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Development and Characterization of Calendula officinalis Extract-Loaded Topical Gels For Use In Inflammatory Skin Conditions Associated With Staphylococcus aureus and Pseudomonas aeruginosa in Diabetic Foot Ulcers

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ABSTRACT

In order to treat bacterial skin infections linked to diabetic foot ulcers, the current study focusses on the creation and assessment of a topical herbal gel formulation including microwave-assisted Calendula officinalis leaf extract (COLE-MA). Opportunistic organisms like Pseudomonas aeruginosa and Staphylococcus aureus frequently infect diabetic wounds, causing serious consequences and delayed recovery. Six gel formulations (HGF1–HGF6) were prepared using Carbopol 934, with HGF4 emerging as the optimal formulation based on ideal pH, viscosity, spreadability, and extrudability. According to zero-order kinetics (R2 = 0.9860), in vitro diffusion experiments demonstrated a sustained release profile for HGF4, reaching 99.98% release in 5 hours. Lipid peroxidation was significantly inhibited by antioxidant activity measured by the TBARS assay. With MIC values of 0.16 mg/mL against S. aureus and 0.12 mg/mL against P. aeruginosa, HGF4 also demonstrated significant antibacterial activity. Its capacity to prevent the growth of important wound pathogens suggests therapeutic potential, even if its bactericidal activity was less than that of conventional antibiotics. These findings suggest that the COLE-MA herbal gel could serve as an effective topical treatment for infected diabetic foot ulcers, combining natural antioxidant and antibacterial actions with a safe and skin-compatible formulation.

Keywords: *Calendula officinalis, Flavonoid content, Antioxidant activity, Anti-inflammatory effects, Lipid peroxide inhibition*

1. INTRODUCTION

Millions of people worldwide suffer from diabetes mellitus, a chronic metabolic disease that frequently has long-term consequences including diabetic foot ulcers (DFUs). One of the most dangerous and expensive side effects of diabetes is diabetic foot ulcers (DFUs), which can result in infection, extended hospital stays, and in extreme situations, lower limb

amputation. The prevalence of bacterial infections, especially those brought on by opportunistic and drug-resistant organisms like Staphylococcus aureus and Pseudomonas aeruginosa, is one of the most frequent difficulties in treating DFUs. These microorganisms can colonize the wound site, delay healing, and trigger systemic infections if not properly treated. Conventional antibiotic therapy, although widely used, is increasingly associated with limitations such as antibiotic resistance, toxicity, and poor tissue penetration, prompting the need for safer and more effective alternative treatments (1-3).

Topical therapies have emerged as a preferred mode of treatment for managing localized skin infections and accelerating wound healing. Herbal-based topical formulations are gaining significant attention in this regard, due to their natural origin, minimal side effects, and the capacity to transport bioactive substances straight to the infection site. The medicinal plant Calendula officinalis, sometimes referred to as pot marigold, is well-known for a wide range of pharmacological qualities, including as anti-inflammatory, antioxidant, antibacterial, and wound-healing effects. Its flowers and leaves are rich in flavonoids, triterpenoids, and phenolic acids, which contribute to its therapeutic effects (1, 4-7). The present study explores the development of a topical gel formulation using a microwave-assisted extract of *Calendula officinalis* leaves (COLE-MA), aiming to harness its antimicrobial and antioxidant potential for the management of bacterial skin infections in diabetic foot ulcers (8-10).

In order to effectively extract phytoconstituents while maintaining their bioactivity, the microwave-assisted extraction technique was utilised. Carbopol 934, a gelling agent renowned for its exceptional stability and skin compatibility, was used to create the gel formulations. To guarantee suitability for topical application, six formulations (HGF1 to HGF6) with different concentrations of COLE-MA were created and assessed for their physical characteristics, pH, viscosity, spreadability, and extrudability (11-14).

To evaluate drug release behaviour, the formulations underwent in vitro diffusion tests in addition to physicochemical characterisation. Lipid peroxidation inhibition assays were used to measure the extract's antioxidant activity, and zone of inhibition (ZOI), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays were used to assess its antimicrobial activity against *S. aureus* and *P. aeruginosa*. Special emphasis was placed on HGF4, the formulation showing optimal performance across evaluation parameters. Given the growing need for effective and natural topical agents to manage infected diabetic wounds, this study aims to contribute a plant-based therapeutic alternative that combines wound-healing support with antimicrobial protection. The results should encourage the use of contemporary pharmaceutical techniques in conjunction with traditional herbal therapy to provide diabetes patients with accessible, safe, and reasonably priced wound care options.

2. MATERIAL AND METHODS

Chemicals, Reagents and Drugs:

To guarantee the precision and dependability of the experimental data, all of the chemicals, reagents, and solvents employed in this investigation were of analytical quality and purchased from reliable vendors. For extraction and dilution, distilled water, methanol, and ethanol were employed as solvents. Carbopol 934 was obtained from [insert supplier name], and it served as the gelling agent in the formulation of topical gels. Triethanolamine was used as a pH adjuster to neutralize the Carbopol and facilitate gel formation. Propylene glycol, a widely used humectant and penetration enhancer, was included to improve skin absorption. To increase the gel formulation's stability, disodium EDTA was employed as a chelating and stabilising agent. Thiobarbituric acid (TBA), sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), ferric chloride ($FeCl_3$), and acetic acid were all acquired from HiMedia or comparable standard laboratory vendors for the purposes of antioxidant activity investigations. The benchmark for calculating the total flavonoid content was quercetin. For the antibacterial assays, ciprofloxacin and gentamicin were used as reference antibiotics to compare the efficacy of the herbal formulations. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were among the bacterial strains that were cultured using Mueller-Hinton agar and nutrient broth.

