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Original Article

Synergistic Effect of *Quercus incana* Fruit Extract Incorported Chitosan in Accelerating Wound Healing and Inflammation Reduction

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This study investigates the wound healing and skin allergy applications of Quercus incana (QI) fruit extract incorporated with chitosan (CS) using a freeze-drying method. The extract was prepared by aqueous extraction and combined with CS to enhance its therapeutic properties. The formulation was characterized using Fourier-transform infrared spectroscopy to confirm the interaction between the QI extract and CS. The contact angle study revealed the surface wettability of the composite material. Anti-inflammatory assays demonstrated that QI-CS outperformed CS in reducing inflammation, likely due to the synergistic effects of the bioactive phenolic compounds from QI, which inhibit pro-inflammatory cytokines. Histopathological analysis of zebrafish wound healing indicated superior tissue regeneration and reduced inflammation in QI-CS-treated groups, with more organized collagen fibers and enhanced fibroblast proliferation. Gene expression analysis confirmed that QI-CS significantly up-regulated genes associated with extracellular matrix remodelling (MMP-13, MMP-9) and inflammation (IL-1β, TNF-α), further supporting its potential for improved wound healing. These findings suggest that QI-CS has superior therapeutic potential compared to CS, making it a promising candidate for applications in skin creams and inflammation-related conditions.

Introduction

Skin allergies are a common issue affecting a wide range of individuals, causing discomfort and, in severe cases, lasting damage to the skin [1]. These conditions often result from environmental allergens, contact dermatitis, or other irritants, leading to symptoms such as itching, swelling, redness, and rashes. It is mainly divided into two categories: allergic contact dermatitis (ACD) and irritant contact dermatitis (ICD) [2]. About 80% of instances of ICD are caused by direct chemical or physical harm to the skin barrier, usually as a result of friction, soaps, and solvents. Topical corticosteroids are the first-line therapy for both ICD and ACD, aiming to reduce inflammation and alleviate symptoms [3]. In cases where topical treatments are insufficient, systemic therapies such as oral corticosteroids or immunosuppressants may be considered. However, these treatments can have side effects, and there is a growing interest in exploring natural alternatives with fewer adverse effects. This has led to growing interest in alternative, natural treatments that can offer effective solutions without the adverse effects associated with pharmaceutical interventions.

Chitosan (CS) is a natural polysaccharide derived from the deacetylation of chitin, which is abundantly found in the exoskeletons of crustaceans. It is composed of β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine units [4], [5]. One of the notable properties of chitosan is its solubility in acidic solutions due to the protonation of amino groups, which imparts a positive charge, enabling it to bind to negatively charged surfaces such as mucosal membranes. This characteristic contributes to its

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bioadhesive properties, making it useful in drug delivery systems [5]. Additionally, chitosan exhibits biodegradability and biocompatibility, essential for biomedical applications [6]. Its antimicrobial activity against a range of bacteria and fungi has been documented, attributed to its ability to disrupt microbial cell membranes. In wound management, chitosan-based dressings have been shown to promote hemostasis, accelerate healing, and reduce infections. Moreover, chemical modifications of chitosan, such as thiolation and quaternization, have been explored to enhance its mechanical properties and functional versatility, expanding its potential applications in tissue engineering and regenerative medicine [7].

Quercus incana (QI), or grey oak, is a plant traditionally used in folk medicine for its medicinal properties. The tree is rich in polyphenolic compounds, particularly tannins and flavonoids, known for their anti-inflammatory, antioxidant, and antimicrobial activities [8]. These bioactive compounds are believed to play a key role in soothing inflamed or irritated skin, reducing oxidative stress, and preventing infections such as common issues in allergic reactions [9]. The combination of these two bioactive compounds, QI and CS (QI-CS), offers a promising new approach to treating skin allergies. By combining the anti-inflammatory, antioxidant, and antimicrobial properties of QI with the moisturizing, protective, and healing effects of chitosan, this combination aims to address both the symptoms and the underlying causes of allergic skin conditions. Such a formulation could reduce inflammation, soothe irritated skin, and expedite healing, while also preventing secondary infections, making it a potentially superior alternative to conventional treatments. This study could offer a natural, safe, and effective alternative to conventional treatments for skin allergies. The aim is to develop a safe, natural remedy for managing allergic skin reactions effectively.

