the donor side and the diffusion cell sealed with parafilm. The receptor fluid (15 mL) was maintained at 37 ± 0.5°C under continuous magnetic stirring (200 rpm). Aliquots (1 mL) were withdrawn at 0, 1, 2, 4, 6, 8, 12, and 24 hours, replacing with fresh receptor fluid to maintain sink conditions. Curcumin content was determined by validated HPTLC method. Cumulative percentage drug release was calculated. Drug release kinetics were fitted to zero-order, first-order, Higuchi, and Korsmeyer-Peppas models using PCP Disso v3 software [20].

Stability Studies: Accelerated stability studies of the optimised polyherbal gel were conducted in a validated stability chamber (Thermolab, India) at 40°C ± 2°C / 75% RH ± 5% RH in accordance with ICH Q1A(R2) guidelines. Gel samples (50 g in

sealed amber glass jars) were stored at accelerated conditions and withdrawn at time points 0, 1, 2, 3, and 6 months. Long-term stability studies were simultaneously initiated at 25°C ± 2°C / 60% RH ± 5% RH with sampling at 0, 3, and 6 months (with continuation to 12 months). At each time point, samples were evaluated for: appearance and organoleptic properties; pH; viscosity; spreadability; drug content (HPTLC — curcumin, nimbolide, eugenol); and antimicrobial activity against S. aureus ATCC 25923 (zone of inhibition, agar well diffusion). Microbial limit testing (total viable count, yeast and mould count, absence of specified organisms) was performed at 0, 3, and 6 months to confirm preservative efficacy. One-way ANOVA with Tukey's post-hoc test was used to determine statistical significance of changes at each time point ($p < 0.05$ considered significant) [21].

Primary Skin Irritation Study: Primary skin irritation potential of the optimised polyherbal gel was assessed using the Draize patch test method on healthy adult albino rabbits (New Zealand White, male, 2.0–2.5 kg, n=6) in accordance with OECD Guideline 404. All animal studies were conducted after obtaining approval from the Institutional Animal Ethics Committee (IAEC) of ASPM College of Pharmacy, Sangulwadi. Animals were acclimatised for 7 days prior to the study under standard conditions. The dorsal skin of each rabbit was shaved (6 × 8 cm area) 24 hours prior to application using an electric clipper and depilatory cream, inspected for abrasions or dermatological abnormalities, and only animals with intact skin were included. Gel formulation (0.5 g) was applied to one 2.5 × 2.5 cm site under semi-occlusive patch (Johnson & Johnson surgical gauze pad secured with Micropore tape), and the contralateral site received plain Carbopol gel vehicle (negative control). After 24 hours, patches were removed and skin reaction sites cleaned with physiological saline. Erythema and edema scores were recorded at 1, 24, 48, and 72 hours post-patch removal using the Draize primary irritation score scale (0–4 for each endpoint) [22]. Primary Irritation Index (PII) was calculated as:

PII = (Sum of all erythema scores + Sum of all edema scores) / (Number of observations × Number of animals)

Classification: PII 0–0.5 = non-irritant; 0.5–2.0 = mild irritant; 2.0–5.0 = moderate irritant; 5.0–8.0 = severe irritant.

Statistical Analysis: All experimental data were expressed as mean ± standard deviation (SD) from three independent determinations unless otherwise specified. Statistical significance between groups was determined by one-way Analysis of Variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post-hoc test using GraphPad Prism v9.0 software. Pearson's correlation coefficient was calculated to determine relationships between formulation parameters. p-value < 0.05 was considered statistically significant. For drug release kinetic modelling, coefficient of determination (R^2) was used to determine best-fit model.

RESULTS AND DISCUSSION:

Plant Material Quality Control: The plant materials collected and authenticated for the present study met all pharmacopoeial standards for identity, purity, and quality. Authentication certificates from the botanist and herbarium voucher specimen numbers were documented as per standard practice.

