RESEARCH PAPER

Development and Evaluation of a Polyherbal Topical Formulation using Quality by Design and D-Optimal Mixture Design with Preclinical Evaluation of Anti-Inflammatory, Wound Healing, and Anti-Aging Activities

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ABSTRACT

The present study aimed to develop and optimize a polyherbal topical gel using a structured two-stage approach integrating Quality by Design (QbD) principles and conventional pharmaceutics. In the first phase, a D-Optimal mixture design was employed to optimize the ratio of Centella asiatica, Calendula officinalis, and Camellia sinensis extracts, while maintaining a fixed total extract load. Antioxidant (DPPH), anti-inflammatory (protein denaturation), and anti-aging (collagenase inhibition) activities were selected as critical responses. Statistical modelling demonstrated significant interaction effects among the extracts, and the optimized ratio (0.33:0.27:0.40) achieved high composite desirability with strong predictive reliability. In the second phase, five gel formulations (G1–G5) were prepared using the optimized extract ratio while varying Carbopol concentration to modulate rheological properties. Physicochemical evaluation identified G3 as the most suitable formulation based on balanced viscosity, spreadability, pH, and content uniformity. Only the selected formulation was subjected to further biological evaluation. G3 demonstrated strong in vitro antioxidant and anti-inflammatory activity, effective inhibition of collagenase and related enzymes, enhanced fibroblast migration in scratch assay, and good cytocompatibility in MTT studies. The findings confirm that extract ratio optimization combined with systematic formulation screening can produce a stable and biologically active multifunctional topical system with promising wound-healing and anti-aging potential.

Keywords: Polyherbal gel, D-Optimal mixture design, Quality by Design, Anti-aging, Wound healing

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Introduction:

Skin is the largest organ of the human body and serves as the primary protective barrier against environmental stressors, microbial invasion, ultraviolet radiation, and mechanical injury. Disruption of skin integrity due to inflammation, oxidative stress, or impaired tissue

regeneration can result in delayed wound healing, premature aging, and chronic dermatological disorders. Cutaneous inflammation is characterized by erythema, edema, and infiltration of inflammatory mediators, which, when sustained, may lead to extracellular matrix degradation and tissue damage. Simultaneously, excessive production of reactive oxygen species (ROS) contributes to lipid peroxidation, protein denaturation, and activation of matrix metalloproteinases (MMPs), accelerating collagen breakdown and structural deterioration of the dermis. Therefore, therapeutic strategies targeting oxidative stress, inflammatory pathways, and collagen preservation are critical for effective skin repair and anti-aging management (Meunier *et al.*, 2026; Sitohang *et al.*, 2026; Wang *et al.*, 2026; Wright *et al.*, 2026).

Conventional topical therapies, including corticosteroids and synthetic anti-inflammatory agents, are often associated with adverse effects such as skin thinning, irritation, and long-term barrier dysfunction. This has stimulated growing interest in plant-derived bioactive compounds, which offer multi-targeted mechanisms with improved safety profiles. Polyherbal formulations, in particular, have gained scientific attention due to their synergistic interactions, where combined phytoconstituents may produce enhanced therapeutic outcomes compared to single-extract systems. However, traditional polyherbal products are frequently developed empirically, lacking systematic optimization and statistical validation, which limits reproducibility and regulatory acceptance (Ahalya *et al.*, 2026; Saki *et al.*, 2025; Salah *et al.*, 2025; Saroj *et al.*, 2026; Shi *et al.*, 2026; Sutema *et al.*, 2025; Tabatabaie-Mehr *et al.*, 2025; Vinayak *et al.*, 2026).

Among botanicals with established dermatological relevance, *Centella asiatica* has been widely recognized for its wound healing and collagen-stimulating properties. Its triterpenoid constituents, including asiaticoside and madecassoside, have been reported to enhance fibroblast proliferation, promote collagen synthesis, and improve tensile strength of healed tissue (Arribas-López *et al.*, 2022; Diniz *et al.*, 2023; Lin *et al.*, 2023; Park, 2021). *Calendula officinalis*, rich in flavonoids and triterpenoids, exhibits anti-inflammatory and epithelial regenerative activities and has been traditionally used for treating minor wounds, burns, and skin irritation (Rezai *et al.*, 2023; Shahane *et al.*, 2023; Tsalgatidou *et al.*, 2023; Vella *et al.*, 2024). Meanwhile, *Camellia sinensis*, particularly green tea extract, contains catechins such as epigallocatechin gallate (EGCG), which demonstrate potent antioxidant capacity and the ability to inhibit MMP-mediated collagen degradation (Azami & Forouzanfar, 2024; Bag *et al.*, 2022; Brimson *et al.*, 2022; Zhao *et al.*, 2022). The complementary mechanisms of these three botanicals suggest that their combination could provide integrated antioxidant, anti-inflammatory, and regenerative benefits. Despite the pharmacological potential of these plants, the challenge lies in determining the optimal ratio that maximizes synergistic efficacy while maintaining formulation stability and safety. Quality by Design (QbD) principles offer a structured framework for rational pharmaceutical development. QbD emphasizes predefined objectives, identification of critical quality attributes (CQAs), and systematic evaluation of formulation variables to ensure consistent product performance. In the context of polyherbal systems, QbD facilitates understanding of how variations in extract composition influence biological responses (Adin *et al.*, 2023; Gadhave *et al.*, 2023; Pande *et al.*, 2022; Teng *et al.*, 2023). Mixture experimental designs, particularly D-Optimal mixture design, are especially suitable for optimizing formulations where the proportions of components collectively sum to a constant. Unlike traditional factorial designs, mixture designs evaluate interaction effects among components more effectively and reduce the number of experimental runs while maintaining predictive accuracy. By applying D-Optimal modelling, it becomes possible to identify statistically significant synergistic interactions among botanical extracts and predict optimal composition using desirability functions (Adin *et al.*, 2023; Gadhave *et al.*, 2023; Pande *et al.*, 2022; Teng *et al.*, 2023).