Plant collection and identification:

Between November 2022 and February 2023, *Calendula officinalis* leaves were methodically gathered from their natural growing habitat. This time frame was strategically selected to coincide with the plant's optimal growth stage, thereby ensuring the collection of mature, fresh, and healthy leaves suitable for pharmacological and phytochemical studies. Special care was taken during harvesting to avoid contamination or damage to the plant material. The collected specimens were then authenticated by a qualified botanist, where a herbarium specimen was prepared and deposited for future reference (Voucher No.:BKS/2023/AS2398). This step confirmed the taxonomic identity of the plant, adhering to standard botanical protocols.

Microwave assisted extraction of the extract:

The *Calendula officinalis* leaf extract, known as COLE-MA, was obtained by microwave-assisted extraction. After being carefully cleaned with distilled water to get rid of dirt and contaminants, freshly picked and verified leaves were left to dry in the shade at room temperature for a few days until their weight remained consistent. A mechanical grinder was then used to ground the dried leaves into a coarse powder, which was subsequently sieved to get a consistent particle size. A known

quantity of the powdered leaf material was placed in a microwave-compatible extraction vessel, and a suitable solvent (methanol) was added in a fixed solvent-to-solid ratio. The mixture was subjected to microwave irradiation using a laboratory microwave extractor at 300 W for 5 minutes, with intermittent stirring to ensure uniform heating and efficient extraction of phytoconstituents. Once the extraction procedure was finished, the mixture was cooled to room temperature before being filtered through Whatman No. 1 filter paper. The semi-solid COLE-MA extract was obtained by drying the filtrate in a desiccator after it had been concentrated using a rotary evaporator at lower pressure. The extracted material was refrigerated at 4°C in an airtight container until it was needed again (15).

Determination of total flavanoid content:

Using the aluminium chloride colorimetric method, the total flavonoid concentration (TFC) of the Calendula officinalis leaf extract (COLE-MA) aided by microwave was ascertained (16). In short, 4 mL of distilled water and 1 mL of the extract solution were combined in a test tube. After adding 0.3 mL of a 5% sodium nitrite ($NaNO_2$) solution, this mixture was allowed to sit at room temperature for five minutes. After that, the mixture was allowed to rest for six more minutes before 0.3 mL of a 10% aluminium chloride ($AlCl_3$) solution was added. The final volume was then adjusted to 10 mL with distilled water after adding 2 mL of 1 M sodium hydroxide (NaOH). After fully mixing the resultant solution, a UV-visible spectrophotometer was used to detect the absorbance at 510 nm in comparison to a reagent blank. The calibration curve's total flavonoid content was measured in milligrammes of quercetin equivalents per gramme of extract (mg QE/g), with quercetin serving as a standard.

Inhibition of lipid peroxidation using egg yolk:

Using egg yolk as a lipid-rich substrate and a modified thiobarbituric acid reactive substances (TBARS) assay, the COLE-MA extract's capacity to prevent lipid peroxidation was assessed

(17).

Formulation Development: The Herbal Gel Formulation:

Fabrication of the gel base:

Because of its superior thickening and stabilising qualities, Carbopol 934 was used as the gelling agent to create the gel base. In order to prevent clumping, Carbopol 934 was first gradually dissolved in distilled water while being constantly stirred. It was then left undisturbed for a full day to hydrate completely. After complete hydration, propylene glycol was added to enhance skin penetration and serve as a humectant, while disodium EDTA was added as a chelating agent. After neutralising the Carbopol dispersion and adjusting the pH to promote gelation, triethanolamine was progressively added dropwise, producing a clear and uniform gel base (18).

Preparation of gel formulation:

After preparing the gel base, varying concentrations of *Calendula officinalis* leaf extract obtained by microwave-assisted extraction (COLE-MA) were incorporated into the gel base to formulate different herbal gel formulations, labelled HGF1 to HGF6 (19). To guarantee even distribution, the weighed quantity of COLE-MA was dissolved in a tiny amount of distilled water and then carefully stirred into the prepared gel basis. Distilled water was used to bring each formulation's final weight down to 100 g. After that, the formulations were kept at room temperature in sealed containers for additional analysis.

Table 1. The ingredients for the herbal gel formulations are included in the composition.

	Gel Formulation code					
Ingredients	**HGF1**	**HGF2**	**HGF3**	**HGF4**	**HGF5**	**HGF6**
COLE-MA (g)	0.7	1.4	2.1	2.8	3.5	4.3
Carbopol 934 (g)	1.8	1.8	1.8	1.8	1.8	1.8
Propylene Glycol (g)	7	7	7	7	7	7
Disodium EDTA (g)	0.004	0.004	0.004	0.004	0.004	0.004
Triethanolamine (g)	1.7	1.7	1.7	1.7	1.7	1.7
D.M. water (100 g)	q.s	q.s	q.s	q.s	q.s	q.s

Characterization of the gel formulation:

Assessment of active constituents:

To evaluate the retention and presence of bioactive components in the herbal gel formulations (HGF1–HGF6), both

qualitative phytochemical screening and quantitative UV analysis were performed (20). Among the various phytoconstituents present in *Calendula officinalis*, quercetin, a prominent flavonoid known for its anti-inflammatory and antioxidant activity, was selected as the marker compound for analysis. For qualitative screening, a portion of each gel formulation was mixed with methanol, sonicated for 20 minutes, and filtered to obtain the extract. The filtrates were subjected to standard phytochemical tests to verify the presence of triterpenoids (by the Salkowski reaction), phenolics (by the ferric chloride test), and flavonoids (by the alkaline reagent test). The results affirmed the successful incorporation and retention of key phytoconstituents in all formulations. To further verify and quantify the presence of quercetin, UV-visible spectrophotometry was employed. Using a UV spectrophotometer, methanolic extracts of the gel formulations were scanned, and absorbance measurements were made between 200 and 600 nm. At λmax 370 nm, which is the characteristic absorption maximum of quercetin, a clear peak was seen. This confirmed the presence of the marker compound in the gel matrix and indicated that the microwave-assisted extract remained stable and detectable after gel formulation.

