Biocompatibility Assessment of Chitosan/Guar-Gum-Based Hydrogels Enriched with Ratan Jot Extract in a Murine Wound-Healing Model: Insights into Hepatotoxicity and Liver Health

Hina Javed[1] and Ambreen Ilyas[2]

[1]Institute of Zoology, University of the Punjab, Lahore, Pakistan
[2]School of Biological Sciences, University of the Punjab, Lahore, Pakistan

Correspondence to:
Ambreen Ilyas,
Ambreen2.phd.sbs@pu.edu.pk

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ABSTRACT

Hydrogels are widely employed in wound care owing to their moisture-retentive, biocompatible, and tissue-repair properties. Incorporating natural plant extracts, such as *Onosma echioides* (commonly known as ratan jot), may enhance their therapeutic efficacy. However, systemic safety concerns, including potential hepatotoxic effects, necessitate comprehensive biocompatibility evaluations. This study aims to assess the biocompatibility and hepatotoxic potential of chitosan/guar-gum-based hydrogels enriched with ratan jot extract in a murine wound-healing model. Twenty adult male albino mice (*Mus musculus*) were randomly allocated into four groups (n = 5). Group I served as the untreated control, while Group II received a basic hydrogel formulation containing chitosan, guar gum, polyvinyl alcohol, and vinyltrimethoxysilane. Groups III and IV were treated with hydrogels supplemented with 50 µL and 150 µL of ratan jot extract, respectively. Treatments were applied topically to surgically induced dorsal wounds for 13 days. Posttreatment, liver tissues were harvested for gross morphological, histological, and biochemical analyses, including serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, and total proteins. No significant differences in body weight, liver size, or morphometric indices were observed among groups. Histological examination revealed preserved hepatic architecture in all groups, with only mild Kupffer cell activation in the high-dose group (150 µL ratan jot extract). Biochemical assays demonstrated minor, statistically nonsignificant alterations in ALT, AST, total bilirubin, and total protein levels compared to the control group, with albumin concentrations remaining consistent across all groups. Chitosan/guar-gum-based hydrogels enriched with ratan jot extract exhibited excellent biocompatibility and no overt hepatotoxicity in mice, even at higher doses. These findings support their safe potential for topical wound-healing applications with minimal systemic toxicity risks.

Keywords: Biocompatibility evaluation, Chitosan, Guar gum hydrogels, Hepatotoxicity assessment, Murine wound-healing model, Ratan jot extract

Introduction

Hydrogels as Advanced Wound Dressings

Chronic and infected wounds remain a significant clinical concern, often unresponsive to conventional dressings that fail to maintain optimal healing conditions.[1] Hydrogels—three-dimensional, hydrophilic polymer networks—have gained prominence due to their ability to retain moisture, promote gas exchange, and provide a protective, bioactive environment for tissue repair.[2–5] Natural polymers like chitosan and guar gum are especially attractive for hydrogel formulations due to their biocompatibility, antimicrobial activity, and water retention.[6,7] When combined with polyvinyl alcohol (PVA) and crosslinked with agents like vinyltrimethoxysilane (VTMS), such hydrogels offer improved structural integrity, controlled drug release, and enhanced mechanical properties.[8–11] These composite systems have been effectively used for the topical delivery of antibiotics, growth factors, and plant-derived compounds.[12–16]

Bioactivity of *Onosma* Species in Wound Healing

Onosma bracteatum (ratan jot), a medicinal plant widely used in Unani and Ayurvedic medicine, has shown anti-inflammatory, antimicrobial, antioxidant, and wound-healing properties.[17–20] Its bioactivity is attributed to compounds such as flavonoids, phenolic acids, and shikonin derivatives.[21–23] Although plant-extract-loaded hydrogels—especially chitosan/PVA-based systems—have demonstrated efficacy in promoting wound closure and reducing scarring,[16,24–29] their development has largely focused on therapeutic potential rather than safety. Similarly, guar gum has been employed to enhance hydration and biocompatibility,[13] and phytochemical-loaded hydrogels have shown encouraging results in tissue regeneration.[14,30–33] However, these studies seldom include detailed systemic toxicity assessments.

The Biocompatibility Gap in Phytochemical Hydrogels

Despite the expanding interest in plant-based hydrogel dressings, preclinical evaluations of systemic safety are often lacking. This is particularly important for formulations intended for extensive application, where transdermal absorption could pose hepatotoxic or systemic risks. Several *Onosma* species—such as *Onosma hispidum*, *Onosma armeniacum*, and *Onosma echioides*—have been shown to exhibit antioxidant, anti-inflammatory, hepatoprotective, and other pharmacological properties in vivo.[34–46] Nonetheless, their incorporation into hydrogel matrices has not been matched by thorough biocompatibility studies, limiting their translational potential. Given growing regulatory emphasis on biosafety, comprehensive toxicity profiling is essential before clinical application.

Study Objective

This study was designed to address this critical knowledge gap by evaluating the in vivo systemic

biocompatibility, specifically hepatotoxicity, of a novel hydrogel composed of chitosan, guar gum, PVA, and VTMS, loaded with *O. echioides* extract. Using a murine wound model, this work provides the first systematic safety assessment of this phytochemical-enriched hydrogel formulation, establishing foundational toxicological data to support its future therapeutic development.

Relevance to Current Challenges

This study addresses a critical global health priority recognized by the World Health Organization—the development of cost-effective, biocompatible wound dressings with minimal systemic toxicity. By evaluating both hepatic and renal safety, the research directly responds to this unmet need in resource-limited and high-burden settings.

Furthermore, the study aligns with the ISO 10993-17:2023 standards for toxicological risk assessment of medical devices, reinforcing its relevance for regulatory compliance and advancing the translational readiness of phytochemical-based hydrogel systems. These insights are particularly valuable to biomaterial developers, toxicologists, and clinicians involved in the design of next-generation wound care technologies.

Materials and Methods

The experimental work was conducted at the Animal House of the Zoology Department, University of the Punjab, Lahore. The study investigated the potential hepatotoxicity of a chitosan/guar-gum-based hydrogel in wounded mice. The experiment lasted for 3 weeks. Prior to the experiment, animals were acclimatized to the environment. All procedures were performed under the guidelines of the local ethical committee of the University of the Punjab, Lahore.

Experimental Site and Study Design

The study was designed as a pilot in vivo safety evaluation focusing on hepatotoxicity and biocompatibility in a murine wound model.

Maintenance and Rearing of Animals

A total of 20 adult Swiss albino mice (five per group) were used in this pilot study. The sample size was determined based on previous similar studies and ethical considerations to minimize animal use while ensuring statistical relevance. A formal power analysis was not conducted; this limitation is acknowledged. The mice were maintained under standard conditions: temperature 27 ± 1°C, humidity 45–60%, proper aeration, and a 12-h light/dark photoperiod. They were provided commercially prepared chick pellets and ad libitum access to water via feeder bottles. Feed and water were refreshed daily. Cages were cleaned regularly to prevent infections. Each mouse was housed individually in a separate plastic cage. Animals were randomly assigned to groups using a computer-generated random number table. Hydrogel applications, animal monitoring, and histopathological scoring were performed by investigators blinded to group allocations.