TABLE 2: QUALITY CONTROL PARAMETERS OF DRIED PLANT MATERIALS

Parameter	*Azadirachta indica*	*Curcuma longa*	*Curcuma longa*	*Aloe vera* (gel)	*Centella asiatica*
Moisture content (% w/w)	5.4 ± 0.3	6.1 ± 0.4	5.8 ± 0.2	4.2 ± 0.3	6.4 ± 0.5
Total ash (% w/w)	8.2 ± 0.4	6.4 ± 0.3	10.2 ± 0.5	3.8 ± 0.2	9.6 ± 0.4
Acid-insoluble ash (% w/w)	1.4 ± 0.1	0.8 ± 0.1	1.6 ± 0.2	0.6 ± 0.1	1.8 ± 0.2
Alcohol-soluble extractive (% w/w)	16.8 ± 0.8	28.4 ± 1.2	22.6 ± 0.9	38.2 ± 1.6	14.4 ± 0.6
Water-soluble extractive (% w/w)	12.4 ± 0.6	18.6 ± 0.8	28.4 ± 1.1	48.6 ± 2.1	22.8 ± 0.9

All moisture content values were below the pharmacopoeially acceptable limit of 8% w/w, confirming adequate drying and suitability for extract preparation. Total ash values were within pharmacopoeial limits for each respective plant material. The high water-soluble extractive of *Aloe vera* (48.6% w/w) reflected the high content of water-soluble polysaccharides (acemannan) and amino acids characteristic of the inner leaf gel.

Extract Preparation: Percentage Yield:

TABLE 3: PERCENTAGE YIELD OF STANDARDISED PLANT EXTRACTS (SOXHLET, 70% ETHANOL)

Plant	Weight of Powder Used (g)	Weight of Dry Extract Obtained (g)	% Yield (w/w)
Azadirachta indica (leaves)	500	82.4 ± 3.1	16.5 ± 0.6
Curcuma longa (rhizome)	500	124.8 ± 4.6	24.9 ± 0.9
Curcuma longa (leaves)	500	98.6 ± 3.8	19.7 ± 0.8
Aloe vera (freeze-dried gel)	500	194.2 ± 6.4	38.8 ± 1.3
Centella asiatica (whole plant)	500	76.2 ± 2.9	15.2 ± 0.6

The highest percentage yield was obtained from *Aloe vera* lyophilised gel (38.8 ± 1.3%), reflecting the high content of water-soluble polysaccharides and carbohydrates in the inner leaf gel. *Curcuma longa* rhizome yielded the second highest extract (24.9 ± 0.9%), attributed to the high curcuminoid and carbohydrate content of the rhizome. *Azadirachta indica* and *Centella asiatica* showed comparatively lower yields (16.5% and 15.2% respectively), consistent with published data for 70% ethanol extraction. All extracts were freely soluble in 70% ethanol and showed characteristic odour and colour: *Azadirachta indica* (dark green, bitter odour), *Curcuma longa* (deep orange-yellow, characteristic turmeric odour), *Curcuma longa* (dark brown-green, clove-like odour), *Aloe vera* (pale yellow, characteristic mucilaginous), *Centella asiatica* dark brown-green, characteristic earthy odour).

Phytochemical Screening:

TABLE 4: PRELIMINARY PHYTOCHEMICAL SCREENING OF PLANT EXTRACTS

Phytochemical Group	*Azadirachta indica*	*Curcuma longa*	*Curcuma longa*	*Aloe vera*	*Centella asiatica*
Alkaloids	+	−	+	−	−
Carbohydrates	+	+	+	++	+
Saponins	+	−	+	++	++
Tannins	++	+	++	+	+
Flavonoids	++	+	++	+	+
Sterols/Terpenoids	++	++	++	+	++
Anthraquinones	−	−	−	++	−
Cardiac glycosides	+	−	+	−	−

(++ = strongly positive; + = positive; − = absent).

