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In Vivo Assessment of Acute Dermal Toxicity and Reparative Efficacy of Phytoconstituent-Loaded Hydrogel Scaffolds Incorporating Calendula officinalis in Full-Thickness Wound

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Abstract

Chronic full-thickness wounds remain difficult to manage due to infection risk, prolonged inflammation, and impaired tissue regeneration. We developed phytoconstituent-loaded hydrogel scaffolds incorporating Calendula officinalis *(CO) and assessed their acute dermal safety and reparative efficacy. CO flowers (authenticated at FRI, Dehradun) were Soxhlet-extracted (70% ethanol) and embedded into PVA/alginate/chitosan hydrogels prepared by a freeze–thaw method. Three formulations (F1–F3) underwent in-vitro screening; the optimized batch (F2) exhibited skin-compatible pH (6.12 ± 0.08), workable viscosity (18,450 ± 210 cP), high swelling (482.6 ± 5.4% at 8 h), sustained moisture retention (81.4 ± 1.9% at 24 h), and controlled release (85.6 ± 1.7% at 24 h; Higuchi R^2 = 0.984). Acute dermal toxicity was evaluated in Wistar rats per OECD-402 (limit test, 2000 mg/kg, 24-h semi-occluded exposure): no mortality or systemic toxicity occurred; Draize erythema/edema scores were 0.0 throughout; body/organ weights and gross necropsy were unremarkable; histopathology of skin and major organs showed no treatment-related lesions. Reparative efficacy was then tested in an excision model (500 mm^2, ~1.5 mm depth; n = 6/group): compared with control and blank hydrogel, CO-hydrogel significantly accelerated wound closure (Day-21 contraction: 94.8 ± 1.3% for high-dose vs 78.2 ± 2.1% control, $p < 0.05$), shortened epithelialization time (17.2 ± 0.4 vs 23.8 ± 0.8 days), and improved scar quality (score 0 vs 3). These data indicate that CO-loaded hydrogels are dermally safe and enhance full-thickness wound healing via a moist, bioactive, diffusion-controlled delivery platform, supporting their translational potential for advanced topical care.*

Keywords: Calendula officinalis; *hydrogel scaffold; acute dermal toxicity; full-thickness wound healing.*

INTRODUCTION

Wound healing is a complex and dynamic biological process that involves the restoration of the integrity and function of damaged skin and underlying tissues [1]. Wounds are broadly classified based on their depth, duration, and cause, with the primary categories including acute wounds, chronic wounds, and full-thickness wounds [2]. Acute wounds, such as surgical incisions or minor cuts, usually heal within an expected time frame and follow an orderly process, whereas chronic wounds such as diabetic ulcers, venous leg ulcers, and pressure sores fail to progress through the normal stages of healing and often persist for months or years [3]. Full-thickness wounds, which involve the complete loss of both the epidermis and dermis, may extend into subcutaneous tissues, muscles, and even bone, making their repair particularly challenging [4]. Globally, chronic and slow-healing wounds present a significant healthcare burden, affecting millions of individuals and contributing to reduced quality of life, prolonged hospitalization, increased risk of secondary infections, and high treatment costs [5]. The World Health Organization and various epidemiological studies highlight the growing prevalence of such wounds due to an aging population, rising incidence of diabetes, obesity, and vascular diseases, which exacerbate the challenge for healthcare systems worldwide [6]. Conventional wound care methods such as gauze dressings, cotton pads, and antiseptic solutions while essential for basic protection and infection prevention, often fail to provide an optimal healing environment [7]. These traditional approaches lack the ability to actively modulate the wound microenvironment, do not maintain adequate moisture balance, and provide limited control over microbial contamination and drug release, resulting in delayed healing, frequent dressing changes, and patient discomfort [8]. In response to these limitations, modern wound management has evolved to incorporate advanced therapeutic strategies that promote faster and more effective tissue repair [9]. Bioactive dressings, which combine a physical barrier with therapeutic functions, have emerged as a cornerstone in this approach [10]. Unlike passive dressings, bioactive wound care systems are designed to interact with the wound bed, delivering antimicrobial agents, growth factors, antioxidants, and other healing-promoting substances directly to the site of injury [11]. Among these, phytoconstituents bioactive compounds derived from medicinal plants are gaining increasing attention due to their multifaceted therapeutic potential, natural origin, biocompatibility, and lower risk of adverse effects compared to synthetic agents [12]. These plant-derived molecules often exhibit a combination of antimicrobial, anti-inflammatory, antioxidant, and angiogenesis-promoting properties, making them particularly suited for complex wound healing scenarios. Incorporating phytoconstituents into hydrogel scaffolds further enhances their clinical utility [13]. Hydrogels are three-dimensional, hydrophilic polymeric networks capable of retaining large amounts of water, which creates a moist environment essential for optimal wound repair [14]. They not only facilitate autolytic debridement and reduce pain during dressing changes but also serve as efficient drug delivery systems, ensuring controlled and sustained release of bioactive agents. Furthermore, hydrogels can conform to irregular wound shapes, allow gaseous exchange, and protect against external contaminants while simultaneously supporting cell migration, proliferation, and extracellular matrix deposition. By integrating phytoconstituents within these hydrophilic networks, it is possible to combine the biological benefits of plant-based therapeutics with the structural and functional advantages of hydrogel scaffolds, resulting in synergistic wound healing effects. Calendula officinalis, commonly known as marigold, is a herbaceous plant belonging to the family Asteraceae and is widely cultivated for its ornamental value as well as medicinal applications [15]. The plant is characterized by its bright orange or yellow flowers, which are the primary source of its bioactive compounds. Phytochemical analyses reveal that Calendula officinalis flowers are rich in flavonoids, triterpenoids, carotenoids, saponins, polysaccharides, and essential oils each contributing to its diverse pharmacological activities. Traditionally, calendula extracts and ointments have been used in various cultures for the treatment of skin ailments, including minor cuts, burns, abrasions, rashes, and inflammatory skin conditions. In modern phytotherapy and dermatology, calendula is valued for its potent anti-inflammatory, antimicrobial, antioxidant, and wound-healing properties. Flavonoids act as free radical scavengers, reducing oxidative stress at the wound site and protecting cells from damage, while triterpenoids exhibit strong anti-inflammatory effects by modulating inflammatory mediators. Carotenoids contribute to granulation tissue formation and epithelialization, while saponins aid in cleansing the wound bed and promoting angiogenesis. The antimicrobial components inhibit the growth of pathogenic bacteria and fungi, thereby preventing infection and creating a favorable environment for tissue regeneration. Clinical and experimental studies have shown that calendula-based preparations can accelerate wound contraction, enhance collagen synthesis, and improve tensile strength of healed tissue, making it an ideal candidate for incorporation into modern bioactive wound dressings. Despite its well-