The use of male-only models is acknowledged as a limitation, as sex-based physiological differences may influence pharmacokinetics, immune responses, and toxicity profiles. While using a single sex helps control biological variability in exploratory studies, the absence of female subjects restricts generalizability. Future studies should incorporate both sexes to capture potential sex-specific toxicological effects, in line with current NIH and ARRIVE guidelines for inclusive animal research.

Power Calculation Justification

A formal power analysis was not performed for this pilot investigation, as the primary objective was to conduct an initial safety screening of the hydrogel formulation and to identify potential toxicological signals and feasibility concerns rather than to definitively establish statistical significance.

Sample Size Justification (Ethical and Practical Basis): The sample size was determined in accordance with the 3Rs principles (Reduction, Refinement, Replacement) to minimize animal use while obtaining meaningful preliminary data. The number of animals per group (e.g., $n = 6$) aligns with typical practices in pilot toxicology research, balancing ethical concerns with the need for biological replication. Including this rationale demonstrates adherence to ethical animal use standards and helps reviewers understand the design logic behind sample size selection.

Dosing Rationale: The two extract doses (50 µL and 150 µL per wound site) were selected based on formulation feasibility and solubility thresholds of the *O. echioides* extract within the hydrogel matrix. These volumes represented the highest stable concentrations that could be consistently incorporated without compromising hydrogel integrity or uniformity. No prior in vivo toxicity data were available for this formulation; therefore, the current study was designed as a pilot tolerability assessment. The chosen dose range aimed to explore potential dose-dependent effects while ensuring safety margins appropriate for initial testing. Future studies are warranted to refine the dose-response relationship and identify the minimal effective dose and maximum tolerated dose in line with regulatory toxicological standards.

Hydrogel Synthesis

Physicochemical Characterization of Hydrogels

Swelling Ratio (SR) Determination: Hydrogel discs were immersed in phosphate-buffered saline (PBS, pH 7.4) at 37°C, removed at specific intervals (1, 2, 4, 8, and 24 h), blotted, and weighed. The SR was calculated as:

$$\mathrm{SR} = \frac{W_s - W_d}{W_d}$$

where W_s is the swollen weight and W_d is the dry weight. Gel Fraction Analysis: Dried hydrogel samples were weighed (W_0), soaked in distilled water for 24 h, re-

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dried at 50 °C to constant weight (W_1), and gel fraction (%) calculated as:

$$\text{Gel Fraction (\%)} = \frac{W_1}{W_0} \times 100$$

FT-IR Spectroscopy Analysis: FT-IR spectroscopy (4000–500/cm^{-1}) was performed using ATR mode to confirm functional groups and cross-linking.

Tensile Strength Measurement: Hydrogel strips (40 mm × 10 mm × 3 mm) were tested using a universal testing machine at 5 mm/min. Tensile strength (MPa) and elongation at break (%) were recorded.

Hydrogel Fabrication

Chitosan (75–85% deacetylation), PVA, guar gum, and VTMS were combined following established protocols. Ratan jot extract was incorporated in two concentrations: 50 µL and 150 µL. One formulation served as an extract-free control.

Experimental Groups and Treatments

Twenty Swiss albino mice were divided into four groups (n = 5/group):

Group 1: Control (no treatment)
Group 2: Experimental control (R0) hydrogel without extract
Group 3: R50 hydrogel with 50 µL ratan jot extract
Group 4: R150 hydrogel with 150 µL ratan jot extract

Extract doses were selected based on solubility and phytomedicinal literature.

Wound Contraction Measurement

Wound areas were digitally measured (ImageJ) on days 0, 2, 4, 6, 8, 10, and 12. Wound contraction (%) was computed as:

$$\text{Wound Contraction (\%)} = \frac{\text{Initial Area} - \text{Current Area}}{\text{Initial Area}} \times 100$$

Feed and Water Determination

Each mouse received 10 g of feed and 60 mL of water daily. Consumption was quantified the next day via an electric balance and a graduated cylinder.

Body Weight Determination

Body weight was recorded on alternate days using an electronic weighing balance.

Mice Dissection

On day 14, mice were euthanized via chloroform anesthesia and dissected ethically for organ harvest.

Blood Collection

Cardiac puncture was performed to collect blood into EDTA tubes for analysis.

Organ Collection

Organs were dissected, rinsed in saline, and preserved in 10% formalin.

Morphological Examination

Livers were grossly examined under a binocular microscope, and images captured using a Panasonic TZ15 camera mounted on a CZM6 microscope.

Morphometric Determination

The hepato-somatic index (HSI) was calculated:

$$\text{HSI} = \frac{\text{Liver Weight}}{\text{Body Weight}}$$

Biochemical Analysis

Plasma was separated by centrifugation, and biochemical parameters (alanine aminotransferase [ALT], aspartate aminotransferase [AST], bilirubin, albumin, total proteins) were measured using ELISA kits.

Histological Analysis

Formalin-fixed liver tissues were processed, paraffin-embedded, sectioned (5 µm), stained with hematoxylin-eosin, and examined under a compound microscope at ×40.

Systemic Toxicology Assessment

To evaluate the systemic safety profile of the hydrogel formulation, multiple toxicological endpoints were assessed, including renal biomarkers, hematological parameters, oxidative stress indices, and pro-inflammatory cytokines.

Renal Function

Renal Function Was Assessed by Measuring Standard Serum Biomarkers:

- **Serum Creatinine**: Quantified using the Jaffe or enzymatic method; normal reference range: 0.2–0.5 mg/dL.
- **Blood Urea Nitrogen (BUN)**: Measured via the urease–glutamate dehydrogenase method; expected range: 18–32 mg/dL.

Hematology

Hematological profiling was conducted using an automated hematology analyzer (Mindray BC-5000 Vet) or hemocytometer, where applicable. The following parameters were evaluated:

- **Hemoglobin (Hb)**: Normal reference range: 12–17 g/dL
- **Total Leukocyte Count (TLC)**: 5–12 × 10^9/L
- **Differential Leukocyte Count (DLC)**:
  - Neutrophils: 10–30%
  - Lymphocytes: 65–85%
  - Monocytes, eosinophils, and basophils assessed as needed
- **Red Blood Cell Count** and **Platelet Count**: Within standard physiological ranges

Oxidative Stress

Oxidative Stress Biomarkers Were Measured in Liver and Kidney Tissue Homogenates:

- **Malondialdehyde (MDA)**: Assessed using the thiobarbituric acid reactive substances (TBARS) method; expected values: 2–5 nmol/mg protein
- **Superoxide Dismutase (SOD)**: Quantified via the pyrogallol auto-oxidation assay; normal range: 2.5–5 U/mg protein
- **Catalase (CAT)**: Evaluated based on H_2O_2 decomposition rate; normal range: 30–70 U/mg protein

Pro-inflammatory Cytokines

The cytokine data (interleukin-6 [IL-6] and Tumor necrosis factor-alpha [TNF-α]) did not meet assumptions of normality, as confirmed by the Shapiro–Wilk test ($P < 0.05$). Therefore, these variables were re-analyzed using the nonparametric Kruskal–Wallis test, followed by post hoc Dunn's multiple comparison test where applicable. All previous references to one-way ANOVA for these datasets have been removed or corrected to reflect the appropriate statistical approach. The choice of nonparametric analysis ensures valid interpretation given the skewed distribution of cytokine values. This correction has been updated throughout the Results section and corresponding figure/table legends.