The phytochemical screening results confirmed the presence of expected major phytochemical groups in each extract, consistent with published chemical data. The strong positive reactions for sterols and terpenoids in *Azadirachta indica*, *Curcuma longa*, *Curcuma longa*, and *Centella asiatica* confirmed the presence of the bioactive terpenoid constituents (nimbolide, curcuminoids, eugenol-containing volatile oil, and triterpenoid saponins respectively) that are responsible for the primary antimicrobial activities of these plants. The strongly positive anthraquinone reaction of *Aloe vera* extract confirmed the presence of aloin, emodin, and aloe-emodin, consistent with the known antimicrobial constituents of *Aloe vera* leaf gel. The strongly positive saponin reaction of both *Aloe vera* and *Centella asiatica* extracts is consistent with the high saponin content of these plants (acemannan-associated saponins in *Aloe vera*; asiaticoside and madecassoside in *Centella asiatica*) that contributes to their antimicrobial and wound healing activities.

Quantitative Phytochemical Estimation:

TABLE 5: TOTAL PHENOLIC CONTENT (TPC) AND TOTAL FLAVONOID CONTENT (TFC) OF PLANT EXTRACTS

Plant Extract	TPC (mg GAE/g DE)	TFC (mg QE/g DE)
Azadirachta indica	186.4 ± 8.2	64.8 ± 3.1
Curcuma longa	224.6 ± 9.8	48.2 ± 2.4
Curcuma longa	248.4 ± 10.6	82.4 ± 3.8
Aloe vera	142.8 ± 6.4	28.6 ± 1.4
Centella asiatica	168.2 ± 7.6	44.4 ± 2.2

Curcuma longa exhibited the highest TPC (248.4 ± 10.6 mg GAE/g DE), consistent with its high content of rosmarinic acid, caffeic acid, and phenylpropanoids. *Curcuma longa* showed the second highest TPC (224.6 ± 9.8 mg GAE/g DE), attributed to the high curcuminoid content (curcumin, demethoxycurcumin, bisdemethoxy-curcumin). The TPC and TFC data confirmed the substantial polyphenolic composition of all five extracts, providing a pool of antimicrobial and antioxidant phytoconstituents beyond the specific marker compounds quantified by HPTLC. Correlation between TPC and antimicrobial activity across plant extracts ($r = 0.84$, $p < 0.05$) confirmed that total phenolic content is a significant predictor of antimicrobial potency in this plant series.

HPTLC Method Validation:

TABLE 6: HPTLC VALIDATION PARAMETERS FOR MARKER COMPOUNDS (ICH Q2R1)

Validation Parameter	Curcumin	Nimbolide	Eugenol
Linearity range (ng/band)	100–600	50–300	200–1200
Correlation coefficient (r^2)	0.9991	0.9984	0.9976
LOD (ng/band)	28.4	14.2	52.6
LOQ (ng/band)	86.1	43.1	159.4
Precision — Intraday (% RSD, n=6)	0.82	1.14	1.48
Precision — Interday (% RSD, n=3)	1.16	1.52	1.94
Accuracy — Recovery at 80% (%)	99.4 ± 0.8	98.8 ± 1.1	100.2 ± 1.4
Accuracy — Recovery at 100% (%)	100.2 ± 0.6	99.4 ± 0.9	99.6 ± 1.2
Accuracy — Recovery at 120% (%)	99.8 ± 0.9	100.1 ± 1.0	100.4 ± 1.6
Rf value (mean ± SD)	0.52 ± 0.02	0.64 ± 0.02	0.58 ± 0.02

The HPTLC methods for all three marker compounds met all ICH Q2(R1) validation criteria. Excellent linearity was demonstrated ($r^2 > 0.997$ for all markers). Precision values (% RSD) were well within the ICH-recommended limit of 2.0% for both intraday and interday precision. Accuracy recovery values between 98.8% and 100.4% confirmed the methods' accuracy for reliable quantification of marker compounds in plant extract matrices. The validated LOD and LOQ values confirmed the methods' sensitivity for quantification at the ng/band level, appropriate for quality control of complex polyherbal preparations where individual marker compound content may be low relative to total extract. These validated HPTLC methods were subsequently applied for drug content determination in gel formulations and for stability analysis. Marker Compound Content in Individual Plant Extracts: Curcumin content in *Curcuma longa* dry extract: 4.82 ± 0.18% w/w (consistent with reported range of 3–5%). Nimbolide content in *Azadirachta indica* dry extract: 0.38 ± 0.02% w/w (within reported range of 0.25–0.60%). Eugenol content in *Curcuma longa* dry extract: 6.24 ± 0.28% w/w (within reported range of 4–8% for dried leaf extract).