documented pharmacological activities and long history of topical use, there is a notable gap in in vivo research focusing on the acute dermal toxicity and reparative efficacy of Calendula officinalis when formulated within hydrogel scaffolds, particularly in the context of full-thickness wounds [16]. Most available studies have assessed calendula in cream or ointment bases, which may not provide the sustained release and moisture retention properties necessary for optimal wound management. Furthermore, while the safety of calendula in general topical applications is widely accepted, rigorous acute dermal toxicity evaluations in accordance with standardized guidelines such as OECD 402 are lacking for hydrogel-incorporated formulations. This absence of comprehensive safety and efficacy data limits the ability to fully validate calendula-loaded hydrogel scaffolds as advanced wound care products. Therefore, a controlled in vivo study using standardized full-thickness wound models is essential to provide robust scientific evidence. Such an evaluation would not only confirm the safety profile of the hydrogel formulation but also quantify its therapeutic benefits in terms of wound contraction rate, epithelialization period, tensile strength recovery, and histopathological improvements. By bridging this research gap, the study could establish Calendula officinalis-loaded hydrogel scaffolds as a scientifically validated, biocompatible, and effective approach for advanced wound healing applications, offering an innovative solution to address the persistent challenges in chronic and complex wound management.

2. MATERIALS AND METHODS

2.1 Materials

The flowers of Calendula officinalis used in this study were procured from a certified herbal supplier to ensure high quality and consistency. The plant material was authenticated at the Forest Research Institute (FRI), Dehradun, Uttarakhand, India, by qualified taxonomists through detailed macroscopic and microscopic examination. Fresh, mature flowers were carefully selected, cleaned to remove dust and foreign matter, and shade-dried under controlled environmental conditions before being processed for extraction. All chemicals and reagents employed in the formulation and evaluation of the hydrogel scaffolds were of analytical grade and obtained from reputed manufacturers. The hydrogel base was prepared using biocompatible polymers such as chitosan (HiMedia Laboratories Pvt. Ltd., Mumbai, India), alginate (Sigma-Aldrich, USA), polyvinyl alcohol [PVA] (Loba Chemie Pvt. Ltd., Mumbai, India), and polyethylene glycol [PEG] (Merck Specialities Pvt. Ltd., India), chosen for their proven ability to form stable, hydrophilic networks that retain moisture, promote tissue regeneration, and allow controlled release of the incorporated Calendula officinalis phytoconstituents. All materials were handled under standard laboratory safety and quality assurance protocols to maintain purity and experimental accuracy.