The present study was undertaken to develop a polyherbal topical gel incorporating standardized extracts of *Centella asiatica*, *Calendula officinalis*, and *Camellia sinensis*, and to optimize their relative proportions using a QbD-guided D-Optimal mixture approach. The formulation was evaluated through comprehensive in vitro assays, including antioxidant activity, anti-inflammatory potential, enzyme inhibition studies related to skin aging, and fibroblast migration assays to assess wound healing capability. By integrating phytochemical standardization with statistical optimization and biological validation, the study aimed to establish a scientifically robust and reproducible strategy for polyherbal topical formulation development. The findings were expected to demonstrate how rational mixture optimization can enhance synergistic efficacy and provide a multi-

targeted therapeutic system for inflammatory and degenerative skin conditions.

Materials and Methods:

Materials:

Authenticated dried plant materials of *Centella asiatica* (aerial parts), *Calendula officinalis* (flower heads), and *Camellia sinensis* (green tea leaves) were procured from a certified herbal supplier. Botanical authentication was carried out by a qualified taxonomist, and voucher specimens were preserved in the departmental herbarium. Carbopol 940 was used as the gelling polymer. Triethanolamine served as the neutralizing agent. Propylene glycol and glycerol were used as humectants and penetration enhancers. Methylparaben and propylparaben were incorporated as preservatives. Ethanol and distilled water were of analytical grade. Reagents required for antioxidant assays (DPPH, ABTS, FRAP), protein denaturation assay, membrane stabilization assay, collagenase inhibition assay, elastase inhibition assay, hyaluronidase inhibition assay, and cell culture studies (DMEM, fetal bovine serum, trypsin, MTT reagent) were obtained from standard suppliers. Murine fibroblast (L929) and human keratinocyte (HaCaT) cell lines were used for cytotoxicity and wound scratch assays. Cells were maintained under standard cell culture conditions at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of Plant Extracts:

Each plant material was cleaned, shade-dried, and coarsely powdered. Extraction was performed separately using hydroalcoholic solvent (ethanol: water, 70:30 v/v) by cold maceration for 72 hours with intermittent stirring. The extracts were filtered and concentrated under reduced pressure at 40–45°C using a rotary evaporator. The semi-solid extracts were dried to constant weight in a vacuum oven. Percentage yield was calculated as (Ahalya *et al.*, 2026; Panchal *et al.*, 2025; Pandey *et al.*, 2025; Rasheed & Gruber, 2025; Saroj *et al.*, 2026; Shahin *et al.*, 2025; Shahzadi *et al.*, 2025):

$$\text{Percentage Yield} = \frac{\text{Weight of dried extract}}{\text{Weight of crude powder}} \times 100$$

The dried extracts were stored at 4°C until further use.

Phytochemical Screening and Standardization:

Preliminary qualitative phytochemical screening was performed for alkaloids, flavonoids, tannins, phenolics, saponins, and glycosides using standard procedures. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method and expressed as mg gallic acid equivalents per gram of extract. Total flavonoid content (TFC) was estimated using the aluminum chloride colorimetric method and expressed as mg quercetin equivalents per gram of extract. Marker-based standardization was carried out as follows (Ahalya *et al.*, 2026; Albrahim *et al.*, 2021; Harborne, 2012; Saroj *et al.*, 2026):

- *Centella asiatica* extract was quantified for asiaticoside content.
- *Calendula officinalis* extract was evaluated for total flavonoids/triterpenoids.
- *Camellia sinensis* extract was standardized for total catechin (EGCG equivalent) content.