In-vitro Antimicrobial Activity of Individual Plant Extracts:

Disc Diffusion Results:

TABLE 7: ZONE OF INHIBITION (MM) OF PLANT EXTRACTS BY DISC DIFFUSION

Extract (100 µg/disc)	*S. aureus* ATCC 25923	MRSA (clinical)	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231	*T. rubrum* ATCC 28188
Azadirachta indica	22.4 ± 0.8	19.8 ± 0.6	16.4 ± 0.6	14.2 ± 0.8	18.6 ± 0.6	20.4 ± 0.8
Curcuma longa	20.2 ± 0.6	18.4 ± 0.8	14.8 ± 0.6	12.6 ± 0.6	16.8 ± 0.8	14.2 ± 0.6
Curcuma longa	24.6 ± 0.8	21.2 ± 0.8	18.2 ± 0.8	15.4 ± 0.6	20.4 ± 0.8	18.6 ± 0.6
Aloe vera	14.8 ± 0.6	12.4 ± 0.6	12.2 ± 0.4	10.8 ± 0.6	16.4 ± 0.6	12.4 ± 0.4
Centella asiatica	12.4 ± 0.4	10.8 ± 0.6	10.4 ± 0.4	9.2 ± 0.4	12.2 ± 0.6	10.8 ± 0.4
Mupirocin 5 µg/disc	26.8 ± 0.8	18.2 ± 0.6	—	—	—	—
Ciprofloxacin 5 µg/disc	—	—	28.4 ± 0.8	24.6 ± 0.8	—	—
Fluconazole 25 µg/disc	—	—	—	—	22.4 ± 0.6	10.2 ± 0.6*
Terbinafine 30 µg/disc	—	—	—	—	—	28.4 ± 0.8
Negative control	—	—	—	—	—	—

(**T. rubrum* resistance to fluconazole confirmed by zone < CLSI breakpoint)

All five plant extracts demonstrated measurable antimicrobial activity against all tested pathogens, confirming the broad-spectrum antimicrobial profile required for the polyherbal gel formulation. *Curcuma longa* consistently showed the largest zones of inhibition, particularly against Gram-positive organisms (*S. aureus*: 24.6 mm; MRSA: 21.2 mm), consistent with the high eugenol content of this extract and the documented potency of eugenol against Staphylococci. *Azadirachta indica* showed notably strong activity against *T. rubrum* (20.4 mm), consistent with the documented antidermatophytic activity of neem terpenoids against dermatophytes. Critically, all five plant extracts showed antimicrobial activity against MRSA the most clinically challenging target was comparable to or exceeded the activity of mupirocin (which showed only 18.2 mm zone against the clinical MRSA isolate, compared to 21.2 mm for *Curcuma longa*). This is consistent with the published literature documenting that standard mupirocin shows reduced activity against MRSA clinical isolates compared to MSSA 63. The significantly stronger activity of plant extracts against MRSA compared to mupirocin represents a clinically important finding supporting the therapeutic rationale for the polyherbal gel formulation.

Broth Microdilution MIC Results:

TABLE 8: MINIMUM INHIBITORY CONCENTRATIONS (MG/ML) OF PLANT EXTRACTS

Extract	*S. aureus* ATCC 25923	MRSA	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231	*T. rubrum* ATCC 28188
Azadirachta indica	0.5	1.0	2.0	4.0	1.0	0.5
Curcuma longa	1.0	2.0	4.0	8.0	2.0	4.0
Curcuma longa	0.25	0.5	1.0	2.0	0.5	1.0
Aloe vera	4.0	8.0	8.0	16.0	4.0	8.0
Centella asiatica	8.0	16.0	16.0	32.0	8.0	16.0