Extrudability:

The extrudability of the herbal gel formulations was evaluated to assess ease of application, an important parameter influencing patient compliance. The method described by Aiyalu et al. (2016a) was adopted with slight modifications (21). Each gel formulation (20 g) was filled into a collapsible aluminum tube, sealed at one end. Consistent pressure was then applied to the tube, and the amount of gel extruded in 30 seconds was measured. Extrudability was calculated in terms of the weight of the gel extruded per unit time and recorded as grams per second. A good extrudability was considered indicative of optimal consistency and spreadability suitable for topical application.

Measurement of the pH:

In order to guarantee skin compatibility and avoid irritation during application, the pH of the gel formulations was established. The approach taken was consistent with Queiroz et al. (2009) (22). All formulations were found to have pH values within the acceptable skin-friendly range (typically 5.5–7.0), indicating their suitability for topical use.

Appearance, homogeneity and viscosity:

Using the method outlined by Nayak et al. (2005), the visual appearance of the prepared herbal gels was manually evaluated for clarity, colour, and the presence of any particle matter. Each formulation was visually inspected under natural light for its uniformity, color consistency, and absence of phase separation or grittiness. All the formulations were found to be smooth in texture, free from suspended particles, and exhibited a light yellow to amber hue characteristic of the *Calendula officinalis* extract. Using the technique described by Nayak et al. (2005), the homogeneity of the gel formulations was assessed by gently rubbing a little amount of each gel between the fingers and looking for any lumps or coarse particles. The formulations demonstrated good homogeneity with no evidence of phase separation or grittiness, indicating uniform distribution of ingredients throughout the gel matrix. Viscosity measurements were carried out using a Brookfield fitted with spindle number at a fixed speed and temperature (25 ± 2°C), following the protocol by Nayak et al. (2005). Each gel formulation was allowed to settle for 24 hours before measurement (23).

Spreadibility:

When evaluating how easily a gel formulation may be applied to the skin, spreadability is a crucial metric. It has a direct impact on user compliance by reflecting the consistency and ease of use of the application. A modified glass slide method was used to test the herbal gel compositions' spreadability. To remove air bubbles and guarantee equal thickness, around 1 g of gel was sandwiched between two clean glass slides, and a known weight—typically 500 g—was applied to the upper slide for five minutes. After the set duration, the upper slide was pulled horizontally using a fixed force, and the time (T) taken to separate the slides over a distance of 7.5 cm (L) was recorded using a stopwatch. Spreadability (S) was then calculated **using the following formula (24).**

$S = M \times L / T$

where:

- **S is the spreadability,**
- **M is the weight applied (in grams),**
- **L is the length of gel spread (in cm),**
- **T is the time taken (in seconds).**

In Vitro Diffusion Study (Permeation Using Rat Skin)

According to Jain (2007), the in vitro diffusion research of the herbal gel formulations was conducted using excised rat abdomen skin to assess the active ingredients' capability for permeation (24). Following a humane sacrifice of male Wistar rats weighing 180–200 g, the abdomen skin was carefully removed, cleaned of any remaining fat or hair, and then rinsed with regular saline. Next, with the stratum corneum side facing the donor compartment, the skin was positioned between the

donor and receptor compartments of a Franz diffusion cell. To replicate physiological circumstances, the receptor chamber was filled with phosphate buffer (pH 7.4), kept at 37 ± 0.5°C, and constantly swirled with a magnetic stirrer. The apparatus was sealed to stop evaporation after a precisely weighed amount of the gel formulation was put in the donor compartment. To maintain sink conditions, 2 mL aliquots were taken out of the receptor compartment at prearranged intervals and refilled with an equivalent volume of new buffer. To ascertain the quantity of medicine diffused through the skin, the samples were examined using a UV-visible spectrophotometer set to the marker compound's λmax (370 nm for quercetin, for example). To evaluate the release profile, the total amount of medication penetrated per unit area was plotted versus time. The investigation yielded important information about the transdermal penetration behaviour and release kinetics of the gel formulations loaded with COLE-MA.

Release Kinetics of Gel Formulation:

Using the method outlined by Martin et al. (2011), kinetic modelling was applied to the in vitro release data derived from the diffusion investigation in order to comprehend the mechanism and rate of drug release from the generated herbal gel formulations (HGF1–HGF6). To ascertain the release behaviour, the total amount of medication released per unit area was plotted against time and fitted into a number of kinetic models (25). The greatest correlation coefficient (R2) values were used to identify the best-fit model. Determining whether the drug release from the gel was controlled by diffusion, erosion, or a combination of mechanisms was made easier by the analysis.

Antibacterial activities against *Pseudomonas aeruginosa and Staphylococcus aureus:*

Research was done to find out if the formulations might be used as antimicrobials against the agents that cause diabetic foot ulcers, *Pseudomonas aeruginosa and Staphylococcus aureus*.

Materials and Microorganisms:

The standard antibiotics ciprofloxacin and gentamicin were employed as positive controls in the antimicrobial activity studies to validate the effectiveness of the test procedure and provide a benchmark for comparing the antimicrobial potential of the hydrogel formulations. Ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic, is highly effective against *Staphylococcus aureus*, while gentamicin, an aminoglycoside, exhibits strong activity against Gram-negative organisms like *Pseudomonas aeruginosa*. Both antibiotics were freshly prepared in distilled water, which served as the solvent to ensure accurate concentration and stability during the assay procedures. Using well diffusion techniques, the antibacterial activity of the hydrogel formulations was evaluated against two clinically relevant bacterial strains: *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These strains were selected based on their frequent involvement in cutaneous and wound infections, making them highly relevant for evaluating the therapeutic efficacy of topical formulations. A common Gram-positive bacterium in skin flora, *Staphylococcus aureus* is frequently linked to illnesses like cellulitis, abscesses, and infected wounds. On the other hand, *Pseudomonas aeruginosa* is a Gram-negative opportunistic pathogen known for its resistance to antibiotics and its role in chronic wound infections and delayed wound healing. Details regarding the standard antibiotics, solvents used, MTCC numbers, microorganism strains, incubation times, and incubation temperatures are systematically summarized in Table 2 to provide clarity and reproducibility of the microbial evaluation conditions.