Materials and Methods

Chitosan, acetic acid glacial, and Sodium hydroxide were procured from Sisco Research lab (SRL), India. All the chemicals were used without further purification.

Preparation of QI fruit extract

The collected fruits of QI were washed numerous times with distillation water to remove dust, followed by drying at room temperature. The 1 g of fruits were grind into powder, put into 50 ml of methanol, and stirred for 48 h. The solution was filtered and the filtrate was then used for further study [9].

Chemical crosslinking and lyophilisation of QI loaded Chitosan

To prepare the chitosan (CS) films, 1 g of chitosan was dissolved in 40 ml of 1% acetic acid under continuous stirring for 6 hours. Once dissolved, gel-like substance of CS was obtained. Further, 10 ml of QI extract was added to the CS gels and continued stirring for 2 hours. The gels were subjected to cross-linking with NaOH for 12 h and freeze-dried to obtain QI loaded CS films [10].

Characterization

To thoroughly analyze the synthesized samples, various techniques were employed. Field emission scanning electron microscopy (FESEM, CARL ZEISS – SIGMA, Germany) was used to assess material morphology, with gold coating applied for enhanced conductivity. Fourier transform infrared spectroscopy (FT-IR, Bruker Alpha II, Germany) was utilized to examine chemical bonds and functional groups. Additionally, a Contact Angle Goniometer (Ossila, Netherlands) measured the surface wettability.

Anti-inflammatory assay

The protein denaturation assay of QI-CS performed using bovine serum albumin (BSA) to assess anti-inflammatory activity [11]. The experiment was carried out using phsopahte buffer saline (PBS 7.4). Briefly, 3.2 ml of varying concentrations of QI-CS samples added to the 1.8 ml of 1% BSA solution and incubated in water bath at 37 ºC for 15 min. Then the reaction mixture was heated at 70 ºC for 5 min to induce protein denaturation. After cooling the degree of denaturation was measured at 660 nm using a UV–Vis spectrophotometer (JASCO V-730 spectrophotometer). The PBS alone without the sample was used as control. The percentage inhibition of protein denaturation was then calculated, providing insight into the anti-inflammatory potential of QI-CS by assessing its ability to prevent heat-induced protein denaturation [11], [12].

$$\text{Percentage (\%) inhibition} = (Abs_{control} - Abs_{sample} / Abs_{control}) \times 100$$

Where, Abs = absorbance. The experiment was conducted in triplicates to ensure the reliability.

Wound healing Potential in Zebrafish models

Adult zebrafish (Danio rerio), 5–6 months old, were sourced from a local supplier and maintained in a controlled environment under a 14-hour light/10-hour dark cycle, mimicking natural conditions. They were fed a balanced diet twice daily to ensure optimal health, and water quality parameters (pH, temperature, dissolved oxygen) were carefully monitored and maintained within ideal ranges. The tanks were equipped with filtration, aeration, and enrichment items to reduce stress and promote natural behaviors. Wounds were inflicted by anesthetizing the zebrafish in an ice-cold water bath, followed by a standardized incision on the dorsal region with approximately 2 mm in diameter using a clinical dermatology laser. Post-wounding, the fish were placed in fresh, clean water for recovery, with optimal water quality maintained throughout the healing process. Zebrafish were assigned to treatment groups (n = 6), where one group received 25 μg/ml of pure chitosan (CS) and another group received 25 μg/ml of QI-CS samples, while control groups were untreated [13].