Curcuma longa demonstrated the lowest MIC values across all six tested organisms (MIC range: 0.25–2.0 mg/mL), confirming the highest individual antimicrobial potency among the five plants. The MIC of 0.5 mg/mL for *Curcuma longa* against MRSA clinical isolate demonstrates clinically significant antimicrobial potency. *Azadirachta indica* showed excellent activity against *S. aureus* (MIC: 0.5 mg/mL) and *T. rubrum* (MIC: 0.5 mg/mL), consistent with its documented antistaphylococcal and antidermatophytic properties. The relatively higher MIC values for *Centella asiatica* (8–32 mg/mL) against all tested organisms confirm that its primary therapeutic contribution to the polyherbal formulation is wound healing promotion rather than direct antimicrobial activity, though it does contribute synergistically at the concentrations used in the formulation.

Synergy Assessment by Checkerboard Assay:

TABLE 9: FICI VALUES FOR PLANT EXTRACT PAIRS AGAINST MRSA AND *T. RUBRUM*

Extract Pair	FICI Against MRSA	Interpretation	FICI Against *T. rubrum*	Interpretation
Azadirachta indica + *Curcuma longa*	0.31	Synergistic	0.28	Synergistic
Azadirachta indica + *Curcuma longa*	0.38	Synergistic	0.34	Synergistic
Curcuma longa + *Curcuma longa*	0.22	Synergistic	0.26	Synergistic
Azadirachta indica + *Aloe vera*	0.42	Synergistic	0.38	Synergistic
Curcuma longa + *Aloe vera*	0.44	Synergistic	0.40	Synergistic
Triple combination (AZA + CUL + OCS)	0.18	Synergistic	0.14	Synergistic

All tested extract pairs demonstrated synergistic antimicrobial interactions against both MRSA (FICI range: 0.22–0.44) and *T. rubrum* (FICI range: 0.14–0.40), with FICI values consistently well below the synergy threshold of 0.50. The strongest synergistic interaction was observed for the *Curcuma longa* + *Curcuma longa* combination (FICI: 0.22 against MRSA), consistent with the mechanistic synergism between curcumin (FtsZ inhibitor and membrane-active agent) and eugenol (DNA gyrase inhibitor and membrane disruptor). The triple combination of *Azadirachta indica* +

Curcuma longa + *Curcuma longa* demonstrated the strongest overall synergy (FICI: 0.18 against MRSA; 0.14 against *T. rubrum*), confirming that the three-component combination at one-quarter to one-eighth of individual MIC concentrations achieves complete growth inhibition. These synergy data provide the quantitative pharmacodynamic evidence base for the specific plant combination used in the polyherbal gel formulation.

Physicochemical Evaluation of Gel Formulations:

TABLE 10: PHYSICOCHEMICAL EVALUATION PARAMETERS OF POLYHERBAL GEL FORMULATIONS F1, F2, F3

Parameter	F1 (Carbopol 0.5%)	F2 (Carbopol 1.0%)	F3 (Carbopol 1.5%)	Specification
Appearance	Translucent green, homogeneous	Transparent green, homogeneous	Transparent green, homogeneous	Homogeneous, acceptable colour
pH	6.0 ± 0.1	6.1 ± 0.1	6.2 ± 0.1	5.0–6.5
Viscosity (cP, 37°C)	2,840 ± 124	6,180 ± 204	12,640 ± 384	3,000–10,000
Spreadability (g.cm/s)	12.4 ± 0.6	8.2 ± 0.4	4.6 ± 0.2	≥ 6.0
Extrudability (%)	96.8 ± 2.4	94.4 ± 1.8	88.2 ± 2.6	≥ 85%
Drug content — Curcumin (%)	98.4 ± 1.2	98.8 ± 1.0	99.2 ± 0.8	95–105%
Drug content — Nimbolide (%)	97.8 ± 1.4	98.4 ± 1.2	98.6 ± 1.0	95–105%
Drug content — Eugenol (%)	97.2 ± 1.6	97.8 ± 1.4	98.2 ± 1.2	95–105%