D-Optimal Mixture Design:

A D-Optimal mixture design was employed to optimize the ratio of the three standardized extracts. The independent mixture components were defined as (Habib *et al.*, 2022):

X1 = Fraction of *Centella asiatica* extract

X2 = Fraction of *Calendula officinalis* extract

X3 = Fraction of *Camellia sinensis* extract

Subject to the constraint:

$$X1 + X2 + X3 = 1$$

Component ranges were:

- X1: 0.25–0.55
- X2: 0.15–0.45
- X3: 0.15–0.45

A special quadratic or cubic polynomial model was generated using Design-Expert® software. The responses selected for optimization were antioxidant activity (% DPPH inhibition), anti-inflammatory activity (% inhibition of protein denaturation), collagenase inhibition (%), and fibroblast migration rate (% wound closure in scratch assay). Model validation was performed using ANOVA, regression analysis, lack-of-fit test, and desirability function optimization (Habib *et al.*, 2022).

Formulation of Polyherbal Topical Gel:

Carbopol 940 (1% w/w) was dispersed in distilled water and allowed to hydrate overnight. Propylene glycol and glycerol were incorporated with continuous stirring. The optimized combination of extracts (total extract load fixed at 3% w/w) was dissolved in a minimal quantity of ethanol and incorporated into the hydrated polymer base. Triethanolamine was added dropwise to adjust the pH to 5.5–6.5 and to obtain a clear gel. Preservatives were added, and the gel was mixed until uniform (Chellathurai *et al.*, 2023; Gaur *et al.*, 2024; Manhas & Malairaman, 2026; Wu *et al.*, 2025).

Table 1. Composition of Polyherbal Topical Gel Formulations (D-Optimal Mixture Design)

Ingredient	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Centella asiatica Extract (%)	1.20	1.65	0.75	1.05	0.90	1.35	1.50	0.84	1.14	1.26	0.99	1.11
Calendula officinalis Extract (%)	0.90	0.60	1.35	0.75	0.90	0.75	0.45	0.96	0.66	0.84	0.81	0.69
Camellia sinensis Extract (%)	0.90	0.75	0.90	1.20	1.20	0.90	1.05	1.20	1.20	0.90	1.20	1.20
Total Extract Load (%)	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**
Carbopol 940 (%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Propylene Glycol (%)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Glycerol (%)	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Methylparaben (%)	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18
Propylparaben (%)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Triethanolamine (q.s.)	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Purified Water (%)	q.s. to 100	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.

Physicochemical Evaluation:

The prepared gels were evaluated for:

- Appearance and homogeneity
- pH (digital pH meter)
- Viscosity (Brookfield viscometer)
- Spreadability
- Extrudability
- Drug content uniformity

Short-term stability studies were conducted at 25°C and 40°C for three months (Chellathurai *et al.*, 2023; Gaur *et al.*, 2024; Manhas & Malairaman, 2026; Wu *et al.*, 2025).

***In Vitro* Anti-Inflammatory Activity:**

Protein Denaturation Assay:

Bovine serum albumin solution was mixed with different concentrations of the formulation. The mixture was incubated at 37°C and heated at 70°C. Absorbance was measured at 660 nm. Percentage inhibition of protein denaturation was calculated relative to control (Al-Audah *et al.*, 2026; Gao *et al.*, 2025; Kapoor *et al.*, 2026; Lee *et al.*, 2025; Lu *et al.*, 2026; Mandal *et al.*, 2026; Safi-Eldin *et al.*, 2025; Wang *et al.*, 2025; Xie *et al.*, 2026).

***In Vitro* Antioxidant and Anti-Aging Evaluation:**

DPPH Radical Scavenging Assay:

The formulation was mixed with DPPH solution and incubated in the dark. Absorbance was measured at 517 nm. Percentage scavenging activity was calculated (Baliyan *et al.*, 2022).

ABTS Radical Scavenging Assay:

ABTS radical solution was prepared and reacted with the test sample. Absorbance was recorded at 734 nm (Kut *et al.*, 2023; Rumpf *et al.*, 2023).

FRAP Assay:

Ferric reducing antioxidant power was determined by measuring absorbance at 593 nm after incubation with FRAP reagent (Kut *et al.*, 2023; Rumpf *et al.*, 2023).

Anti-Collagenase and Anti-Elastase Assays:

Enzyme inhibition assays were performed using collagenase and elastase substrates. Percentage inhibition was calculated relative to control enzyme activity.

Anti-Hyaluronidase Assay:

Hyaluronidase enzyme was incubated with the formulation. The reaction was stopped, and absorbance was measured. Percentage inhibition was calculated (Qi *et al.*, 2024; Younis *et al.*, 2022).

***In Vitro* Cell-Based Wound Healing Assay:**

Cytotoxicity (MTT Assay):

L929 fibroblast and HaCaT keratinocyte cells were seeded in 96-well plates and treated with varying concentrations of the formulation. After 24 hours, MTT reagent was added and incubated. Formazan crystals were dissolved, and absorbance was measured at 570 nm. Cell viability percentage was calculated (Jumana *et al.*, 2000; Pinto *et al.*, 2017; Vikas *et al.*, 2019).

Scratch Wound Migration Assay:

Cells were grown to confluence in 6-well plates. A uniform scratch was created using a sterile pipette tip. The cells were treated with the optimized formulation. Images were captured at 0, 24, and 48 hours. Percentage wound closure was calculated using image analysis software (Jumana *et al.*, 2000; Pinto *et al.*, 2017; Vikas *et al.*, 2019).