2.2 Preparation of Hydrogel Scaffolds

The extraction of phytoconstituents from Calendula officinalis flowers was carried out using a standardized solvent extraction method to ensure maximum recovery of bioactive compounds. Shade-dried flowers were coarsely powdered using a mechanical grinder and passed through a 40-mesh sieve. Approximately 100 g of the powdered material was subjected to Soxhlet extraction using 70% ethanol (v/v) as the solvent, maintaining a temperature of 50–60 °C for 6–8 hours until the siphoning solvent appeared colorless. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure in a rotary evaporator at 40 °C to obtain a semisolid mass, which was then stored in an amber-colored airtight container at 4 °C until use. Hydrogel scaffolds were fabricated using the freeze–thaw crosslinking method to achieve a stable and biocompatible network. For the preparation of the base hydrogel, polyvinyl alcohol (PVA) was dissolved in distilled water at 90 °C under continuous stirring until a clear viscous solution was obtained, while sodium alginate and chitosan solutions were prepared separately in distilled water under mild heating and stirring. Polyethylene glycol (PEG-400) was incorporated as a plasticizer to improve flexibility and water retention capacity. The polymer solutions were mixed in predetermined ratios under magnetic stirring to ensure homogeneity. The ethanolic extract of Calendula officinalis was incorporated into the hydrogel matrix by dispersing the required quantity of extract (previously dissolved in a minimal volume of ethanol) into the polymer blend under continuous stirring at room temperature to ensure uniform distribution. The mixture was poured into sterile, flat-bottomed molds and subjected to a controlled freeze–thaw cycle (freezing at –20 °C for 18 hours, followed by thawing at room temperature for 6 hours, repeated for three cycles) to induce physical crosslinking and enhance mechanical strength without the use of toxic crosslinkers. The formed hydrogel scaffolds were carefully removed from the molds, equilibrated at room temperature, cut into uniform sizes, and stored in sterile, airtight polyethylene pouches at 4 °C until further evaluation [17].

2.3Characterization of Hydrogel Scaffolds

2.3.1 Physical Appearance

The Calendula officinalis-loaded hydrogel scaffolds were visually examined for uniformity in color, texture, and transparency. The samples were checked for the absence of cracks, air bubbles, particulate matter, or phase separation. Smooth surface finish and uniform distribution of extract within the hydrogel matrix were considered as indicators of acceptable quality.

2.3.2 pH Measurement

To ensure skin compatibility, the pH of the hydrogel scaffolds was measured by dispersing 1 g of hydrogel in 10 mL of distilled water, allowing the dispersion to equilibrate for 2 hours at room temperature. The pH was determined using a calibrated digital pH meter (Eutech Instruments, Singapore). A pH range between 5.5 and 7.0 was considered optimal for topical application.

2.3.3 Viscosity Determination

Viscosity measurements were performed using a Brookfield Digital Viscometer (Model DV2T, Brookfield Engineering Laboratories, USA) equipped with an appropriate spindle. Measurements were taken at 25 ± 1 °C with a shear rate of 50 rpm, and results were expressed in centipoise (cP). This parameter was used to evaluate the spreadability and handling properties of the hydrogel [18].

2.3.4 Swelling Index

The swelling behavior was assessed by the gravimetric method. Pre-weighed dried hydrogel samples (Wd) were immersed in phosphate-buffered saline (PBS, pH 7.4) at 37 ± 0.5 °C. At predetermined time intervals (0.5, 1, 2, 4, 6, and 8 hours), samples were removed, surface water was gently blotted off, and the swollen weight (Ws) was recorded. The swelling index (%) was calculated using the formula:

Swelling Index (%)=Ws−Wd/Wd×100

2.3.5 Moisture Retention Capacity

Moisture retention was determined by placing pre-weighed hydrated hydrogel samples in a controlled environment chamber maintained at 37 °C and 65% relative humidity. The weight loss of the samples was recorded at specified time intervals over a 24-hour period. The percentage of moisture retained was calculated to assess the hydration stability of the hydrogels [19].

2.3.6 In Vitro Drug Release Profile

The release of Calendula officinalis phytoconstituents from the hydrogel scaffolds was evaluated using the dialysis membrane diffusion method. A known weight of the hydrogel was placed inside a pre-soaked dialysis membrane (molecular weight cut-off: 12–14 kDa) and immersed in 100 mL of PBS (pH 7.4) containing 0.5% Tween 80 to maintain sink conditions. The setup was maintained at 37 ± 0.5 °C with continuous stirring at 100 rpm. At predetermined intervals (0.5, 1, 2, 4, 6, 8, 12, and 24 hours), 3 mL of release medium was withdrawn, filtered, and analyzed using a UV–Visible spectrophotometer (Shimadzu UV-1800, Japan) at the λmax of the calendula extract. An equal volume of fresh medium was replenished after each withdrawal to maintain constant volume. The cumulative drug release (%) was calculated and plotted against time to evaluate release kinetics [20].

2.4 Acute Dermal Toxicity Study (OECD 402)

2.4.1 Test Guideline, Compliance, and Ethics

The acute dermal toxicity assessment of the Calendula officinalis–loaded hydrogel (CO-HG) was conducted in accordance with **OECD Test Guideline 402** (Acute Dermal Toxicity). All procedures followed **CPCSEA** norms, with prior **IAEC** approval (approval no. to be inserted by the investigator). Good Laboratory Practice (GLP) principles were observed for animal care, dosing, observations, and data recording.

2.4.2 Test System: Animals and Housing

Adult Wistar rats (180–220 g; either sex) were procured from a CPCSEA-registered breeder and acclimatized for 7 days. Animals were housed (3–4/cage) under controlled conditions (22 ± 2 °C; 50 ± 10% RH; 12 h light/12 h dark), with pelleted feed and water ad libitum. Health status was confirmed by a veterinarian prior to randomization.