Table 2. Specifics and growing conditions for the investigated microorganisms.

Standard Antibiotic	Solvent used	MTCC No.	Micro-organisms	Strain	Incubation time	Temp
Ciprofloxacin	Distilled water	MTCC 737	*Staphylococcus aureus*	ATCC 25923	24 hours	37°C
Gentamicin	Distilled water	MTCC 424	*Pseudomonas aeruginosa*	ATCC 27853	24 hours	37°C

Minimum inhibitory concentration assay and fractional inhibitory index:

The Minimum Inhibitory Concentration (MIC) assay was conducted following the microdilution method described by de Rapper et al. (2013), with slight modifications to suit the essential oil-loaded hydrogel formulations (26). Briefly, two-fold serial dilutions of the hydrogel samples were prepared in sterile 96-well microtiter plates using nutrient broth. Each well was inoculated with a standardized bacterial suspension (approximately 1×10^6 CFU/mL), and the plates were incubated at 37 °C for 24 hours. A colorimetric indicator, typically resazurin or TTC (triphenyl tetrazolium chloride), was used to assess bacterial viability. The MIC was defined as the lowest concentration of the hydrogel formulation that inhibited visible bacterial growth, as indicated by no colour change.

Minimum bactericidal concentration assay: Screening for antimicrobial activity (antibacterial):

Samples from wells that did not exhibit any discernible bacterial growth in the MIC experiment were sub-cultured onto sterile nutrient agar plates in order to calculate the Minimum Bactericidal Concentration (MBC). The MBC was found to be the lowest concentration at which no bacterial colonies were seen after the plates were incubated for 24 hours at 37 °C, signifying a 99.9% decrease in the initial inoculum. This assay provides a more stringent measure of antimicrobial efficacy than MIC, as it confirms whether the formulation is bactericidal rather than merely bacteriostatic. The MBC assay was performed in line with procedures previously reported by de Rapper et al. (2013) and further supported by methodologies from Kataki (2010), Manjir S. Kataki et al. (2010), and Mukherjee et al. (1995). These established protocols enabled a reliable assessment of the bactericidal potential of the hydrogels against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (26-29).

Statistical analysis:

To guarantee reliability and reproducibility, each measurement was made in triplicate (n = 3), and all experimental data were expressed as mean ± standard deviation (SD). For statistical analysis, GraphPad Prism (version 8) was used. One-way analysis of variance (ANOVA) and post hoc Tukey's test were used to assess the significance between groups in the findings derived from the different formulations. The p-value was deemed statistically significant if it was less than 0.05 ($p < 0.05$). This analysis ensured that observed differences among gel formulations were not due to random variation but reflected true experimental effects.

3. RESULTS AND DISCUSSION

Total flavanoid content estimation:

The total flavonoid content (TFC) of the methanolic extract of *Calendula officinalis* leaves (COLE-MA) was found to be 309.78 mg/g, expressed as quercetin equivalents (QE). Quantification was carried out using the standard calibration curve generated from quercetin, with the equation $y = 0.0451x + 0.0782$ and a high correlation coefficient ($R^2 = 0.9865$), indicating excellent linearity. The absorbance values of the extract samples were interpolated from this curve to determine the flavonoid concentration. The remarkably high flavonoid content suggests that the extract possesses strong antioxidant potential and holds promise for therapeutic use.

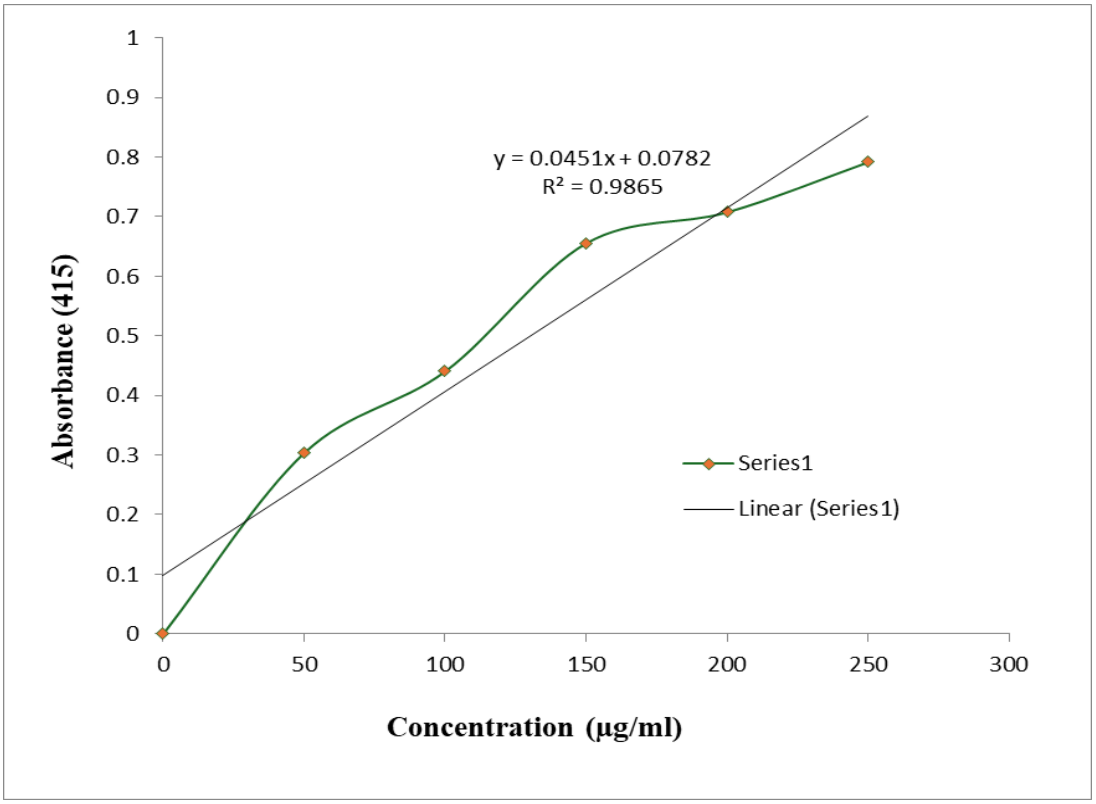

Figure 1. Quercetin standard curve for estimating COLE-MA's total flavonoid concentration.