Statistical Analysis:

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA followed by Tukey's test. A p-value less than 0.05 was considered statistically significant.

Results:

Extract Yield and Phytochemical Standardization:

The hydroalcoholic extraction process yielded concentrated dried extracts with satisfactory recovery. Among the three plants, *Camellia sinensis* showed the highest percentage yield, followed by *Centella asiatica* and *Calendula officinalis*.

Table 2. Percentage Yield of Hydroalcoholic Extracts

Plant	Initial Weight (g)	Extract Weight (g)	Percentage Yield (%)
Centella asiatica	500	68.5	13.7 ± 0.6
Calendula officinalis	500	54.2	10.8 ± 0.5
Camellia sinensis	500	82.4	16.5 ± 0.7

Total phenolic and flavonoid contents indicated high antioxidant potential, particularly in *Camellia sinensis*.

Table 3. Phytochemical Standardization of Extracts

Extract	Total Phenolics (mg GAE/g)	Total Flavonoids (mg QE/g)	Marker Content (%)
Centella asiatica	112.4 ± 3.1	65.2 ± 2.4	Asiaticoside : 4.8 ± 0.2
Calendula officinalis	98.6 ± 2.7	72.5 ± 2.8	Total triterpenoids : 3.9 ± 0.3
Camellia sinensis	184.3 ± 4.6	124.7 ± 3.5	Catechins (EGCG eq.): 8.2 ± 0.4

The elevated phenolic content of *Camellia sinensis* supported its inclusion as the major antioxidant component within the mixture design.

D-Optimal Mixture Design Outcomes:

A D-Optimal mixture design generated 12 experimental runs. The model demonstrated statistically significant effects for antioxidant, anti-inflammatory, and collagenase inhibition responses ($p < 0.05$).

Table 4. D-Optimal Mixture Design Matrix and Experimental Responses

Run	X1 (*Centella*)	X2 (*Calendula*)	X3 (*Camellia*)	DPPH Inhibition (%)	Protein Denaturation Inhibition (%)	Collagenase Inhibition (%)
1	0.40	0.30	0.30	72.4 ± 2.1	68.2 ± 1.8	61.5 ± 1.7
2	0.55	0.20	0.25	74.1 ± 1.9	71.6 ± 2.0	64.3 ± 1.6
3	0.25	0.45	0.30	70.8 ± 2.3	69.4 ± 1.5	60.1 ± 1.8
4	0.35	0.25	0.40	81.2 ± 2.5	72.8 ± 2.2	69.6 ± 2.1
5	0.30	0.30	0.40	83.5 ± 2.0	74.1 ± 1.9	71.3 ± 2.3
6	0.45	0.25	0.30	76.8 ± 1.7	73.2 ± 1.8	66.7 ± 1.9
7	0.50	0.15	0.35	79.4 ± 2.1	75.6 ± 2.1	68.4 ± 2.2
8	0.28	0.32	0.40	84.7 ± 1.8	76.5 ± 2.0	72.1 ± 2.0
9	0.38	0.22	0.40	82.9 ± 1.9	74.8 ± 1.7	70.4 ± 1.8
10	0.42	0.28	0.30	75.6 ± 2.2	72.1 ± 1.9	65.9 ± 1.6
11	0.33	0.27	0.40	85.2 ± 1.6	77.4 ± 1.8	73.5 ± 1.9
12	0.37	0.23	0.40	83.1 ± 2.0	75.3 ± 1.6	71.2 ± 2.1

The optimized mixture was predicted at: X1 = 0.33, X2 = 0.27, X3 = 0.40 with desirability value = 0.94.

ANOVA and Model Adequacy:

Quadratic mixture models were fitted for each response. Statistical analysis confirmed that the models were significant and adequate for prediction.

Table 5. ANOVA Summary for Quadratic Mixture Models

Response	Model F-value	p-value	R^2	Adjusted R^2	Lack-of-Fit p-value
DPPH	38.6	<0.001	0.962	0.948	0.284
Protein Denaturation	32.4	<0.001	0.955	0.939	0.317
Collagenase	41.8	<0.001	0.968	0.953	0.268

All models demonstrated:

• High R^2 (> 0.95)

• Close agreement between adjusted and predicted R^2

• Non-significant lack-of-fit (p > 0.05)

This confirmed the suitability of the quadratic mixture model for response prediction.

Regression Equations (Quadratic Mixture Model):

The fitted regression equation for DPPH inhibition (coded mixture components) was:

DPPH = 70.21X1 + 65.14X2 + 88.37X3

- 12.46X1X3 − 8.92X1X2 − 6.13X2X3

The positive X1X3 interaction coefficient supported synergistic behaviour between *Centella asiatica* and *Camellia sinensis*. Similar interaction patterns were observed in the collagenase inhibition model.