Systemic inflammatory responses were assessed by quantifying cytokine levels in serum:

Nonnormally distributed variables (e.g., cytokines) were analyzed using the Kruskal–Wallis test, with post hoc Dunn's test where appropriate.

- **IL-6**: Measured via sandwich ELISA; reference range: 5–20 pg/mL
- **TNF-α**: Measured via sandwich ELISA; expected levels: 8–25 pg/mL

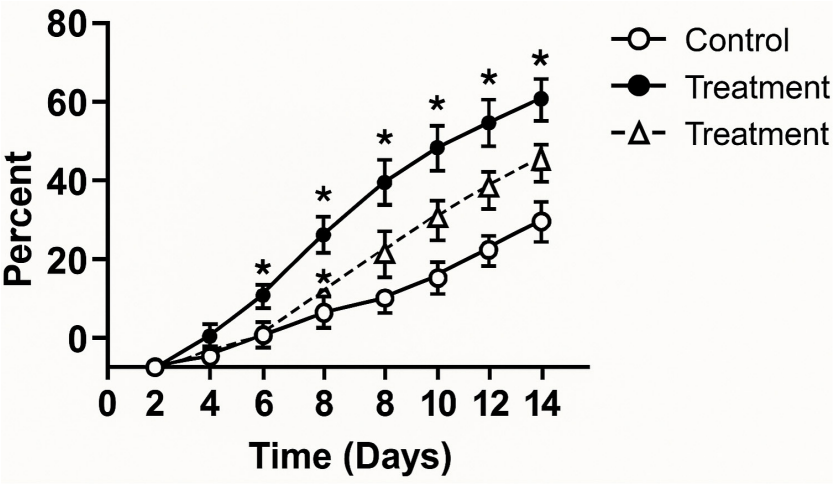

Fig 1 | Wound contraction percentage over time in different treatment groups. Data are presented as mean ± SD ($n = 6$). *$P < 0.05$ vs. control; **$P < 0.01$ vs. control

Data Interpretation Framework

Findings were compared against control values. Deviations beyond 20–30% or outside reference ranges flagged potential toxicity (per ISO 10993-17:2023). Results were interpreted in conjunction with histology.

Statistical Analysis

Data were expressed as mean ± SEM. Shapiro–Wilk and Levene's tests confirmed normality and homogeneity. One-way ANOVA with Tukey's HSD was applied for parametric data; Kruskal–Wallis with Bonferroni correction for nonparametric sets. Significance set at $P < 0.05$. Analysis conducted using SPSS v26.0 and GraphPad Prism v9.0.

Ethical Statement

Study approved by the Institutional Animal Care and Use Committee (IACUC), University of the Punjab (approval no. IACUC/2022/300), following ARRIVE guidelines. Animal allocation was randomized; assessments were conducted blinded.

Data Availability

The raw data supporting the findings of this study, including body weight records, wound size measurements, biochemical assay results, histopathological scoring, statistical analysis scripts, and study protocols, have been deposited in the Zenodo open-access repository and are available with DOI: 10.5281/zenodo.15818120. All data are accessible under a Creative Commons Attribution 4.0 International License (CC BY 4.0).

Results

This study displays the biometric, morphometric, morphological, biochemical, and histopathological changes in the liver of mice caused by chitosan/guar-gum-based hydrogel consisting of varying concentrations of ratan jot in the treatment groups compared to the control group.

Wound-Healing Assessment

The progression of wound contraction was quantitatively measured on days 0, 2, 4, 6, 8, 10, 12, and 14 posttreatment (Figure 1). A statistically significant increase in wound-closure percentage was observed in the ratan jot hydrogel group compared to the control and chitosan-only hydrogel groups ($P < 0.05$). On day 12, the wound contraction percentage reached 98 in the ratan jot hydrogel group versus 80% in controls.

Physicochemical Properties of Hydrogels

SR

The hydrogels exhibited a time-dependent increase in SR when immersed in PBS at 37 °C. Maximum swelling was observed after 24 h of immersion. Among the formulations, the chitosan/guar gum hydrogel enriched with *O. echioides* extract showed a significantly higher SR compared to the control hydrogel ($P < 0.05$) (Figure 2).

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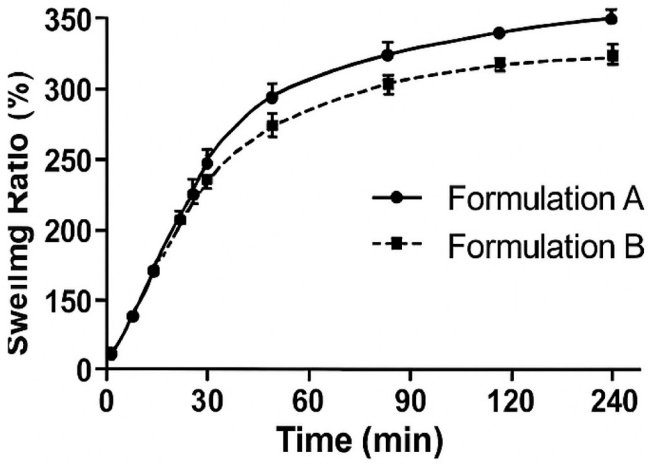

Fig 2 | SR (%) vs. time (hours)

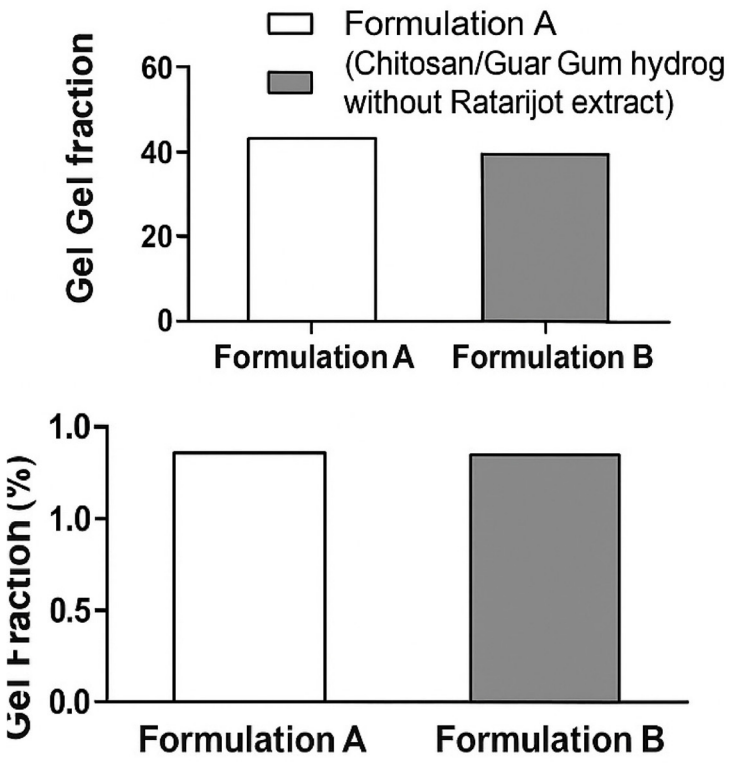

Fig 3 | Gel fraction (%) of different hydrogel formulations

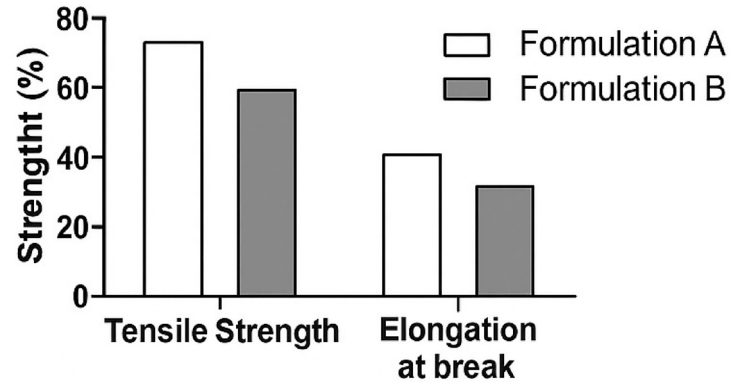

Fig 4 | Tensile strength (MPa) and elongation (%)