Formulation F1 (Carbopol 0.5%) showed adequate spreadability (12.4 g.cm/s) but viscosity below the specification (2,840 cP), indicating insufficient consistency for topical gel application. The low viscosity of F1 would result in inadequate post-application residence time on the skin surface, potentially compromising sustained antimicrobial effect. Formulation F3 (Carbopol 1.5%) produced the highest viscosity (12,640 cP) and lowest spreadability (4.6 g.cm/s), falling below the minimum spreadability specification. The excessive stiffness of F3 would compromise patient acceptability and uniform application. Formulation F2 (Carbopol 1.0%) demonstrated the optimal combination of physicochemical properties: viscosity within specification (6,180 ± 204 cP), adequate spreadability (8.2 ± 0.4 g.cm/s), acceptable extrudability (94.4 ± 1.8%), and pH within the skin-compatible range (6.1 ± 0.1). Drug content for all three marker compounds was within the accepted range of 95–105%, confirming uniform distribution of plant extracts throughout the gel matrix and absence of incompatibility between the Carbopol matrix and plant-derived components. Formulation F2 was selected as the optimised formulation for all further studies based on its compliance with all physicochemical specifications.

***In-vitro* Antimicrobial Activity of Polyherbal Gel Formulations:**

TABLE 11: ZONE OF INHIBITION (MM) OF POLYHERBAL GEL FORMULATIONS BY AGAR WELL DIFFUSION

Formulation / Control	*S. aureus* ATCC 25923	MRSA (clinical)	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*C. albicans* ATCC 10231	*T. rubrum* ATCC 28188
F1 (Carbopol 0.5%)	19.4 ± 0.6	17.8 ± 0.6	14.8 ± 0.6	12.4 ± 0.6	17.4 ± 0.6	18.2 ± 0.6
F2 (Carbopol 1.0%)	22.6 ± 0.8	20.4 ± 0.8	17.2 ± 0.8	14.8 ± 0.6	20.8 ± 0.8	21.4 ± 0.8
F3 (Carbopol 1.5%)	20.2 ± 0.8	18.4 ± 0.8	15.4 ± 0.6	12.8 ± 0.8	18.4 ± 0.8	19.2 ± 0.8
Mupirocin 2% cream	24.4 ± 0.8	16.8 ± 0.6	—	—	—	—
Plain Carbopol gel	0	0	0	0	0	0

All three polyherbal gel formulations demonstrated significant and broad-spectrum antimicrobial activity against all six tested pathogens, confirming that the antimicrobial activity of the plant extracts was retained following formulation in the Carbopol gel matrix. The plain Carbopol gel (negative control) produced no zones of inhibition, confirming that all antimicrobial activity was attributable exclusively to the plant extract components. Formulation F2 produced the largest

zones of inhibition across all pathogens, significantly superior to F1 ($p < 0.05$, ANOVA, Tukey's HSD) and F3 ($p < 0.05$). The higher antimicrobial efficacy of F2 compared to F3 despite equal extract content is attributed to the superior sustained drug release characteristics of the 1.0% Carbopol matrix (demonstrated in drug release studies, Section 5.10), which enabled more effective diffusion of active phytoconstituents from the gel matrix into the agar diffusion medium.

The critically important finding is that F2 (polyherbal gel) produced a zone of 20.4 ± 0.8 mm against MRSA, compared to only 16.8 ± 0.6 mm for the mupirocin 2% cream positive control ($p < 0.05$). This superior anti-MRSA activity of the polyherbal gel compared to the established standard topical antibiotic is explained by the multi-target antimicrobial mechanism of the phytochemical combination — simultaneously targeting bacterial membrane integrity (nimbolide, eugenol), cell division (curcumin-FtsZ), DNA synthesis (eugenol-DNA gyrase), and quorum sensing (curcumin) — versus the single-target mechanism of mupirocin (isoleucyl tRNA synthetase inhibition), for which clinical resistance has been documented in the test strain.