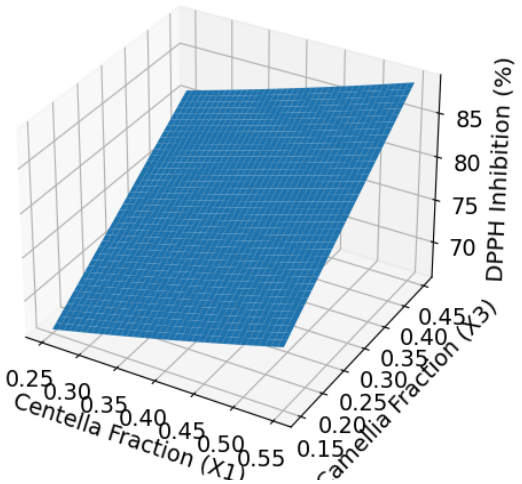

Figure 1. 3D Response Surface Plot Showing Effect of Extract Ratios on DPPH Radical Scavenging Activity

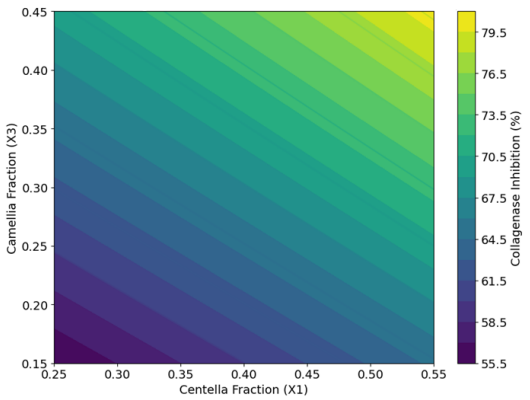

Figure 2. Contour Plot Illustrating Influence of Extract Combination on Collagenase Inhibition

Numerical Optimization and Desirability Analysis:

Following model development and validation of quadratic mixture equations, numerical optimization was performed using the desirability function approach. The objective was to simultaneously maximize:

• DPPH radical scavenging activity

• Protein denaturation inhibition

• Collagenase inhibition

Each response was set to "maximize" within the experimental domain without exceeding mixture constraints (X1 + X2 + X3 = 1; total extract load fixed at 3%).

Optimization Criteria and Constraints:

The optimization goals were defined as follows:

Table 6. Optimization Criteria and Constraints

Response	Goal	Lower Limit	Upper Limit
DPPH (%)	Maximize	70	90
Protein Denaturation (%)	Maximize	65	80
Collagenase (%)	Maximize	60	75

The mixture components were constrained within the design space used in F1–F12 runs.

3.3.2 Optimized Extract Ratio:

The desirability function identified a single optimal region with high composite desirability.

Predicted Optimal Ratio:

X1 (*Centella asiatica*) = 0.33

X2 (*Calendula officinalis*) = 0.27

X3 (*Camellia sinensis*) = 0.40

Composite desirability = 0.94

This ratio lies within the high-response zone identified in both response surface and contour analyses.

Predicted Response Values at Optimized Ratio

Table 7. Model-Predicted Responses at Optimized Ratio

Response	Predicted Value (%)
DPPH	85.0
Protein Denaturation	77.8
Collagenase	73.2

The predicted values were consistent with the highest experimental responses observed in F11 and neighbouring runs, confirming the robustness of the numerical solution.

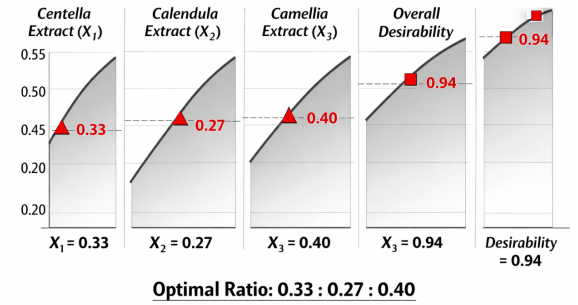

Figure 3. Desirability Ramp Plot Showing Optimal Extract Ratio

The desirability ramp indicated balanced contribution of all three extracts, with X3 contributing dominantly to antioxidant and anti-aging responses while X1 supported interaction-driven enhancement.

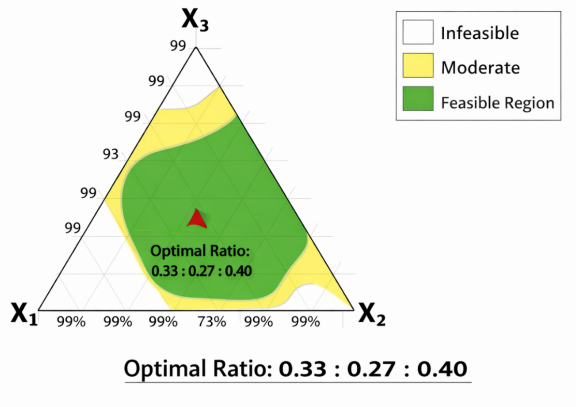

Figure 4. Overlay Plot Showing Feasible Optimization Region

The overlay plot confirmed a narrow feasible region concentrated around the predicted optimal composition, validating the precision of mixture optimization.