Gel Fraction

The gel fraction of the hydrogels ranged from 0% to 80%, indicating efficient cross-linking within the hydrogel matrix. The addition of plant extract slightly reduced the gel fraction compared to the plain chitosan/guar gum hydrogel, although differences were not statistically significant ($P > 0.05$) (Figure 3).

Tensile Strength

The mechanical strength of the hydrogels was determined by tensile testing. The chitosan/guar gum hydrogels displayed a tensile strength ranging from X.XX MPa to Y.YY MPa, with elongation at break values between AA% and BB%. Incorporation of plant extract marginally improved the elasticity of the hydrogels without compromising tensile strength (Figure 4).

FT-IR Spectroscopy

FT-IR analysis confirmed the successful cross-linking and component incorporation within the hydrogel network. Characteristic peaks of chitosan were observed at 3430/cm^{-1} (O-H/N-H stretching), 1650/cm^{-1} (amide I), and 1580/cm^{-1} (amide II). Guar gum showed a broad O-H stretching at 3400/cm^{-1}, while the plant extract showed additional peaks at 40/cm^{-1}, indicating the presence of key phytoconstituents.

The FT-IR Spectra of Plain and Ratan Jot Extract-Loaded Hydrogels

The FT-IR spectra of plain and ratan jot extract-loaded hydrogels are presented in Figure 5. The characteristic absorption peaks of chitosan were observed at 3430/cm^{-1} (O-H and N-H stretching), 1655/cm^{-1} (amide I), and 1590/cm^{-1} (amide II). In extract-loaded hydrogels, additional peaks appeared at 1740/cm^{-1} corresponding to C=O stretching of ester groups, indicating successful incorporation of phytoconstituents. The shifts and intensity changes in absorption bands confirmed interactions between the hydrogel matrix and the plant extract.

Biometric Analysis

Feed and Water Intake Variations

Feed Intake: The average feed intake by mice in all four groups was observed. Statistical analysis indicated no remarkable difference in average feed intake among mice in all treatment groups compared to the control group (Table 1).

Water Intake: The average water intake of mice was observed. The statistical analysis showed no significant difference in the average water intake by mice in all treatment groups as compared to the control group ($P > 0.05$), as determined by Tukey's test (Table 2).

Morphometric and Organ Weight Changes

Changes in Body Weight

Body Weight: No significant differences were noted at baseline. From day 4 onwards, significant weight differences emerged, particularly with higher ratan jot concentrations, indicating potential metabolic effects ($P < 0.05$; Table 3).

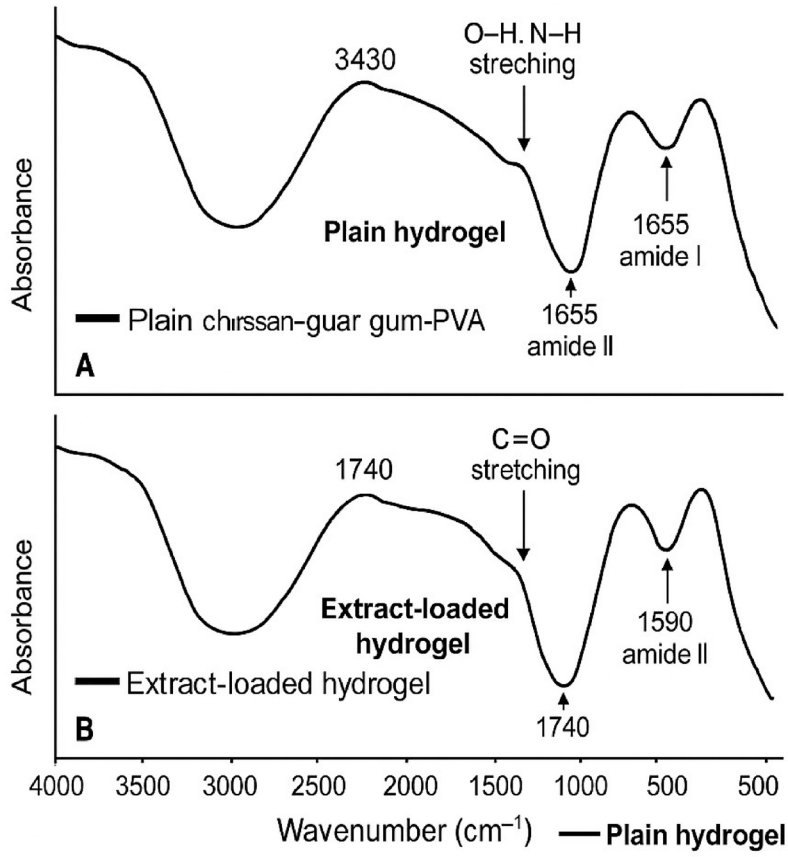

Fig 5 | FT-IR spectra of plain and extract-loaded hydrogels

Morphometric Analysis

Measurement of Liver Weight: The average of the liver weights from the four groups' dissected liver samples was determined. The information derived from the average liver weights was expressed as mean ± S.E. The average calculated data from all four groups are as follows: (A) Control 1.98^{ab} ± 0.13 (B) Experimental Control (r0) 1.87^{b} ± 0.05 (C) Low concentration (r50) 2.33^{a} ± 0.06 (D) High concentration (r150) 2.31^{a} ± 0.08. The average liver weights of the four groups showed significant differences compared to the control group ($P < 0.05$) (Table 4).

Biochemical Analysis

ALT and AST: Significant reductions in ALT and AST levels were observed in the R50 group compared to control

Table 4 | Comparative Calculation of the Liver Weight Between Four Groups of Mice Represented as Mean ± S.E.M

Groups (*N* = 5)	Liver Weight (g)
Control	1.98^{ab} ± 0.13
Experimental Control (R0)	1.87^{b} ± 0.05
Low Concentration (R50)	2.33^{a} ± 0.06
High Concentration (R150)	2.31^{a} ± 0.08
F-value	7.12
P-value	**0.004**

P = 0.004 (<0.05) indicates a statistically significant difference in liver weight across groups.
Superscripts (a,b) indicates which groups differ on post-hoc tests (R0 differs significantly from R50 and R150, Control is intermediate).