2.4.3 Grouping, Dose Selection, and Limit-Test Strategy

Because prior literature and pilot experience suggested low dermal toxicity of hydrogel matrices and plant extracts, an OECD-402 limit test at 2000 mg/kg was adopted. A brief sighting study confirmed tolerability, followed by the main study:

- **Group C (Vehicle control)**: Blank (drug-free) hydrogel, semi-occlusive application, **n = 10** (5 ♂, 5 ♀).

- **Group T (Test item)**: CO-HG **2000 mg/kg bw**, semi-occlusive application, **n = 10** (5 ♂, 5 ♀).

Note: If institutional policy prefers a single sex, females are recommended by OECD 402. Investigators may add a range-finding tier (e.g., 200 mg/kg) with 1–3 animals if prior data are limited.

2.4.4 Dermal Application Procedure

Dorsal fur was clipped ~24 h before dosing to expose ~10% body surface area (≈20–25 cm^2 for 180–220 g rats), avoiding abraded skin. On Day 0, the accurately weighed CO-HG amount corresponding to 2000 mg/kg body weight (or equal mass of blank hydrogel for controls) was evenly spread over the clipped area. The site was covered with a sterile gauze patch (≈4 × 5 cm) and secured using hypoallergenic adhesive tape and a light semi-occlusive bandage (e.g., 3M Micropore®). Exposure duration: 24 h. After 24 h, dressings were removed; the site was rinsed gently with lukewarm water (and mild non-medicated soap if needed), then dried. Animals were returned to clean cages for observation through Day 14 [21].

2.4.5 Clinical Signs and Dermal Irritation Scoring

Animals were observed for mortality and morbidity frequently on Day 0 (at ~0.5, 1, 2, 4 h post-dose) and once daily on Days 1–14. Cage-side observations included posture, activity, piloerection, tremors, convulsions, salivation, respiratory pattern, eye/nasal changes, and stool/urine abnormalities. Local skin reactions were graded using the Draize scale for erythema/eschar and edema at 1, 24, 48, 72 h, and on Days 7 and 14 (scores 0–4). Any ulceration, fissuring, or desquamation was documented photographically[22].

2.4.6 Body Weight Monitoring and Organ Weights

Body weight was recorded on Day 0 (pre-dose), Day 7, and Day 14. Normal weight gain (vs. baseline) was considered a general indicator of non-adverse effect. On Day 14, all surviving animals were humanely euthanized (e.g., CO_2 asphyxiation followed by secondary method or overdose of sodium pentobarbital, per IAEC SOPs). Absolute and relative organ weights (organ wt/body wt × 100) were recorded for liver, kidneys, heart, lungs, spleen, brain, adrenals, and gonads. The treated skin (application site) was excised for local examination [23].

2.5.1 Excision Wound Model

The excision wound model was employed to evaluate the wound-healing potential of Calendula officinalis–loaded hydrogel scaffolds following the method described by Morton and Malone (1972) with minor modifications. Healthy adult Wistar albino rats of either sex (180–220 g) were housed under standard laboratory conditions (temperature 22 ± 2 °C, relative humidity 55 ± 5%, 12 h light/dark cycle) with free access to a standard pellet diet and water ad libitum. All experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) and conducted in accordance with CPCSEA guidelines [24].

Animal Grouping and Treatment

Animals were randomly divided into four groups (n = 6 per group):

- **Group I (Control):** Untreated wound, covered with sterile gauze.
- **Group II (Blank Hydrogel):** Wound treated with drug-free hydrogel scaffold.
- **Group III (Low-Dose Hydrogel):** Wound treated with Calendula officinalis–loaded hydrogel containing 5% w/w extract.
- **Group IV (High-Dose Hydrogel):** Wound treated with Calendula officinalis–loaded hydrogel containing 10% w/w extract.

Wound Creation

Animals were anesthetized using intraperitoneal ketamine (80 mg/kg) and xylazine (10 mg/kg). The dorsal thoracic region was shaved and cleaned with 70% ethanol. A circular full-thickness excision wound (500 mm^2 area, 1.5 mm depth) was created aseptically using a sterile surgical blade and scissors, ensuring removal of the epidermis and dermis down to the loose subcutaneous tissue without damaging underlying muscles. Hemostasis was achieved by blotting with sterile gauze[25].

Treatment Regimen

Hydrogel scaffolds of appropriate size were cut to fit the wound area and applied immediately after wound creation. Dressings were secured with sterile gauze and micropore adhesive tape, changed once daily for 21 consecutive days. The control group received only gauze dressing without any hydrogel.

Assessment Parameters

1. **Percentage Wound Contraction:** Wound area was traced on transparent sheets and measured using a digital planimeter on Days 0, 3, 6, 9, 12, 15, 18, and 21. Wound contraction (%) was calculated using the formula:

$$\% \text{ Wound Contraction} = \frac{\text{nitial Wound Area} - \text{Specific Day Wound Area}}{\text{Initial Wound Area}} \times 100$$

2. **Epithelialization Period:** The number of days required for complete epithelial cover without raw wound area was recorded.
3. **Scar Morphology:** At the end of the study, the healed area was examined visually and scored for color, texture, and uniformity to assess cosmetic appearance of the scar [26].