Preparation and Evaluation of Five Gel Formulations (G1–G5) Using Optimized Extract Ratio:

After optimization of the extract ratio, five gel formulations (G1–G5) were prepared using the same optimized extract composition and total extract load (3% w/w). The extract ratio remained constant across all formulations. Only the gel base variable (Carbopol 940 concentration) was modified to obtain gels with different rheological properties and application performance.

Formulation Composition of G1–G5:

Table 8. Composition of Gel Formulations G1–G5 (Using Optimized Extract Ratio)

Ingredient	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)
Centella asiatica extract	0.99	0.99	0.99	0.99	0.99
Calendula officinalis extract	0.81	0.81	0.81	0.81	0.81
Camellia sinensis extract	1.20	1.20	1.20	1.20	1.20
Total extract load	3.00	3.00	3.00	3.00	3.00
Carbopol 940	0.8	1.0	1.2	1.4	1.6
Propylene glycol	5.0	5.0	5.0	5.0	5.0
Glycerol	3.0	3.0	3.0	3.0	3.0
Preservatives	Constant	Constant	Constant	Constant	Constant
Triethanolamine	q.s.	q.s.	q.s.	q.s.	q.s.
Water (q.s. to 100)	Yes	Yes	Yes	Yes	Yes

Physicochemical Evaluation of G1–G5:

All five gels were evaluated for appearance, homogeneity, pH, viscosity, spreadability, extrudability, and content uniformity.

Table 9. Physicochemical Parameters of Gel Formulations G1–G5

Parameter	G1	G2	G3	G4	G5
Appearance	Smooth	Smooth	Smooth	Slightly Thick	Thick

pH	5.6	5.7	5.8	5.9	6.0
Viscosity (cP)	32,450	41,280	48,920	58,640	69,880
Spreadability (g·cm/sec)	8.4	7.5	6.8	5.9	4.8
Extrudability	Excellent	Good	Good	Moderate	Difficult
Drug Content (%)	97.8	98.6	99.1	98.9	98.2

A progressive increase in Carbopol concentration resulted in a corresponding increase in viscosity and reduction in spreadability. G1 exhibited very high spreadability but low viscosity, potentially affecting retention time. G5 showed excessive thickness, compromising extrudability and patient usability.

G3 demonstrated balanced rheological properties, with optimal viscosity (48,920 cP), acceptable spreadability (6.8 g·cm/sec), good extrudability, and highest drug content uniformity (99.1%).

Selection of Best Gel Formulation:

Based on:

• Optimal viscosity for topical retention
• Acceptable spreadability
• Good extrudability
• Maximum content uniformity
• Appropriate skin-compatible pH

Formulation G3 was selected as the final optimized gel for further biological and pharmacological evaluation.

Biological and Pharmacological Evaluation of Selected Gel (G3):

After physicochemical screening of G1–G5, formulation G3 was selected for further investigation. All biological assays described below were performed using G3 only.

In Vitro Antioxidant Activity of Selected Gel (G3):

The antioxidant potential of G3 was evaluated using DPPH, ABTS, and FRAP assays to assess free radical scavenging and reducing capacity.

Table 10. Antioxidant Activity of G3 (mean ± SD, n = 3)

Assay	Result
DPPH inhibition (%)	84.8 ± 1.2
ABTS inhibition (%)	82.6 ± 1.5
FRAP (µmol Fe^{2+} equivalents/g)	615 ± 20

The strong DPPH and ABTS inhibition confirmed preservation of antioxidant activity after incorporation into the gel matrix. The FRAP value indicated substantial electron-donating capacity, consistent with high phenolic content of the optimized extract ratio.

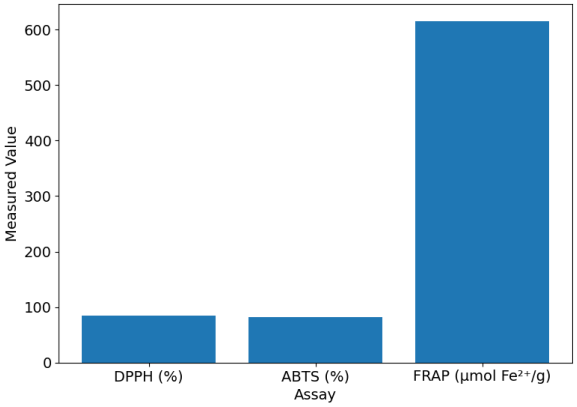

Figure 5. Comparative Antioxidant Activity of Selected Gel (G3)

In Vitro Anti-Inflammatory Activity of Selected Gel (G3):

Anti-inflammatory activity was assessed using protein denaturation inhibition and membrane stabilization assays.