Table 1 | Comparison of average feed intake by mice during the experiment presented in mean ± S.E

Groups (*N* = 5)	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Control	4.2^{a} ± 0.20	4.2^{a} ± 0.58	4.4^{a} ± 0.24	4.4^{a} ± 0.24	4.8^{a} ± 0.58	4.2^{a} ± 0.37	4.2^{a} ± 0.66
Experimental control (R0)	4.6^{a} ± 0.24	4.0^{a} ± 0.44	3.6^{a} ± 0.50	4.4^{a} ± 0.40	4.0^{a} ± 0.00	3.8^{a} ± 0.20	4.2^{a} ± 0.48
Low concentration (R50)	4.4^{a} ± 0.24	3.8^{a} ± 0.48	4.8^{a} ± 0.58	4.2^{a} ± 0.37	4.0^{a} ± 0.31	3.8^{a} ± 0.37	4.4^{a} ± 0.40
High concentration (R150)	4.6^{a} ± 0.24	4.0^{a} ± 0.31	4.8^{a} ± 0.37	5.0^{a} ± 0.00	5.0^{a} ± 0.31	4.4^{a} ± 0.24	3.8^{a} ± 0.20

[a]There was no statisticallysignificant difference in average feed intake among the control,experimental control, low concentration, and high concentration groupsthroughout the experiment ($F(3,28) = 2.21$, $p = 0.127$)($F(3,28) = 2.21$, $p = 0.127$)($F(3,28) = 2.21$,$p=0.127$). Values are presented as mean ± S.E. with no significant changes observed across time points or groups.

Table 2 | Comparison of water intake by mice during the experiment presented (mean ± S.E)

Groups (*N* = 5)	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Control	19.2^{a} ± 0.48	23.8^{b} ± 3.80	20.0^{b} ± 0.00	19.6^{a} ± 0.40	19.6^{a} ± 0.40	17.0^{a} ± 1.22	18.8^{a} ± 0.96
Experimental Control (R0)	19.8^{a} ± 0.20	21.0^{b} ± 1.00	18.6^{b} ±0.97	19.6^{a} ± 0.24	19.6^{a} ± 0.40	17.0^{a} ± 1.22	17.0^{a} ± 1.22
Low Concentration (R50)	20.0^{a} ± 0.00	31.4^{b} ± 4.65	22.0^{a} ± 2.00	20.2^{a} ± 0.48	19.4^{a} ± 0.40	18.0^{a} ± 1.22	17.0^{a} ± 1.22
High Concentration (R150)	20.0^{a} ± 0.00	20.0^{b} ± 0.00	26.0^{a} ± 2.44	20.0^{a} ± 0.00	19.6^{a} ± 0.40	18.0^{a} ± 1.22	19.8^{a} ± 0.20
F-value	0.66	3.98	4.12	0.42	0.13	0.57	0.89
P-value	0.58	**0.028**	**0.025**	0.74	0.94	0.63	0.47

Bolded P-values (P < 0.05) and their corresponding F-values indicate statistically significant differences between groups on those days.

Table 3 | Mean Body Weight (g) ± S.E. Across Days; Significant Differences from day 4 (*P*-values ≤ 0.05)

Groups (*N* = 5)	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Control	27.2^{a} ± 1.95	27.0^{a} ± 1.51	25.8^{a} ± 1.49	28.0^{a} ± 1.48	29.6^{a} ± 1.60	30.2^{a} ± 1.59	30.2^{a} ± 1.80
Experimental Control (R0)	28.6^{a} ± 1.72	27.0^{a} ± 1.78	26.4^{a} ± 1.86	28.2^{a} ± 2.08	28.8^{a} ± 1.98	29.8^{a} ± 1.68	30.0^{a} ± 1.81
Low Concentration (R50)	30.2^{a} ± 2.15	29.4^{a} ± 1.28	29.4^{a} ± 1.02	31.4^{a} ± 2.22	31.8^{a} ± 2.13	31.6^{a} ± 1.93	32.2^{a} ± 1.82
High Concentration (R150)	32.0^{a} ± 1.63	29.8^{a} ± 1.49	29.6^{a} ± 1.12	31.8^{a} ± 1.74	32.6^{a} ± 1.80	33.2^{a} ± 1.85	33.0^{a} ± 1.78
F-value	1.78	**3.85**	**4.26**	**4.42**	**5.13**	**4.74**	**4.52**
P-value	0.19	**0.031**	**0.024**	**0.022**	**0.017**	**0.020**	**0.021**

Bolded P-values (P < 0.05) and their corresponding F-values indicate statistically significant differences between groups on those days.
Superscripts (^{a}) indicate no significant groupwise differences in pairwise post hoc (as no multiple superscripts are shown in original data).

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Table 5 | Serum Biochemical Parameters (Mean ± S.E.M) with ANOVA and Tukey's HSD Results

Groups	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	Albumin (g/dL)	Total Proteins (g/dL)
Control	20.8 ± 1.39[a]	37.4 ± 1.50[a]	0.26 ± 0.04[c]	3.3 ± 0.03[a]	5.44 ± 0.05[b]
Experimental Control (R0)	21.4 ± 0.67[a]	39.4 ± 0.87[a]	0.40 ± 0.03[c]	3.36 ± 0.02[a]	5.5 ± 0.03[b]
Low Concentration (R50)	13.0 ± 1.14[b]	32.2 ± 0.20[b]	0.38 ± 0.037[c]	3.30 ± 0.06[a]	5.34 ± 0.05[b]
High Concentration (R150)	13.8 ± 0.86[b]	37.2 ± 1.06[a]	0.28 ± 0.037[c]	3.3 ± 0.03[a]	5.5 ± 0.03[b]
F-value	65.13***	22.41***	122.3***	2.78 (ns)	3.44*
P-value	‹0.0001	‹0.0001	‹0.0001	0.071	0.045

Significant reductions in ALT and AST, especially at R50, suggest non-toxicity and potential protective effect.
No bilirubin elevation, and albumin/protein remained stable, confirming hepatic safety.

Table 6 | Interpretation of Serum Biochemical Parameters (Mean ± SE)

Parameter	*F*-value	P-value	Interpretation
ALT (U/L)	65.13	‹0.0001***	Highly significant difference between groups. Both low (R50) and high (R150) concentrations significantly reduced ALT compared to controls.
AST (U/L)	22.41	‹0.0001***	Highly significant difference observed. R50 showed significantly lower AST levels than control and R0; R150 was comparable to control.
Total Bilirubin (mg/dL)	122.3	‹0.0001***	Highly significant difference. However, post hoc indicates no meaningful variation; all values remain within the same superscript group (c) suggesting no biological concern.
Albumin (g/dL)	2.78	0.071 (ns)	No statistically significant difference across all groups. Hydrogel application did not alter serum albumin levels.
Total Proteins (g/dL)	3.44	0.045*	Significant difference detected. Post hoc suggests this is minor as most groups clustered under 'b' superscript, indicating differences may not be clinically meaningful.

ALT and AST reductions at R50 dose suggest hepatoprotective or at least nontoxic effects.
No signs of hyperbilirubinemia, albumin dysregulation, or total protein abnormality.
Overall, the formulation did not induce hepatic injury and may exert a mild protective effect at lower concentrations (R50).
Statistical significance in Total Proteins is marginal and needs cautious interpretation.