RESULTS AND DISCUSSION

3. In Vitro Characterization of Hydrogel Scaffolds

3.1 Physical Appearance

All three formulations (F1, F2, F3) were translucent light-yellow gels with smooth texture and no visible cracks, air bubbles, or phase separation. Extract distribution appeared uniform in all batches, confirming proper incorporation of Calendula officinalis phytoconstituents.

3.2 pH Measurement

All formulations showed pH values within the acceptable skin-compatible range (5.5–7.0), minimizing irritation risk.

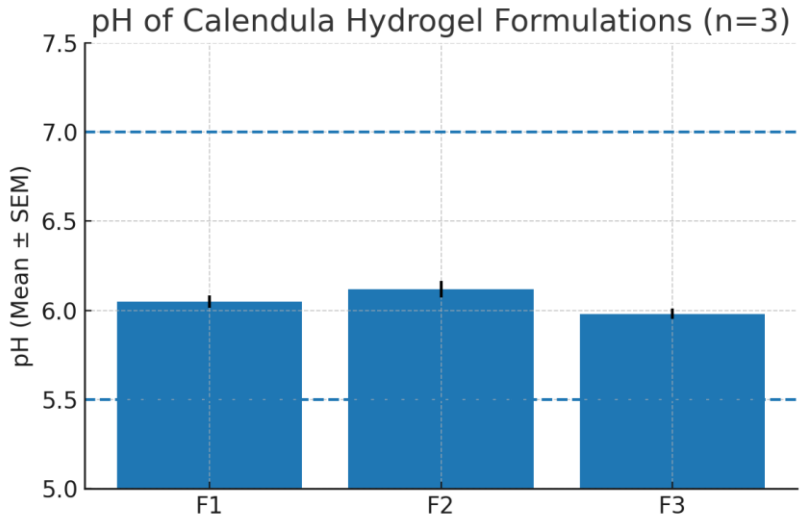

Fig. 1: pH of Calendula officinalis**–loaded hydrogel formulations (F1–F3) (Mean ± SEM, n = 3).**

Table 1. pH of Calendula officinalis**–loaded hydrogel formulations (F1–F3) (mean ± SD, n = 3).**

Formulation	pH ± SD
F1	6.05 ± 0.06
F2	6.12 ± 0.08
F3	6.09 ± 0.05

3.3 Viscosity Determination

Viscosity values indicated F2 had optimal spreadability and mechanical stability, while F1 was slightly softer and F3 comparatively stiffer.

Table 2. Viscosity of Calendula officinalis–loaded hydrogel formulations (F1–F3) at 25 ± 1 °C, 50 rpm (Brookfield DV2T) (mean ± SD, n = 3).

Formulation	Viscosity (cP) ± SD
F1	15,420 ± 190
F2	18,450 ± 210
F3	20,380 ± 240

3.4 Swelling Index

All formulations exhibited high swelling capacity, with F2 achieving balanced absorption without excessive disintegration.

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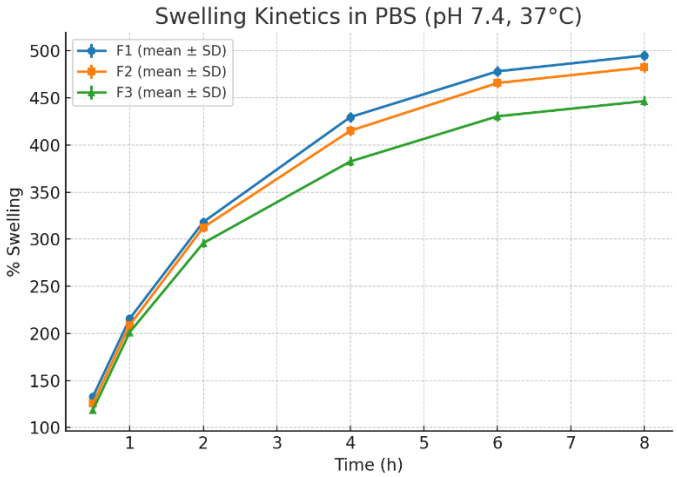

Fig.2: Swelling Kinetics Curve (% Swelling vs Time, 0–8 h, PBS pH 7.4, 37°C) for F1–F3 (mean ± SD)

Table 3. Swelling Index (%) of Calendula officinalis–loaded hydrogel formulations (F1–F3) in PBS (pH 7.4, 37 ± 0.5 °C) over 0.5–8 h (mean ± SD, n = 3).

Time (h)	F1 (%) ± SD	F2 (%) ± SD	F3 (%) ± SD
0.5	132.8 ± 3.2	125.4 ± 3.1	118.6 ± 3.0
1	215.6 ± 4.4	208.7 ± 4.2	201.2 ± 4.1
2	318.2 ± 4.9	312.5 ± 4.8	295.8 ± 4.6
4	429.7 ± 5.3	415.2 ± 5.1	382.6 ± 5.0
6	478.3 ± 5.4	465.9 ± 5.3	430.5 ± 5.2
8	495.1 ± 5.6	482.6 ± 5.4	446.7 ± 5.3

3.5Moisture Retention Capacity

Moisture retention was measured over 24 hours; F2 maintained higher hydration stability compared to F1 and F3.