Table 1: List of primers used to evaluate the gene expression levels during wound healing process

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Accession number
Matrix metalloproteinase-13 (MMP-13)	GAGAAGGTTTGGGCTCTCTATG	TGAGTTGCTGTCTTCCTTGTAG	AF506756
Matrix metalloproteinase-9 (MMP-9)	TTTGCCCTGATCGTGGATAC	GGGAAACCCTCCACGTATTT	AY151254
Interleukin 1β (IL-1β)	TCAAACCCCAATCCACAGAG	TCACTTCACGCTCTTGGATG	AY340959.1
Tumor necrosis factor-α (TNF- α)	AGAAGGAGAGTTGCCTTTACCGCT	AACACCCTCCATACACCCGACTTT	AY427649
β-actin	AATCTTGCGGTATCCACGAGACCA	TCTCCTTCTGCATCCTGTCAGCAA	AF025305

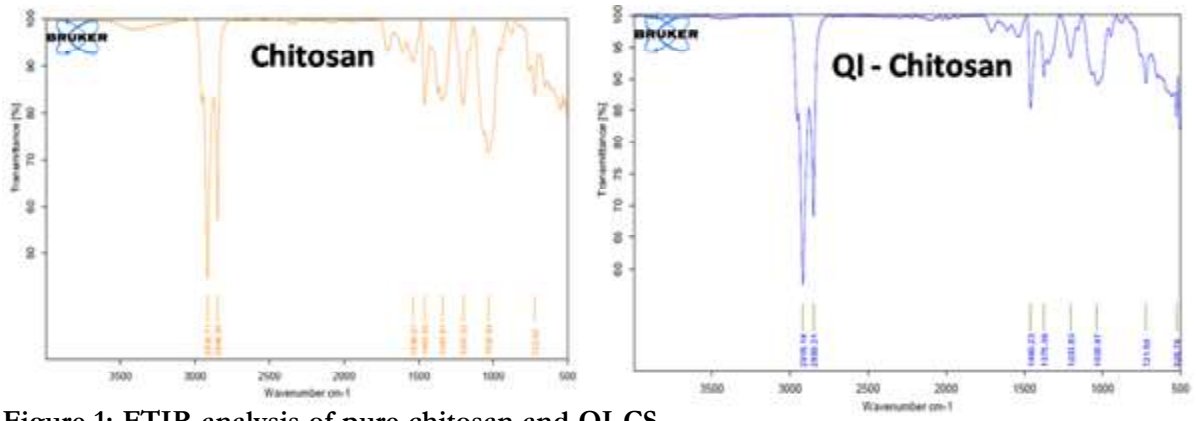

Figure 1: FTIR analysis of pure chitosan and QI-CS

Histology analysis

Zebrafish (*Danio rerio*) from various treatment groups were humanely euthanized at predetermined time intervals after injury, and the injured regions were meticulously dissected. The tissues were then fixed in a 4% paraformaldehyde solution, followed by dehydration through sequential ethanol washes and embedding in paraffin wax. Thin sections, approximately 5-10 µm in thickness, were sliced using a microtome and placed onto glass slides. Hematoxylin and eosin (H&E) staining was applied to examine the tissue histology.

Gene expression during wound healing QI-CS treatment

Wound healing efficiency of the treated and untreated groups were subjected mRNA expression levels at 3rd day post-wound. Tissue samples from the wounded area were collected and stored at –80 °C. The homogenized tissues were subjected to RNA extraction with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was measured using a NanoDrop One spectrophotometer. The cDNA synthesis was carried out using 2 ìg of isolated RNA using the PrimeScript single strand cDNA synthesis kit (TaKaRa®, Tokyo, Japan), following the manufacturer's instructions. The primers used in the study are listed in table 1, with zebrafish **â-actin** serving as the housekeeping gene. Quantitative real-time PCR (qRT-PCR) was performed on a SYBER Green Master Rox (Roche Diagnostics Ltd., Mannheim, Germany) with a 7500 ABI RT-PCR system, Applied Biosystems, Foster City, USA). Relative expression fold changes were calculated using the $2^{-\Delta\Delta CT}$ method [13].

Statistical Analysis

All the samples were analyzed in triplicate, and statistical differences between groups were assessed using one-way ANOVA. The data are expressed in mean ± standard deviation. A p-value < 0.05 was considered statistically significant, with p-value < 0.01 indicating high significance.