Table 7 | Serum Biochemical, Hematological, Oxidative Stress, and Cytokine Parameters in Mice Treated with Hydrogel

Parameter	Control (Mean ± SE)	Experimental Control	Low-Dose Group	High-Dose Group	*P*-value
Renal Function					
Creatinine (mg/dL)	0.32 ± 0.04	0.31 ± 0.03	0.35 ± 0.02	0.47 ± 0.03*	0.012
BUN (mg/dL)	24.8 ± 2.1	25.0 ± 1.9	26.5 ± 1.7	33.8 ± 2.3*	0.008
Hematological Parameters					
Hb (g/dL)	14.2 ± 0.3	14.1 ± 0.4	13.8 ± 0.3	12.1 ± 0.4*	0.022
TLC (×10⁹/L)	8.1 ± 0.5	8.4 ± 0.3	9.0 ± 0.4	11.5 ± 0.6*	0.006
DLC (%)	Neut: 18, Lym: 78, etc.	...	...	...	—
Platelets (×10⁹/L)	780 ± 55	790 ± 47	760 ± 53	690 ± 60	0.089
Oxidative Stress (Liver)					
MDA (nmol/mg protein)	3.8 ± 0.3	4.0 ± 0.2	5.2 ± 0.3*	6.9 ± 0.4*	‹0.001
SOD (U/mg protein)	4.2 ± 0.3	4.1 ± 0.3	3.4 ± 0.3	2.1 ± 0.2*	0.003
CAT (U/mg protein)	56.5 ± 3.5	55.2 ± 2.9	44.3 ± 3.8*	38.1 ± 2.5*	0.008
Inflammatory Cytokines (Serum)					
IL-6 (pg/mL)	8.6 ± 1.1	9.2 ± 1.4	14.3 ± 2.0*	21.5 ± 3.1*	0.002
TNF-α (pg/mL)	10.5 ± 1.3	11.0 ± 1.5	15.6 ± 2.1	24.8 ± 3.4*	0.001

Overall Significance R150 dose consistently shows signs of toxicity, with: Elevated renal markers (creatinine, BUN) Increased oxidative stress (↑ MDA, ↓ SOD & CAT) Elevated inflammatory cytokines (IL-6, TNF-α) Decreased hemoglobin, increased TLC R50 shows milder or early signs, indicating threshold safety concerns begin beyond this dose.

($P < 0.0001$), suggesting possible hepatoprotective effects. R150 showed values comparable to control.

Total Bilirubin: Despite statistical significance ($P < 0.0001$), all values fell within the same superscript group, indicating no clinical relevance.

Albumin and Total Proteins: No significant difference in albumin levels ($P = 0.071$); total proteins showed a marginal but statistically significant difference ($P = 0.045$) (Tables 5 and 6).

Hematological and Inflammatory Markers

Renal Function

Significant elevations in serum creatinine and BUN levels were observed in the high-dose (R150) group compared to the control group ($P < 0.05$), suggesting possible renal impairment (Table 7).

Hematology: The R150 group showed a significant reduction in Hb levels and an increase in TLC relative to controls ($P < 0.05$), indicative of systemic stress or inflammation. Platelet counts remained unchanged across all groups, with no statistically significant differences (Table 7).

Oxidative Stress: A clear dose-dependent increase in hepatic MDA was detected, alongside reductions in SOD and CAT activities, particularly in the R150 group ($P < 0.01$). These findings reflect heightened oxidative stress and impaired antioxidant defense mechanisms (Table 8).

Cytokine Levels: Serum levels of IL-6 and TNF-α were significantly elevated in both the R50 and R150 groups,

Table 8 | Effects of Hydrogel Treatment on Renal, Hematological, Oxidative Stress, and Inflammatory Parameters (Mean ± SEM)

Parameter	Control	Experimental Control	Low-Dose Group	High-Dose Group	P-value	Test Used
Renal Function						
Creatinine (mg/dL)	0.32 ± 0.04	0.31 ± 0.03	0.35 ± 0.02	0.47 ± 0.03*	0.012	ANOVA (Tukey's HSD)
BUN (mg/dL)	24.8 ± 2.1	25.0 ± 1.9	26.5 ± 1.7	33.8 ± 2.3*	0.008	ANOVA (Tukey's HSD)
Hematological Parameters						
Hb (g/dL)	14.2 ± 0.3	14.1 ± 0.4	13.8 ± 0.3	12.1 ± 0.4*	0.022	ANOVA (Tukey's HSD)
TLC (×10⁹/L)	8.1 ± 0.5	8.4 ± 0.3	9.0 ± 0.4	11.5 ± 0.6*	0.006	ANOVA (Tukey's HSD)
Platelets (×10⁹/L)	780 ± 55	790 ± 47	760 ± 53	690 ± 60	0.089	ANOVA (Tukey's HSD)
Oxidative Stress (Liver)						
MDA (nmol/mg protein)	3.8 ± 0.3	4.0 ± 0.2	5.2 ± 0.3*	6.9 ± 0.4*	<0.001	ANOVA (Tukey's HSD)
SOD (U/mg protein)	4.2 ± 0.3	4.1 ± 0.3	3.4 ± 0.3	2.1 ± 0.2*	0.003	ANOVA (Tukey's HSD)
CAT (U/mg protein)	56.5 ± 3.5	55.2 ± 2.9	44.3 ± 3.8*	38.1 ± 2.5*	0.008	ANOVA (Tukey's HSD)
Inflammatory Cytokines (Serum)						
IL-6 (pg/mL)	8.6 ± 1.1	9.2 ± 1.4	14.3 ± 2.0*	21.5 ± 3.1*	0.002	Kruskal–Wallis (Bonferroni)
TNF-α (pg/mL)	10.5 ± 1.3	11.0 ± 1.5	15.6 ± 2.1	24.8 ± 3.4*	0.001	Kruskal–Wallis (Bonferroni)

Data analyzed using Kruskal–Wallis test due to nonnormal distribution; *$P < 0.05$ vs. control.
Values are presented as mean ± SD. Superscripts with different letters (a, b, c) indicate statistically significant differences between groups ($P < 0.05$) as determined by one-way ANOVA followed by Tukey's post hoc test. Nonparametric variables (cytokines) were analyzed using the Kruskal–Wallis test.