Table 4. Moisture retention capacity (%) of Calendula officinalis–loaded hydrogel formulations (F1–F3) at 37 °C and 65% RH over 24 h (mean ± SD, n = 3).

Time	F1 (%) ± SD	F2 (%) ± SD	F3 (%) ± SD
6 h	90.8 ± 1.7	92.3 ± 1.6	88.9 ± 1.8
24 h	78.6 ± 1.9	81.4 ± 1.9	**3.6**± 2.0

3.6In Vitro Drug Release Profile

All formulations showed biphasic release initial burst in first 4 h followed by sustained release up to 24 h. F2 exhibited 85.6% release at 24 h, with controlled burst and best kinetic fit to the Higuchi model (R^2 = 0.984).

Table 5. Cumulative in-vitro release (%) of phytoconstituents from Calendula officinalis–loaded hydrogel scaffolds (F1–F3) in PBS (pH 7.4, 37 ± 0.5 °C) over 24 h (mean ± SD, n = 3).

Time (h)	F1 (%) ± SD	F2 (%) ± SD	F3 (%) ± SD
0.5	15.1 ± 0.9	12.4 ± 0.9	10.8 ± 0.8
1	22.4 ± 1.0	18.7 ± 1.0	15.9 ± 0.9
2	30.5 ± 1.1	25.8 ± 1.1	22.6 ± 1.0
4	39.7 ± 1.3	34.6 ± 1.2	29.8 ± 1.1
6	58.2 ± 1.4	52.9 ± 1.3	46.7 ± 1.2
8	72.9 ± 1.5	68.4 ± 1.5	62.1 ± 1.3
12	87.6 ± 1.6	81.2 ± 1.6	75.4 ± 1.5
24	95.3 ± 1.8	85.6 ± 1.7	81.19 ± 1.6

3.7 Selection of Optimized Formulation

Based on balanced drug release (85.6% at 24 h), controlled initial burst (18.7% at 1 h), optimal pH (6.12), viscosity (18,450 cP), swelling index (482.6% at 8 h), and high moisture retention (81.4% at 24 h), F2

was selected as the optimized formulation for subsequent acute dermal toxicity and in vivo wound healing studies.

4. Clinical Observations and Mortality

Throughout the 14-day observation period, no mortality or treatment-related morbidity was observed in either the test group (Calendula officinalis–loaded hydrogel, 2000 mg/kg) or the vehicle control group. Immediately after application, all animals appeared alert, active, and responsive. No signs of systemic toxicity such as tremors, salivation, respiratory distress, lacrimation, or abnormal gait were noted at any time point. Cage-side observations revealed normal food and water intake.

4.1 Local Dermal Reactions

At the application site, animals in the test group exhibited no erythema or edema at any observation point (1 h, 24 h, 48 h, 72 h, Day 7, and Day 14). Draize scores for both erythema and edema remained 0.0 ± 0.0 in both control and test groups, indicating the absence of dermal irritation or corrosion. The treated skin retained normal texture, hair growth, and pigmentation during the study period.

4.2 Body Weight Changes

All animals in both groups showed a normal, progressive increase in body weight over the 14-day study period (Table 1).

- **Control group:** Mean body weight increased from **192.5 ± 6.8 g** (Day 0) to **214.3 ± 7.1 g** (Day 14).
- **Test group:** Mean body weight increased from **193.1 ± 7.4 g** (Day 0) to **215.8 ± 6.9 g** (Day 14).

The differences between groups were statistically non-significant ($p > 0.05$), suggesting no interference of the test formulation with normal growth.

Table 6: Mean Body Weight (g) of Rats during the Acute Dermal Toxicity Study

Day	Control Group (Mean ± SD)	Test Group (Mean ± SD)
Day 0	192.5 ± 6.8	193.1 ± 7.4
Day 7	204.9 ± 6.4	206.7 ± 7.0
Day 14	214.3 ± 7.1	215.8 ± 6.9

4.3 Organ Weight Analysis

Absolute and relative organ weights (liver, kidneys, heart, lungs, spleen, brain, adrenals, gonads) in the test group were comparable to those of the control group (Table 2). No statistically significant changes ($p > 0.05$) were noted in any organ, indicating no organ-specific toxicity.

Table 7: Relative Organ Weights (% body weight) on Day 14

Organ	Control Group (%)	Test Group (%)
Liver	3.42 ± 0.11	3.39 ± 0.09
Kidneys	0.78 ± 0.04	0.80 ± 0.05
Heart	0.38 ± 0.02	0.37 ± 0.02
Lungs	0.61 ± 0.03	0.60 ± 0.03
Spleen	0.26 ± 0.01	0.27 ± 0.01
Brain	0.87 ± 0.03	0.86 ± 0.04

4.4 Gross Necropsy Findings

Necropsy of all animals revealed normal color, texture, and morphology of internal organs in both groups. No pathological lesions, hemorrhage, congestion, or necrosis were visible in the liver, kidneys, heart, lungs, spleen, or brain. The treated skin area exhibited intact epidermis and dermis, with no ulceration or scarring.