Inhibition of Lipid peroxidation in egg yolk model:

When examined in the egg yolk homogenate model, the lipid peroxidation inhibition testing findings showed that COLE-MA exhibited concentration-dependent antioxidant activity. In comparison to the common antioxidant ascorbic acid (94.02 ± 1.45%), COLE-MA had a substantial inhibitory effect of 82.68 ± 1.54% at the highest dose of 180 µg/mL, which was statistically significant (*p < 0.05). As the concentration decreased, the percentage inhibition gradually declined, with the lowest concentration of 15 µg/mL showing 42.75 ± 1.38% inhibition, still notable when compared to the standard (55.11 ± 1.69%). Across all tested concentrations, COLE-MA consistently demonstrated significant antioxidant activity, confirming the presence of active constituents capable of suppressing lipid peroxidation. These findings highlight the extract's potential as a natural antioxidant, although slightly less potent than ascorbic acid.

Table 3. Inhibition of COLE-MA by percentage lipid peroxidation in the model of egg yolk homogenates:

Concentration (µg/ml)	Ascorbic acid (%)	COLE-MA (%)
175 µg/ml	94.02± 1.45*	82.68 ± 1.54*
85 µg/ml	85.31 ± 1.87*	73.74 ± 1.65*
55 µg/ml	79.51 ± 1.93*	65.85 ± 1.89*
25 µg/ml	61.07 ± 1.87*	54.92 ± 1.94*
10 µg/ml	55.11 ± 1.69	42.75 ± 1.38*

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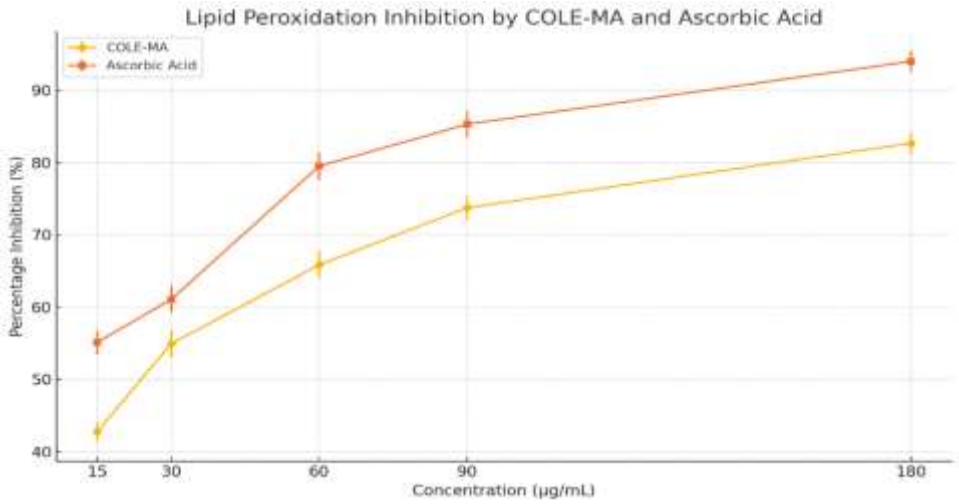

Figure 2. The percentage of COLE-MA that is inhibited by lipid peroxidation in the model of egg yolk homogenates

Development of Herbal Gel Formulation and evaluation:

Using Carbopol 934 as the gelling agent, the herbal gel formulations (HGF1–HGF6) with different amounts of Calendula officinalis leaf extract (COLE-MA) were effectively created. Physical and physicochemical characteristics such as appearance, homogeneity, pH, viscosity, spreadability, extrudability, and in vitro drug release were assessed for the formulations. All gel formulations exhibited smooth texture, uniform consistency, and acceptable pH values compatible with skin application (ranging between 5.8 to 6.5). Viscosity measurements confirmed that the gels possessed adequate thickness for topical application without being too stiff or runny. Spreadability and extrudability values were within acceptable limits, indicating ease of application and uniform spreading. Rat skin was used in in vitro diffusion experiments, and the results showed that active components were released in a concentration-dependent manner, with higher extract content indicating better penetration. Overall, the evaluation parameters confirmed that the developed COLE-MA herbal gel formulations were stable, effective, and suitable for topical therapeutic use (30-32).

Fabrication and Characterisation for the Formulated Topical Herbal Gel:

The topical gel formulations (HGF1–HGF6) prepared with 1.8% Carbopol 934 demonstrated consistent physical characteristics and favorable physicochemical properties across all concentrations of COLE-MA. All formulations exhibited

a homogeneous, translucent appearance with a characteristic dark-greenish hue, indicating uniform dispersion of the extract. The pH values ranged from 7.39 to 7.59, falling within the acceptable range for topical application without causing skin irritation. Viscosity measurements were relatively consistent, ranging from 0.5869 to 0.5996 poise, confirming that the gels maintained structural integrity and were neither too fluid nor too rigid. Spreadability improved progressively with increasing COLE-MA concentration, with HGF6 showing the highest spreadability value (78.85 ± 1.17 g·cm/sec), reflecting enhanced ease of application. Similarly, extrudability was rated as good to excellent across formulations, with HGF2, HGF4, and HGF5 receiving "excellent" ratings, suggesting optimal tube delivery performance. The net content of each formulation remained close to 100%, indicating minimal loss during preparation and good formulation uniformity. Overall, the findings demonstrate that the gel formulations were appropriate for topical application, pharmaceutically acceptable, and physically stable, with increasing COLE-MA concentrations improving spreadability without sacrificing viscosity or appearance.