In-vitro Drug Release Study:

TABLE 12: CUMULATIVE % DRUG RELEASE (CURCUMIN) FROM FORMULATIONS F1, F2, F3 (FRANZ CELL, 24 H)

Time (h)	F1 (Carbopol 0.5%)	F2 (Carbopol 1.0%)	F3 (Carbopol 1.5%)
1	18.4 ± 0.8	12.2 ± 0.6	8.4 ± 0.4
2	32.6 ± 1.2	21.4 ± 0.8	14.8 ± 0.6
4	54.8 ± 1.6	38.6 ± 1.2	26.4 ± 0.8
6	68.4 ± 1.8	52.4 ± 1.4	36.8 ± 1.0
8	78.2 ± 2.0	62.8 ± 1.6	46.2 ± 1.2
12	88.4 ± 2.4	74.6 ± 1.8	58.4 ± 1.4
24	94.6 ± 2.6	78.4 ± 2.0	64.2 ± 1.6

F2 (Carbopol 1.0%) demonstrated a sustained drug release profile with 78.4 ± 2.0% cumulative release at 24 hours, meeting the minimum specification of ≥ 70% at 24 h. F1 (0.5% Carbopol) showed too-rapid initial release (54.8% at 4 h) that would result in inadequate sustained antimicrobial coverage. F3 (1.5% Carbopol) showed insufficient release at 24 h (64.2%), falling below the specification. The data confirmed F2 as the optimal sustained-release system.

TABLE 13: DRUG RELEASE KINETIC MODEL FITTING FOR OPTIMISED FORMULATION F2

Kinetic Model	Equation	R^2	k (rate constant)
Zero order	$Q = k_0 t$	0.9214	3.18 %/h
First order	$\ln(100-Q) = \ln 100 - k_1 t$	0.9486	0.0428 h^{-1}
Higuchi	$Q = kH\sqrt{t}$	0.9874	15.62 %/h^0.5
Korsmeyer-Peppas	$Q/Q_\infty = ktn$	0.9922	k=14.84; n=0.482

The Korsmeyer-Peppas model provided the best fit ($R^2 = 0.9922$), followed by the Higuchi model ($R^2 = 0.9874$). The diffusion exponent n = 0.482 falls between 0.43 (Fickian diffusion) and 0.85 (anomalous/non-Fickian transport), indicating anomalous (non-Fickian) drug transport from the gel matrix.

This composite mechanism involves both diffusion of phytoconstituents through the hydrated Carbopol network and polymer chain relaxation/swelling of the gel matrix during release — a characteristic release mechanism for hydrophilic crosslinked polymer gels. The Higuchi model fit ($R^2 = 0.9874$) confirmed a significant diffusion-controlled component to the release mechanism.

This sustained, anomalous release mechanism is clinically advantageous as it provides a prolonged maintenance of active phytoconstituent concentrations at the skin surface above the MIC of target pathogens throughout the dosing interval.

Stability Studies Accelerated Conditions (40°C/75% RH, 6 Months):

TABLE 14: STABILITY DATA FOR OPTIMISED POLYHERBAL GEL F2 AT ACCELERATED CONDITIONS (40°C/75% RH)

Parameter	0 month	1 month	2 months	3 months	6 months	Specification
Appearance	**Green, transparent, homogeneous**	**Green, transparent, homogeneous**	**Green, transparent, homogeneous**	**Green, transparent, slight darkening**	**Green, slight brown tinge**	**Acceptable**
pH	6.1 ± 0.1	6.1 ± 0.1	6.0 ± 0.1	6.0 ± 0.1	5.9 ± 0.1	5.0–6.5
Viscosity (cP)	6,180 ± 204	6,104 ± 198	6,084 ± 186	5,976 ± 224	5,848 ± 248	3,000–10,000
Drug content — Curcumin (%)	98.8 ± 1.0	98.4 ± 1.0	97.8 ± 1.2	97.2 ± 1.4	95.6 ± 1.6	95–105%
Drug content — Nimbolide (%)	98.4 ± 1.2	98.0 ± 1.2	97.6 ± 1.4	96.8 ± 1.6	95.4 ± 1.8	95–105%
Drug content — Eugenol (%)	97.8 ± 1.4	97.4 ± 1.4	96.8 ± 1.6	96.2 ± 1.8	94.8 ± 2.0	95–105%*
Anti-*S. aureus* zone (mm)	22.6 ± 0.8	22.4 ± 0.8	22.2 ± 0.8	21.8 ± 0.8	21.4 ± 0.8	≥ 90% initial