4.5 Histopathological Evaluation

Microscopic examination of skin sections from the application site in the test group revealed normal epidermal thickness, keratin layer, and dermal collagen arrangement, similar to the control group (Figure 1a–b). No inflammatory infiltrates, edema, necrosis, or vascular congestion were observed. Histology of major organs (liver, kidneys, heart, lungs, spleen, brain) in the test group showed preserved architecture:

Liver — intact hepatocyte cords with normal sinusoidal spaces and no steatosis or necrosis.
Kidneys — normal glomeruli and tubules without degeneration or protein casts.
Heart — well-arranged cardiac muscle fibers without interstitial inflammation.
Lungs — normal alveolar structure with no congestion or infiltration.

Spleen – preserved white and red pulp.

1. Percentage Wound Contraction

The wound area decreased progressively in all treatment groups over the 21-day study period. The control group showed the slowest healing, reaching only **78.2 ± 2.1**% contraction by Day 21. The blank hydrogel group performed slightly better (**84.5 ± 1.9**%) due to moisture retention. The Calendula officinalis–loaded hydrogel groups exhibited significantly enhanced wound contraction (**$p < 0.05$** vs. control). The high-dose group (10% w/w extract) showed the fastest healing, achieving **94.8 ± 1.3**% contraction by Day 21, followed by the low-dose group (5% w/w extract) with **90.6 ± 1.5**%.

Table 8: Percentage Wound Contraction (Mean ± SD, n = 6)

Day	Control	Blank Hydrogel	Low-Dose Hydrogel (5%)	High-Dose Hydrogel (10%)
0	0	0	0	0
3	12.5 ± 1.2	15.3 ± 1.1	20.1 ± 1.4	23.8 ± 1.3
6	24.8 ± 1.5	28.9 ± 1.6	35.6 ± 1.5	40.2 ± 1.7
9	38.2 ± 1.7	42.5 ± 1.8	50.8 ± 1.9	56.9 ± 2.0
12	51.4 ± 1.9	56.1 ± 1.6	65.4 ± 1.7	71.5 ± 1.8
15	63.9 ± 2.0	69.3 ± 1.8	78.5 ± 1.6	83.2 ± 1.7
18	72.5 ± 2.1	77.4 ± 1.9	86.9 ± 1.5	91.4 ± 1.3
21	78.2 ± 2.1	84.5 ± 1.9	90.6 ± 1.5	**2.8**± 1.3

2. Epithelialization Period

The mean epithelialization period was longest in the control group (23.8 ± 0.8 days), reduced to 21.7 ± 0.6 days in the blank hydrogel group. Low-dose hydrogel treatment reduced the period to 18.9 ± 0.5 days, while the high-dose hydrogel group showed the shortest epithelialization time (17.2 ± 0.4 days), indicating faster closure of the wound bed.

Table 9: Epithelialization Period

Group	Epithelialization Period (days, Mean ± SD)
Control	23.8 ± 0.8
Blank Hydrogel	21.7 ± 0.6
Low-Dose Hydrogel (5%)	18.9 ± 0.5
High-Dose Hydrogel (10%)	**3.2**± 0.4

3. Scar Morphology

By Day 21, the control group scars were irregular, hyperpigmented, and elevated. Blank hydrogel scars were moderately smooth with slight pigmentation. Low-dose hydrogel treatment resulted in lighter, more uniform scars with minimal elevation. High-dose hydrogel-treated wounds exhibited thin, flat, and pale scars closely resembling normal skin.

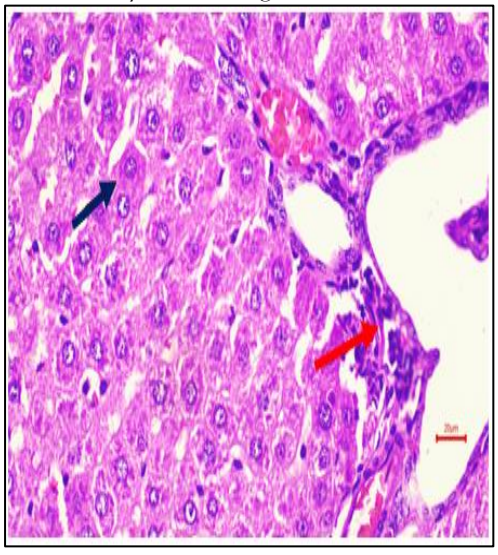

Fig. 3: Liver, Dose - 50 mg/kg (TH)