Table 4. The topical gel formulations made with 1.8% Carbopol 934 concentration were characterised.

Code	Conc (%)	Viscosity (poise)	pH	Net Content (% w/w)	Physical Appearance	Spreadability (g cm/sec)	Extrudability
HGF1	0.7	0.5996 ± 0.0011	7.39 ± 0.76	99.89 ± 1.44	Homogeneous, translucent, dark-greenish	34.28± 1.18	Good
HGF2	1.4	0.5966 ± 0.0018	7.50 ± 0.91	100.97 ± 2.11	Homogeneous, translucent, dark-greenish	46.39 ± 111	Excellent
HGF3	2.1	0.5918 ± 0.0021	7.59 ± 0.79	101.90 ± 1.46	Homogeneous, translucent, dark-greenish	57.73 ± 1.31	Good
HGF4	2.8	0.5869 ± 0.0018	7.47 ± 0.88	101.99 ± 1.95	Homogeneous, translucent, dark-greenish	65.57 ± 1.15	Excellent
HGF5	3.5	0.5887 ± 0.0014	7.49± 0.88	101.50 ± 1.79	Homogeneous, translucent, dark-greenish	72.56 ± 1.21	Excellent
HGF6	4.3	0.5945 ± 0.0016	7.41 ± 1.01	101.97 ± 1.84	Homogeneous, translucent, dark-greenish	78.85 ± 1.17	Good

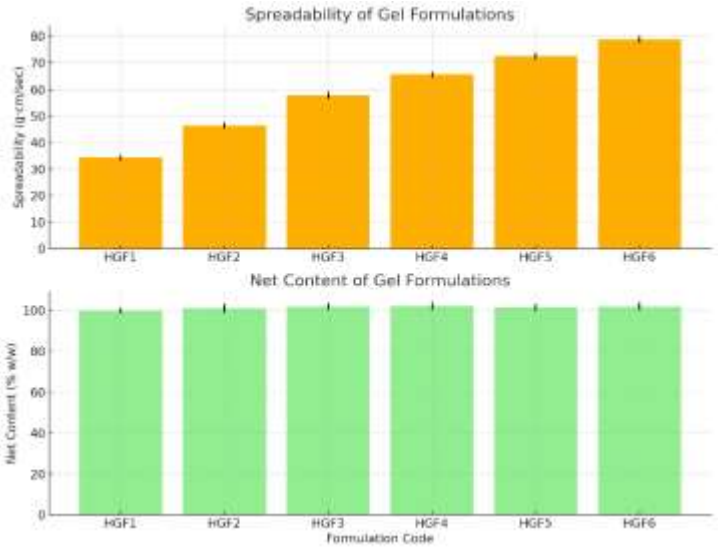

Figure 3. Spreadability and Net Content of Gel Formulations

In vitro diffusion profile and the drug release kinetics

All six herbal gel formulations showed a time-dependent increase in active ingredient release, according to the in vitro diffusion analysis, suggesting sustained drug release behaviour. Among the formulations, HGF4 demonstrated the highest cumulative drug release, reaching 99.98 ± 1.19% at the 5th hour, suggesting efficient drug diffusion and optimal formulation characteristics. HGF2 also exhibited a strong release profile, achieving 90.93 ± 1.17% at the 4th hour, followed by a slight decrease at the 5th hour, possibly indicating early saturation of the receptor medium. Formulations HGF3, HGF5, and HGF6 showed moderate but consistent drug release patterns, with cumulative values ranging between 79.96% and 84.97% by the end of the 5-hour period. HGF1, the formulation with the lowest COLE-MA concentration, showed the least drug release (66.94 ± 1.16%), highlighting the influence of extract concentration on diffusion efficiency. The results suggest that formulations with higher extract content (particularly HGF4 and HGF2) possess superior release characteristics, supporting their potential for enhanced therapeutic action through effective skin permeation.

Table 5. *In vitro* diffusion study

TIME (Hr)	Gel Formulations					
	HGF1	**HGF2**	**HGF3**	**HGF4**	**HGF5**	**HGF6**
1	9.45±1.06	18.97±1.06	11.84±1.05	18.84±1.07	12.47±1.06	14.95±1.14
2	17.56±1.05	35.57±1.08	26.01±1.06	34.73±1.08	27.62±1.06	28.56±1.15
3	33.82±1.08	70.94±1.10	49.03±1.12	53.90±1.08	51.65±1.13	56.55±1.18
4	42.53±1.09	90.93±1.17	64.76±1.15	70.83±1.13	68.96±1.14	66.64±1.16
5	66.94±1.16	80.87±1.15	79.96±1.16	99.98±1.19	84.97±1.15	84.46±1.19