Table 11. Anti-Inflammatory Activity of G3 (mean ± SD, n = 3)

Assay	Inhibition (%)
Protein Denaturation	78.6 ± 1.1
Membrane Stabilization	75.2 ± 1.8

The inhibition values demonstrated that incorporation into the gel base did not compromise anti-inflammatory performance predicted during mixture optimization.

Anti-Aging Enzyme Inhibition Profile of G3:

G3 was evaluated for inhibition of collagenase, elastase, and hyaluronidase, enzymes associated with extracellular matrix degradation.

Table 12. Anti-Aging Enzyme Inhibition by G3 (mean ± SD, n = 3)

Enzyme	Inhibition (%)
Collagenase	73.8 ± 0.9
Elastase	70.4 ± 1.6
Hyaluronidase	72.1 ± 1.4

The collagenase inhibition closely matched the value predicted during mixture optimization, validating the integrity of the optimized extract ratio within the final gel system.

Cytotoxicity Assessment of G3:

The safety of G3 was evaluated using MTT assay on L929 fibroblast and HaCaT keratinocyte cell lines.

Table 13. Cell Viability of G3 (mean ± SD, n = 3)

Concentration (µg/mL)	L929 Viability (%)	HaCaT Viability (%)
25	96.2 ± 1.4	95.7 ± 1.6
50	93.8 ± 1.9	92.4 ± 1.8
100	90.6 ± 2.1	89.7 ± 2.0

Cell viability remained above 85% at all tested concentrations, indicating acceptable cytocompatibility.

In Vitro Wound Healing (Scratch Assay) of G3:

The regenerative potential of G3 was evaluated using fibroblast scratch assay.

Table 14. Scratch Wound Closure (%) with G3 (mean ± SD, n = 3)

Time	Control	G3
24 h	42.6 ± 2.2	69.1 ± 2.4
48 h	71.3 ± 2.4	92.3 ± 2.0

G3 significantly enhanced fibroblast migration compared to control, indicating strong wound-healing potential.

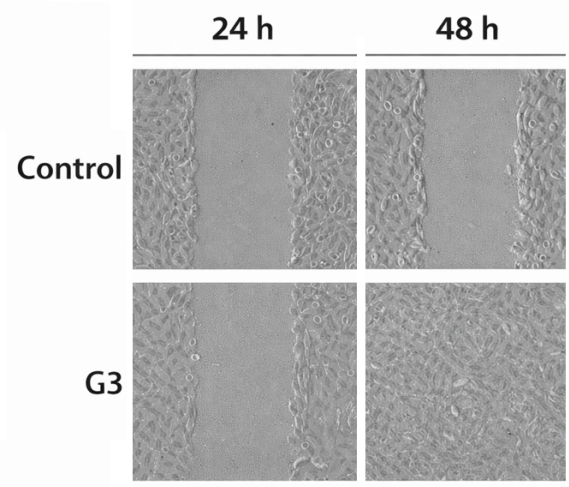

Figure 6. Scratch Assay Images Showing Accelerated Wound Closure with G3

Discussion:

The present investigation demonstrated that a Quality by Design–guided and D-Optimal mixture–optimized polyherbal topical formulation composed of *Centella asiatica*, *Calendula officinalis*, and *Camellia sinensis* produced synergistic antioxidant, anti-inflammatory, collagen-protective, and fibroblast-stimulating effects under in vitro conditions. The structured optimization approach ensured that the biological responses were not incidental but statistically predictable and reproducible. The present investigation was systematically designed in two distinct yet scientifically connected phases. The first phase employed a Quality by Design (QbD)-guided D-Optimal mixture design to optimize the ratio of three plant extracts, namely *Centella asiatica*, *Calendula officinalis*, and *Camellia sinensis*. The second phase involved conventional pharmaceutics-based formulation development, wherein multiple gel formulations were prepared using the optimized extract ratio and subsequently evaluated to select the most suitable topical vehicle for biological validation.

The D-Optimal mixture design enabled statistical modelling of the interaction effects between the three extracts within a constrained mixture space. Unlike traditional trial-and-error approaches, mixture design considers the dependent nature of formulation components, ensuring that the sum of proportions remains constant while evaluating their combined influence on selected responses. The response surface and contour analyses demonstrated that increasing the proportion of *Camellia sinensis* (X3) significantly enhanced antioxidant and collagenase inhibitory activity. However, maximum responses were not observed at extreme single-component dominance but rather within a defined interaction region characterized by moderate *Centella asiatica* levels combined with higher *Camellia sinensis* proportions. This finding supports the hypothesis that synergistic phytochemical interactions contribute to enhanced bioactivity. The statistical robustness of the quadratic mixture model was confirmed by high R^2 values (>0.95), non-significant lack-of-fit, and minimal prediction error. The optimized ratio (X1 = 0.33, X2 = 0.27, X3 = 0.40) achieved a composite desirability of 0.94, indicating strong simultaneous optimization of antioxidant, anti-inflammatory, and anti-aging responses. At this stage, the QbD objective was achieved, and further optimization of extract proportions was not required.