Results and Discussion

Functional group analysis by FTIR

The FTIR spectra of pure chitosan and the QI fruit extract-chitosan composite formulation is shown in figure 1, which revealed the distinct differences, indicating successful incorporation of the extract into the chitosan matrix. The pure CS spectrum exhibited characteristic peaks at 3445 cm^{-1} (–OH and $–NH_2$ stretching), 1645 cm^{-1} (amide I band), and 1066 cm^{-1} (C–O stretching) [5]. The FTIR spectrum of the QI fruit extract-chitosan composite showed additional peaks corresponding to the functional groups present in the extract, such as phenolic –OH groups around 3300 cm^{-1} and C=C stretching at 1600 cm^{-1}, indicating the presence of polyphenolic compounds [14]. The shift and intensity changes in the peaks further confirmed the interaction between the CS and the QI fruit

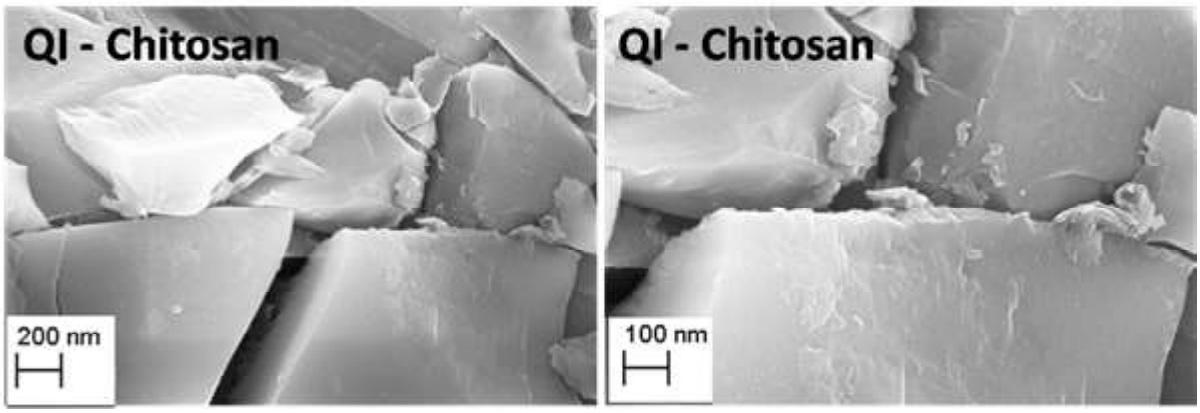

Figure 2: FESEM images of freeze-dried QI fruit extract incorporated chitosan formulation

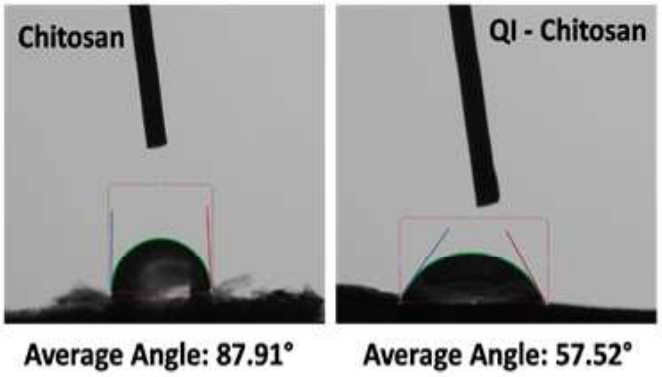

Figure 3: Contact angle analysis of pure CS and QI fruit extact-CS

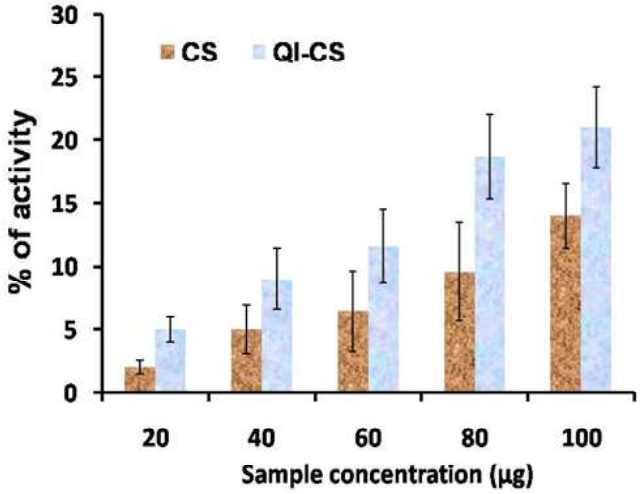

Figure 4: Anti-inflammatory activity of QI-CS samples

extract, suggesting the formation of a stable composite. These results indicate that the incorporation of Quercus incana fruit extract into chitosan enhances its bioactive properties, which may contribute to improved wound healing and anti-inflammatory effects.