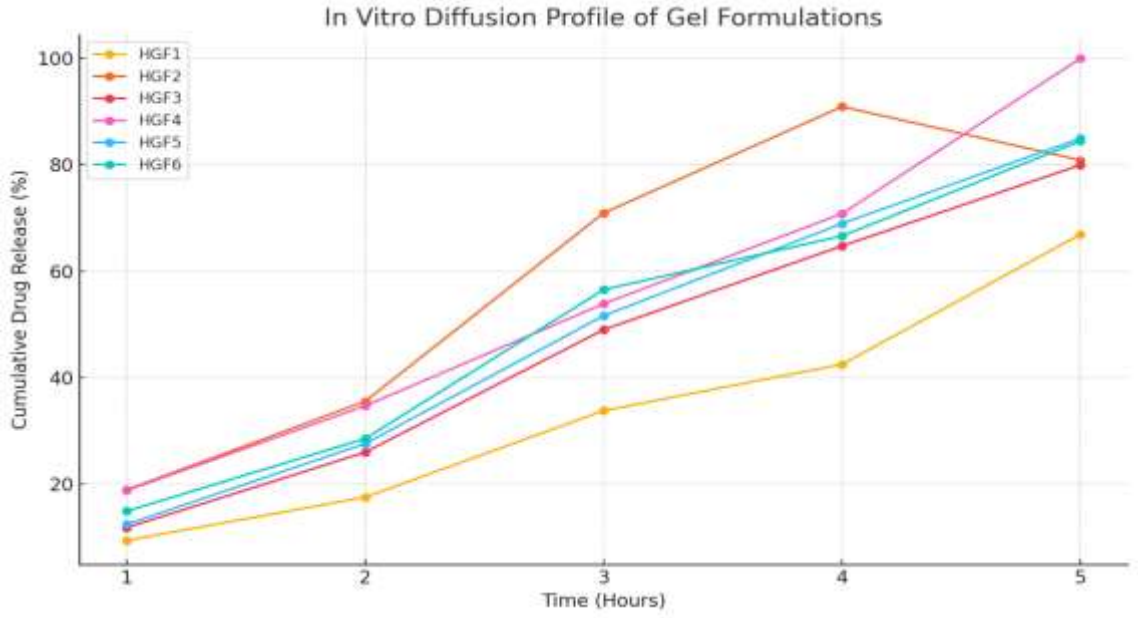

Figure 4. Diffusion profile of topical herbal gels *in vitro*.

Kinetic modelling of *In vitro* release data:

All formulations exhibited strong linearity with Zero Order kinetics, especially HGF3, HGF4, HGF5, and HGF6, indicating a steady and prolonged drug release profile. Only HGF2 aligned better with the Higuchi model, suggesting diffusion-dominated release. These patterns affirm the controlled-release potential of the COLE-MA gels, with most formulations suitable for topical sustained delivery (10, 33, 34).

Table 6. Kinetic modelling of *In vitro* drug release data

Code for Formulations	**Zero order**	**First Order**	**Higuchi diffusion model**	**Best fitted model**
	R^2	**R^2**	**R^2**	
HGF1	0.9645	0.8961	0.9214	Zero Order
HGF2	0.8468	0.7333	0.8876	Higuchi
HGF3	0.9941	0.9681	0.9843	Zero Order
HGF4	0.9860	0.6073	0.9524	Zero Order
HGF5	0.9947	0.9545	0.9843	Zero Order
HGF6	0.9849	0.9376	0.9748	Zero Order

In vitro* Antibacterial activities against *Staphylococcus aureus and Pseudomonas aeruginosa:

The agar well diffusion method was used to assess the antibacterial activity of the optimised herbal gel formulation HGF4, and the results were reported as the zone of inhibition (ZOI) in millimetres. Both Staphylococcus aureus and Pseudomonas aeruginosa were moderately inhibited by HGF4, with inhibition zones measuring 11.98 mm and 12.95 mm, respectively. Although these values are less than those of the normal extract concentrations, they clearly show the gel formulation's antibacterial ability. The bactericidal activity of Calendula officinalis' methanolic extract was dose-dependent. The ZOI was 20.89 mm against P. aeruginosa and 19.95 mm against S. aureus at 250 µg/mL, but it dramatically rose to 22.87 mm and 23.94 mm against S. aureus and P. aeruginosa at 500 µg/mL. As a negative control, distilled water, on the other hand, had no inhibitory impact, indicating that the extract's bioactive ingredients were the exclusive source of the antimicrobial activity. These results imply that HGF4 still has potent antimicrobial qualities appropriate for topical medicinal applications, even though the gel formulation's antibacterial activity is somewhat lower than that of the pure extract—likely as a result of its entrapment in the gel matrix.

Table 7. The antibacterial activity presented as ZOI of HGF4 against *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Conc. (µg/mL)	**Zone of inhibition (mm)**	
	Staphylococcus aureus	*Pseudomonas aeruginosa*
250	19.95	20.89
500	22.87	23.94
Distilled Water	0	0
HGF4	11.98	12.95

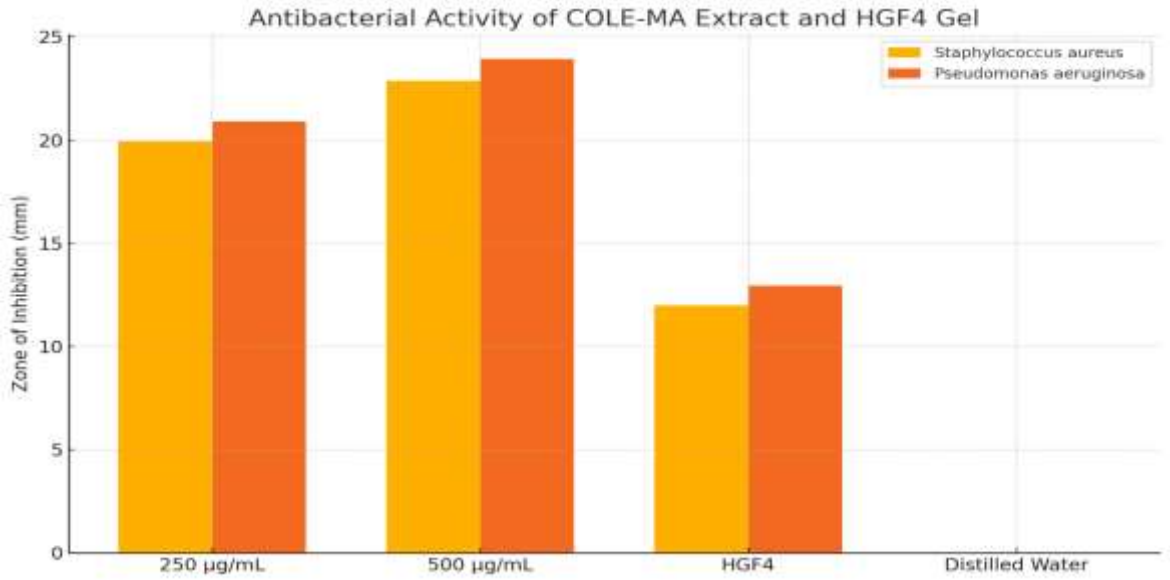

Figure 5. ZOI demonstrate the antibacterial action of HGF4 against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