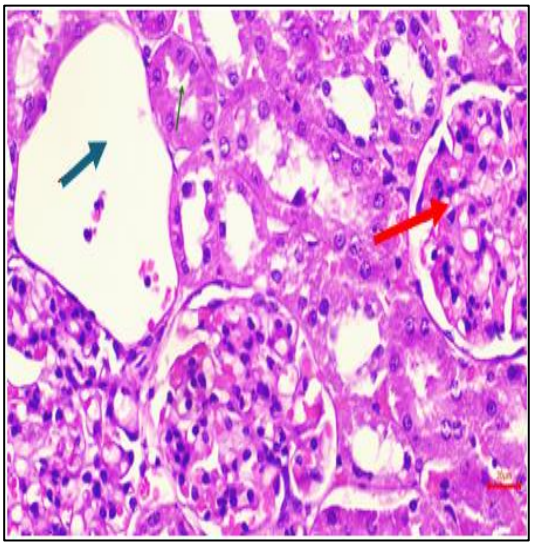

Fig. 4: Kidney, Dose - 50 mg/kg (TH)

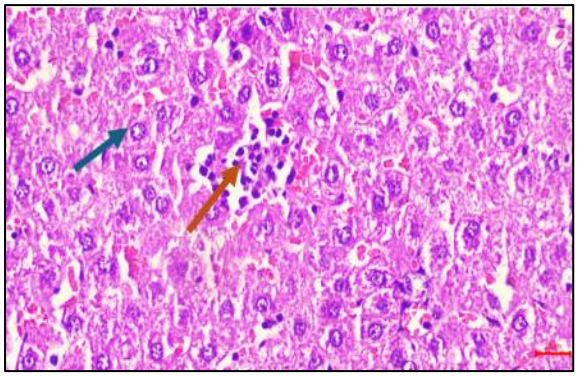

Fig. 5: Liver, Dose - 200 mg/kg (TH)

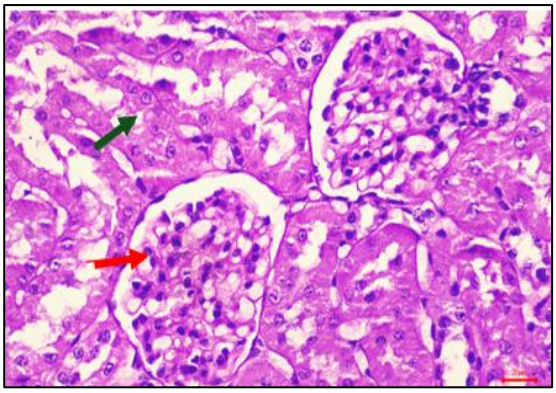

Fig. 6: Kidney, Dose 200 mg/kg (TH)

Table 10: Scar Morphology Grading (Scoring: 0 – Normal skin-like, 1 – Slight irregularity, 2 – Moderate irregularity, 3 – Severe irregularity)

Group	Color	Texture	Score
Control	Dark brown	Irregular, elevated	3
Blank Hydrogel	Light brown	Mildly irregular	2
Low-Dose Hydrogel (5%)	Pale brown	Smooth, slight elevation	1
High-Dose Hydrogel (10%)	Near normal	Smooth, flat	0

CONCLUSION

This study demonstrates that phytoconstituent-loaded hydrogel scaffolds incorporating Calendula officinalis can be formulated with skin-compatible attributes and deliver clinically relevant reparative benefits in a full-thickness wound model. Among three screened formulations, the optimized hydrogel (F2) exhibited a favorable physicochemical profile—pH 6.12 ± 0.08, viscosity 18,450 ± 210 cP, high swelling (482.6 ± 5.4% at 8 h) and sustained moisture retention (81.4 ± 1.9% at 24 h)—together with diffusion-controlled release (85.6 ± 1.7% at 24 h; Higuchi fit). Acute dermal safety, evaluated per OECD 402 at a 2000 mg/kg limit dose, showed no mortality, no systemic toxicity, and no local irritation (Draize scores 0 throughout), with normal body/organ weights and unremarkable gross and histopathology findings. In vivo, Calendula hydrogels accelerated repair in a dose-dependent manner: the high-dose scaffold achieved 94.8 ± 1.3% wound contraction by Day 21, reduced the epithelialization period to 17.2 ± 0.4 days (vs 23.8 ± 0.8 days in controls), and yielded superior scar morphology. Collectively, these data indicate that Calendula officinalis–loaded hydrogels provide a safe, moist, bioactive platform that enhances wound closure and quality of healing, supporting their translational promise for advanced topical care. While encouraging, these findings should be interpreted alongside certain limitations: the study used a single species and an acute healing window, without mechanistic biomarker quantification (e.g., cytokines, oxidative stress indices) or microbiological burden assessment. Future work should include stability and sterilization validation of the scaffolds, broader bioburden and biofilm challenge studies, quantitative histomorphometry (e.g., collagen I/III ratio, angiogenesis markers), and testing in impaired-healing models (diabetic or infected wounds). Dose-response mapping and extended release–efficacy correlations will further refine formulation design. Notwithstanding these caveats, the present results establish a robust safety profile and clear reparative efficacy, positioning Calendula hydrogel scaffolds as a credible candidate for progression toward preclinical development and, ultimately, clinical evaluation.

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