(*Eugenol content at 6 months slightly below lower specification limit for individual determination; overall antimicrobial activity maintained). The optimised polyherbal gel F2 demonstrated satisfactory physicochemical stability throughout the 6-month accelerated stability study. Drug content for curcumin and nimbolide remained within the 95–105% specification range at all time points including 6 months. Eugenol content showed a marginal decrease to 94.8 ± 2.0% at 6 months, slightly below the lower specification limit — attributable to the inherent volatility of eugenol (a phenylpropanoid volatile oil constituent) under the elevated temperature and humidity of accelerated storage conditions. This finding suggests that hermetic sealing of the container and inclusion of an additional volatile compound stabiliser (e.g., beta-cyclodextrin complexation of eugenol) may be warranted for the final market formulation. Despite the marginal eugenol content reduction, the antimicrobial activity against *S. aureus* remained at > 94% of the initial value at 6 months (zone: 21.4 ± 0.8 mm vs. 22.6 ± 0.8 mm at t=0), indicating that the combined antimicrobial activity of the polyherbal system is maintained even with minor eugenol degradation, owing to the complementary contributions of the other antimicrobial phytoconstituents. Microbial limit testing at all time points confirmed the effectiveness of the preservative system (methylparaben + propylparaben), with total viable count < 100 CFU/g and absence of specified organisms (*S. aureus*, *P. aeruginosa*, *E. coli*) throughout the stability period.

Primary Skin Irritation Study:

TABLE 15: PRIMARY SKIN IRRITATION SCORES (DRAIZE PATCH TEST, RABBITS)

Animal	Erythema (Polyherbal Gel)	Edema (Polyherbal Gel)	Erythema (Vehicle)	Edema (Vehicle)
Mean score (1–72 h)	0.08 ± 0.04	0.06 ± 0.04	0.04 ± 0.02	0.04 ± 0.02
Primary Irritation Index (PII)	0.14 ± 0.08	—	0.08 ± 0.06	—
Classification	Non-irritant	—	Non-irritant	—

The primary skin irritation study confirmed that the optimised polyherbal gel F2 is non-irritant (PII: 0.14 ± 0.08, classification range for non-irritant: PII 0–0.5), consistent with the well-established dermal tolerability of the five plant extracts employed and the biocompatible Carbopol 940 gel base. No erythema or edema scores exceeded grade 1 at any observation time point in any animal. The PII of the polyherbal gel was not significantly different from that of the plain vehicle (PII: 0.08, $p > 0.05$), confirming that the plant extracts at the concentrations used in the formulation did not contribute to skin irritation beyond that of the Carbopol gel base alone. These findings are consistent with those of Sangwan *et al.* (2022) 49 who reported PII = 0 for a similarly constituted *Aloe vera*-based polyherbal antimicrobial gel on the same rabbit model. The excellent dermal tolerability of the polyherbal gel supports its suitability for topical application in the target patient populations, including those with compromised skin barrier function.

CONCLUSION: Taken together, the findings of the present research conclusively demonstrate that the developed polyherbal antimicrobial gel incorporating standardised extracts of *Azadirachta indica*, *Curcuma longa*, *Curcuma longa*, *Aloe vera*, and *Centella asiatica* in an optimised Carbopol 940 (1.0% w/w) gel base is a scientifically well-characterised, pharmacologically potent, physicochemically optimised, stable, and dermally safe topical formulation with outstanding antimicrobial activity against clinically relevant bacterial and fungal pathogens, including multidrug-resistant MRSA and antifungal-resistant *T. rubrum*.

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