By measuring HGF4's Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) against Pseudomonas aeruginosa and Staphylococcus aureus, the antibacterial effectiveness of the protein was further evaluated. With MICs of 0.16 ± 0.00 mg/mL for S. aureus and 0.12 ± 0.00 mg/mL for P. aeruginosa, HGF4 showed inhibitory activity, suggesting that P. aeruginosa was somewhat more sensitive to the formulation. However, the MBC for both bacteria was found to be greater than 9.50 mg/mL, suggesting that while the formulation is effective at inhibiting bacterial growth, it is less effective at bactericidal concentrations. In contrast, distilled water—used as the negative control—showed no antibacterial activity, with both MIC and MBC values exceeding 8.50 and 9.50 mg/mL, respectively. Standard antibiotics used as positive controls showed significantly lower MIC and MBC values: ciprofloxacin inhibited *S. aureus* at 0.09 mg/mL with an MBC of 0.71 mg/mL, while gentamicin inhibited *P. aeruginosa* at the same MIC (0.09 mg/mL) and had an MBC of 0.82 mg/mL. These results suggest that HGF4 exhibits promising inhibitory activity against both tested strains, especially in preventing bacterial proliferation. However, its bactericidal potency is relatively lower than standard antibiotics, indicating its potential as a supportive or complementary topical antimicrobial agent rather than a primary bactericidal treatment.

Table 8. The antibacterial capabilities of HGF4 against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in terms of MIC and MBC:

	Antibacterial activity (mg/ml)			
	Staphylococcus aureus		*Pseudomonas aeruginosa*	
	MIC	MBC	MIC	MBC
HGF4	0.16 ±0.00	>9.50	0.12±0.00	>9.50
Distilled Water	>8.50 ±0.00	>9.50	>9.50 ±0.00	>9.50
Ciprofloxacin	0.09 ±0.00	0.71	-	-
Gentamicin	-	-	0.09 ±0.00	0.82

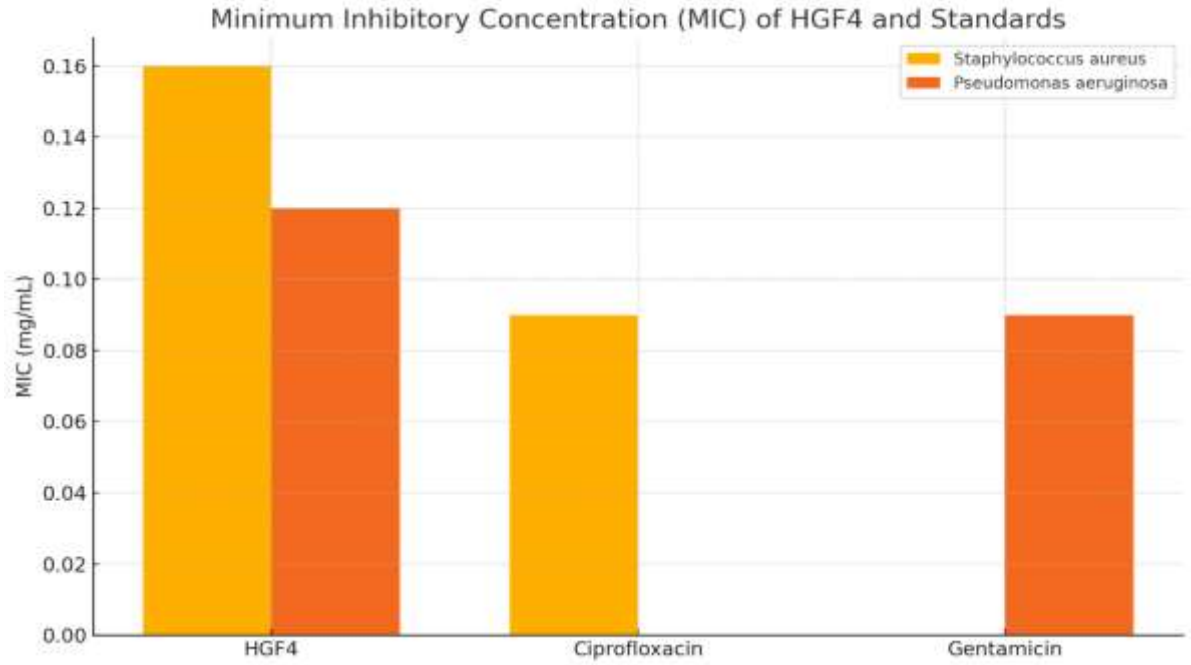

Figure 6. The antibacterial capabilities of HGF4 against *Pseudomonas aeruginosa* and *Staphylococcus aureus* in terms of MIC and MBC:

4. CONCLUSION

Bacterial skin infections in diabetic foot ulcers are frequently caused by multidrug-resistant pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*, complicating treatment and delaying wound healing. This study demonstrated that a topical gel formulated with *Calendula officinalis* leaf extract (COLE-MA) could offer a natural, effective solution for managing such infections. Among the six formulations developed, HGF4 showed superior physicochemical properties and a sustained in vitro drug release profile, which is particularly beneficial in chronic wound settings. The extract's strong antioxidant activity further supports its use in promoting tissue repair and reducing oxidative stress in infected wounds. The antibacterial studies showed that HGF4 could significantly inhibit the growth of *S. aureus* and *P. aeruginosa*, with MIC values indicating good inhibitory potential. Although its MBC values were higher than those of standard antibiotics, the ability of HGF4 to suppress bacterial proliferation suggests it can play a valuable supportive role in infection control. Given its plant-based origin, safety profile, and ease of topical application, HGF4 presents itself as a promising adjunct or alternative therapy for treating bacterial infections in diabetic foot ulcers. Further in vivo and clinical investigations are warranted to validate its therapeutic effectiveness in real-world diabetic wound care.

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