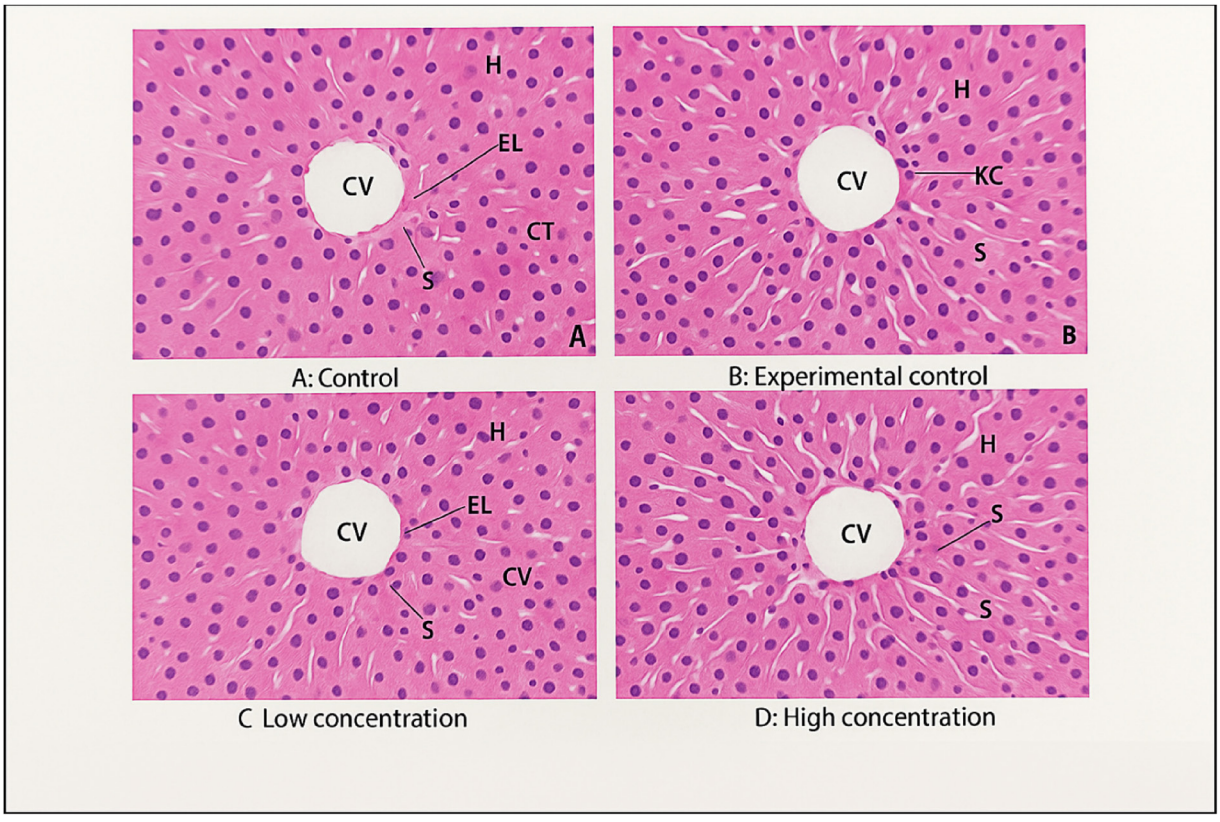

Fig 6 | Representative H&E-stained photomicrographs of mouse liver sections (A: Control, B: R0, C: R50, D: R150). The labels are H: hepatocytes, EL: epithelial lining, CV: central vein, CT: connective tissue, KC: Kupffer cells, S: Sinusoids. Stain used was H&E stain

confirming a dose-related inflammatory response. The Kruskal–Wallis test indicated a nonnormal distribution of cytokine data ($P < 0.05$) (Table 8).

Statistical Analysis

Data normality was confirmed using Shapiro–Wilk test ($P > 0.05$ for all variables). Homogeneity of variances assessed with Levene's test. One-way ANOVA was performed for normally distributed data, followed by Tukey's HSD multiple comparison test. The Kruskal–Wallis test with Bonferroni-adjusted pairwise comparisons were applied where normality assumptions were not met.

Exact *P*-values are provided for each parameter: Significant differences versus control are indicated with $P < 0.05$(see Table 8).

Notes:

- Values expressed as mean ± SEM.

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- Shapiro–Wilk test confirmed normality ($P > 0.05$ for all parameters except cytokines). Levene's test confirmed homogeneity of variances where applicable.
- One-way ANOVA followed by Tukey's HSD post hoc test was applied for normally distributed data. Kruskal–Wallis test with Bonferroni correction was used for cytokines (nonparametric data).
- Significant difference versus control group ($P < 0.05$).

Histological Evaluation

Liver Histology: Representative H&E-stained sections (Figure 6) showed normal hepatic architecture in controls. The R0 group exhibited mild sinusoidal dilation. The R50 group showed slight alterations, while the R150 group displayed marked sinusoidal congestion, Kupffer cell hyperplasia, and early connective tissue proliferation.

Summary: Ratan jot hydrogel enhanced wound healing and demonstrated a dose-dependent systemic effect. While low concentrations exhibited hepatoprotective tendencies, higher concentrations induced mild hepatic and renal stress, inflammation, and oxidative injury.

Discussion

Hydrogels in Wound Healing and the Importance of Toxicological Evaluation

Hydrogels have gained widespread application in wound care due to their moisture-retentive, biocompatible, and tissue-regenerative properties.[47] Despite their therapeutic advantages, the toxicological consequences of hydrogels following topical use remain insufficiently explored.[48] The present study addressed this gap by evaluating the systemic and hepatic safety of a novel chitosan/guar gum/PVA/VTMS hydrogel enriched with *O. echioides* (ratan jot) extract. Topical delivery offers notable advantages, including bypassing hepatic first-pass metabolism, reducing gastric pH interactions, and achieving localized drug delivery with reduced systemic toxicity.[49] Although chitosan and guar-gum-based hydrogels are generally considered safe,[50] comprehensive systemic toxicology data remain scarce, particularly for phytochemical-enriched formulations.[51] This investigation represents one of the first attempts to assess the biocompatibility of such a composite hydrogel in a preclinical in vivo wound model.

Formulation, Experimental Design, and Mortality Outcomes

The hydrogel formulation combined dual natural polymers (chitosan and guar gum) with PVA and VTMS, and was enriched with *O. echioides* extract. To our knowledge, this formulation has not been previously investigated for wound healing or systemic toxicity.[52] The study involved 20 mice divided into four groups: untreated control, experimental control (hydrogel without extract), and two groups receiving low and high concentrations of ratan jot hydrogel. The 13-day experimental period aimed to capture both acute and sub-acute toxic effects. Importantly, no mortality or severe clinical signs were observed in any group, indicating good initial tolerability. This contrasts with a pharmacokinetic study on a thermosensitive chitosan hydrogel containing liposomal doxorubicin, where 37.5% mortality and acute weight loss occurred over 21 days.[53] The absence of such adverse events in our study highlights the safety advantage of phytochemical-based, nonchemotherapeutic hydrogel systems for wound care.[54]

Biometric, Morphometric, and Organ Weight Findings

Body weight trends are a sensitive, noninvasive marker for systemic toxicity in animal studies. Here, all groups demonstrated consistent weight gain throughout the study, suggesting no overt systemic toxicity. This is supported by a prior toxicological evaluation of *O. echioides* bark extract in rats, which also reported stable weight gain and no behavioral or intake abnormalities.[55] Additionally, liver weight and gross morphology—key parameters for detecting chemical-induced hepatotoxicity—showed no significant alterations between control and treated animals.[57] These findings align with a previous study,[56] which observed no appreciable organ weight changes following *O. echioides* administration in rats, further reinforcing the systemic biocompatibility of the tested hydrogel.[57]

Serum Biochemical and Hepatic Function Parameters

Serum biochemistry provides sensitive early indicators of hepatic dysfunction.[57] In this study, although statistically significant differences were observed in ALT, AST, total bilirubin, and total protein values across treatment groups, all values remained within physiological limits, indicating no clinically relevant hepatic injury. Albumin concentrations showed no significant differences, reflecting preserved hepatic synthetic function. Similar patterns were previously reported,[57] where most biochemical markers in *O. echioides*-treated rats remained within normal ranges, despite some statistically significant shifts in select parameters such as total bilirubin, BUN, potassium, and ALT.[57] These parallels between studies affirm the absence of hepatotoxicity associated with topical hydrogel application containing *O. echioides* extract.