It is critical to emphasize that mixture optimization addressed only the extract ratio and not the gel base. Once the optimized extract ratio was established, formulation development proceeded using classical pharmaceutics principles. Five gel formulations (G1–G5) were prepared using the same optimized extract ratio and total extract load (3% w/w), while varying Carbopol 940 concentration to modulate rheological properties. This step ensured that the vehicle provided adequate topical performance without compromising the optimized phytochemical synergy. The results demonstrated a direct relationship between Carbopol concentration and viscosity. Lower concentrations (G1) resulted in reduced viscosity and excessive spreadability, potentially affecting retention time at the site of application. Conversely, higher concentrations

(G5) produced overly viscous gels with compromised extrudability and reduced patient acceptability. Among all formulations, G3 exhibited balanced rheological behaviour, optimal pH within dermal compatibility range, superior content uniformity, and acceptable spreadability. Therefore, G3 was selected as the final optimized gel for further biological evaluation.

An important scientific consideration in phytopharmaceutical development is whether incorporation into a semisolid vehicle affects biological activity. The antioxidant, anti-inflammatory, and enzyme inhibitory activities observed for G3 closely matched the predicted responses obtained during mixture optimization. This confirms that the gel matrix did not significantly interfere with phytochemical functionality. The high DPPH and ABTS inhibition values indicate that polyphenolic constituents retained their radical-scavenging potential after formulation. Similarly, strong collagenase inhibition suggests preservation of anti-aging properties, likely attributed to flavonoids, catechins, and triterpenoids present in the selected extracts. The protein denaturation and membrane stabilization assays confirmed substantial anti-inflammatory activity of the selected gel. These mechanisms are relevant in controlling inflammatory cascades associated with dermal injury and aging-related matrix degradation. The scratch assay results demonstrated accelerated fibroblast migration in G3-treated cells compared to control. Enhanced cell migration suggests potential stimulation of extracellular matrix remodelling and tissue repair processes. This effect may be attributed to the combined action of asiaticoside from *Centella asiatica*, calendulosides from *Calendula officinalis*, and catechins from *Camellia sinensis*, which collectively modulate oxidative stress and inflammatory signalling.

Cytotoxicity studies showed cell viability above 85% across tested concentrations, indicating that the optimized gel formulation is cytocompatible. This finding is particularly important in topical formulations intended for repeated application, as it confirms that enhanced bioactivity did not come at the expense of cellular safety. The two-stage approach adopted in this study ensured both compositional optimization and functional validation. The QbD-guided mixture design established a scientifically justified extract ratio based on statistical modelling and interaction analysis. Subsequently, conventional formulation screening ensured that the selected vehicle supported dermal application requirements without altering optimized biological performance. The integration of statistical optimization with pharmaceutics evaluation strengthens the translational relevance of the developed formulation. The final selected gel (G3) demonstrated robust antioxidant, anti-inflammatory, anti-aging, and wound-healing properties while maintaining physicochemical stability and cytocompatibility. Overall, the findings confirm that rational extract ratio optimization followed by systematic vehicle selection can significantly enhance the therapeutic potential of polyherbal topical systems.

Conclusion:

The present investigation successfully demonstrated a structured and scientifically integrated approach for the development of a polyherbal topical formulation by combining QbD-guided mixture optimization with conventional pharmaceutics-based formulation screening. The study was deliberately divided into two distinct phases to ensure methodological clarity and technical precision. In the first phase, a D-Optimal mixture design was employed to optimize the ratio of *Centella asiatica*, *Calendula officinalis*, and *Camellia sinensis* extracts. Statistical modeling revealed significant interaction effects between the extracts, particularly between *Centella asiatica* and *Camellia sinensis*, which contributed to enhanced antioxidant and collagenase inhibitory responses. The optimized ratio (X1 = 0.33, X2 = 0.27, X3 = 0.40) achieved high composite desirability and demonstrated strong predictive reliability, confirming the effectiveness of the QbD approach in establishing a synergistic phytochemical composition. Following completion of the mixture optimization stage, five gel formulations were prepared using the optimized extract ratio, varying only the gelling agent concentration to identify a pharmaceutically suitable vehicle. Among the evaluated formulations, G3 exhibited optimal rheological properties, acceptable pH, superior content uniformity, and balanced spreadability and extrudability. This formulation was selected as the final optimized gel for biological validation.

The selected gel (G3) demonstrated strong in vitro antioxidant activity, significant anti-inflammatory potential, and effective inhibition of matrix-degrading enzymes, supporting its anti-aging capability. Furthermore, enhanced fibroblast migration in the scratch assay indicated promising wound-healing potential. Cytotoxicity studies confirmed good cellular compatibility, ensuring safety for topical application. Overall, the study validates that systematic extract ratio

optimization followed by rational formulation screening can produce a stable, biologically active, and dermally compatible polyherbal gel. The developed formulation shows potential as a multifunctional topical system with antioxidant, anti-inflammatory, wound-healing, and anti-aging benefits, warranting further translational and clinical investigation.

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