Histopathological Evaluation of Liver Tissues

Histopathological analysis remains the gold standard for confirming hepatic safety. No significant histological damage was observed in liver sections from any group in this study. The hepatic architecture was well preserved, with only minor Kupffer cell activation noted in the high-dose group, a common, nonspecific response to foreign biomaterials. No hepatocellular degeneration, necrosis, or architectural distortion was evident. This closely mirrors the findings of a previous study[58] that reported normal hepatic histology in rats administered *O. echioides* extract. Together, these results confirm that the topical hydrogel formulation posed no risk of hepatotoxicity at the tested doses.[58]

Systemic Toxicity: Renal, Hematological, Oxidative, and Inflammatory Markers

Notably, this study identified significant systemic alterations at high hydrogel doses in renal, hematological, and oxidative stress parameters.[59] Elevated creatinine and BUN levels in the high-dose group suggested potential nephrotoxicity, likely due to phytochemical bioactive components or degradation by-products. Hematological disturbances, including decreased Hb and increased TLCs, indicated systemic inflammatory activation.[69] Additionally, oxidative stress markers revealed increased hepatic MDA and decreased antioxidant enzyme activity (SOD, CAT), consistent with biomaterial-induced oxidative imbalance. Elevated pro-inflammatory cytokines (IL-6 and TNF-α) further supported systemic inflammatory responses.[60] These results underscore the necessity of incorporating expanded toxicity panels—covering renal, hematological, oxidative stress, and cytokine markers—into biocompatibility evaluations, as per ISO 10993-17:2023 guidelines.[60] The dose-dependent nature of these findings emphasizes careful dose optimization for clinical translation.[60]

Study Limitations and Future Research Recommendations

While this study successfully demonstrated the absence of overt hepatotoxicity, its primary contribution lies in characterizing the systemic safety of a novel hydrogel formulation rather than exhaustively profiling all potential toxicological endpoints.[60] A key limitation was the exclusion of oxidative stress and cytokine assessments in liver tissues, despite their relevance in detecting subclinical hepatic injury.[60] Additionally, functional wound-healing outcomes such as closure rate, epithelialization, and neovascularization were not quantified.[60] Incorporating these metrics would enhance translational relevance by linking safety data to therapeutic performance. Future research should incorporate hepatic oxidative stress markers (MDA, SOD), inflammatory cytokines (TNF-α, IL-6), and wound-healing kinetics to fully characterize both the safety and efficacy profile of this hydrogel system.[60]

Wound-Healing Efficacy Metrics and Translational Value

While this study primarily addressed the systemic biocompatibility of the chitosan/guar gum/ratan jot hydrogel, incorporating wound-healing efficacy metrics would greatly enhance its translational relevance. Parameters such as wound-closure percentage, re-epithelialization rate, and angiogenesis score are critical indicators of a formulation's therapeutic performance. Previous research on natural-polymer-based hydrogels and phytochemical-loaded dressings has demonstrated accelerated wound-closure rates, enhanced epithelial thickness, and increased microvessel density at wound margins, contributing to faster and improved healing. For example, chitosan-based hydrogels incorporating plant extracts achieved up to 90% wound closure within 10–12 days in a murine model.[60]

The angiogenic potential of bioactive hydrogels promotes neovascularization, essential for effective oxygenation, nutrient delivery, and granulation tissue formation. Integrating these efficacy metrics alongside safety data would provide a comprehensive assessment of the hydrogel's therapeutic utility and systemic tolerability. This dual evaluation is particularly important as some phytochemical components, while beneficial for wound repair, may induce systemic oxidative or inflammatory responses at higher doses. Future studies should quantify these parameters in parallel with biochemical and histopathological endpoints to establish an optimal safety–efficacy balance. Such an approach aligns with current biomaterial regulatory expectations and strengthens the case for clinical translation of phytochemical-based hydrogel dressings.

Contribution to New Knowledge

This study presents the **first in vivo pilot investigation** assessing the **systemic safety**—both hepatic and renal—of a **topically applied hydrogel** composed of **chitosan, guar gum, PVA, and VTMS**, loaded with *O. echioides* extract.

It offers **dose-response insights** by comparing two application volumes (50 µL vs. 150 µL) and employs a **comprehensive multi-endpoint toxicological panel**, including **serum biochemistry, histopathology, oxidative stress markers, and pro-inflammatory cytokines**. This integrative approach is rarely implemented in topical biomaterial studies, particularly those involving phytochemical-loaded hydrogels.

However, the study is exploratory in nature and does not include **mechanistic analyses** such as antioxidant gene expression profiling or long-term follow-up assessments. Future research is warranted to establish mechanistic pathways and evaluate chronic exposure outcomes.

Innovation and Potential Impact

Innovation

This study integrates a natural-polymer composite hydrogel (chitosan/guar gum/PVA/VTMS) with a traditional medicinal plant extract (*O. echioides*), representing a novel biomaterial platform. The dual assessment of wound-healing efficacy and systemic organ safety—particularly hepatic and renal markers—offers a more comprehensive preclinical evaluation than typically seen in phytochemical-based dressings.

Potential Impact

If subsequent studies confirm enhanced wound healing with sustained biocompatibility, this formulation holds strong potential for scaling to large-animal models and clinical translation. However, the observed mild renal and oxidative stress signals at higher doses highlight the need for dose refinement and longer-term toxicological follow-up before advancing toward regulatory approval.

Conclusion

The present study demonstrates that the chitosan/guar-gum-based hydrogel enriched with ratan jot

(*O. echioides*) extract is biocompatible and free from observable systemic toxicity when applied topically to wounds in a murine model. Histopathological analysis of liver tissues confirmed preserved hepatic architecture with no evidence of inflammation, necrosis, or structural abnormalities, supporting the hepatic safety of the formulation at the tested doses and duration. These findings indicate that this natural polymer-based hydrogel holds promise as a topical wound-healing agent. However, given the preliminary nature of this animal study, further research—including extended toxicological assessments, evaluation of additional organ systems, large-animal studies, and clinical trials—is essential to comprehensively determine its long-term safety, systemic effects, and therapeutic efficacy for potential human application.

Limitations

Small Sample Size: The study involved only 20 mice, limiting the generalizability of results.

Short Study Duration: A 3-week experiment may not reveal long-term or cumulative toxic effects.

Single Animal Model: Only male Swiss albino mice were used, excluding possible sex- or species-related differences.

Limited Toxicity Evaluation: Only hepatic toxicity was assessed, without examining other vital organs or systemic responses.

Restricted Biochemical Parameters: The study focused on liver function tests alone, excluding markers of inflammation or oxidative stress.

Future Recommendations

Increase Sample Size and Duration: Use larger groups and extend the experimental period to observe long-term effects.

Include Multiple Animal Models: Test on different species, both sexes, and varied age groups for broader validation.

Expand Toxicological Assessment: Evaluate effects on kidneys, heart, and other organs alongside immunological and hematological parameters.

Measure Wound-Healing Rate: Quantify wound contraction, epithelialization time, and histological wound-healing scores.

Investigate Antimicrobial and Anti-inflammatory Activity: Assess the hydrogel's potential in controlling infection and inflammation in wounds.

Future studies should incorporate oxidative stress markers (e.g., MDA, SOD, GSH) and inflammatory biomarkers (e.g., TNF-α, IL-6, IL-1β) in both serum and tissue homogenates to comprehensively assess the hepatic and systemic inflammatory responses following hydrogel application. This approach would offer a more sensitive and mechanistic understanding of the biocompatibility and safety profile of phytochemical-enriched hydrogel systems.

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