Morphological analysis by FESEM

FESEM analysis was conducted to examine the morphological characteristics of QI-loaded chitosan and given in figure 2. The SEM images revealed a distinct flaky-like structure, indicating a well-distributed and uniform morphology. The flaky nature of the material may contribute to improved surface interactions, potentially increasing its bioavailability and effectiveness in topical ointments for skin allergies. The presence of a layered and irregular surface could facilitate better adherence and controlled release of active ingredients, making QI-CS a promising candidate for dermatological applications. These findings highlight the importance of FESEM analysis in understanding the microstructural properties of pharmaceutical biomaterials.

Contact Angle analysis

Contact angle analysis was performed to evaluate the spreadability and surface interaction of pure chitosan and QI-chitosan formulations as displayed in figure 3. The results indicate that pure CS exhibited an average contact angle of 87.91°, suggesting good spread ability without excessive dripping or running. In contrast, QI-chitosan showed a reduced contact angle of 57.52°, indicating enhanced surface wettability [15]. A lower contact angle is beneficial in topical applications as it improves surface coverage, enhances antibacterial efficacy by preventing bacterial adhesion, and promotes moisture retention. The improved spread ability of QI-CS is advantageous for effective application on the skin, ensuring better interaction with bacterial membranes and facilitating pathogen removal. These findings suggest that QI-CS may offer superior therapeutic potential in skin creams by optimizing surface properties and increasing antimicrobial effectiveness.

Anti-inflammatory assay

The anti-inflammatory assay results (figure 4) clearly demonstrate that both pure CS and QI-CS exhibit concentration-dependent activity. At each concentration, QI-CS significantly outperformed pure CS in terms of reducing inflammation, indicating the synergistic effects of the polyphenolic compounds from QI when incorporated with chitosan. The enhanced anti-inflammatory activity of QI-CS

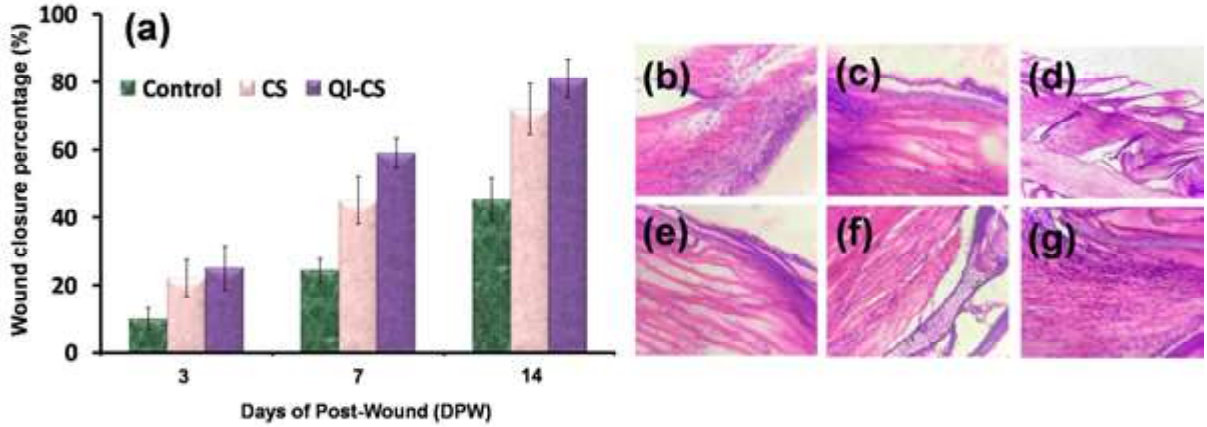

Figure 5: (a) Wound closure percentage in zebrafish at different time points; H & E staining images of (b) control, (c) CS and (d) QI-CS at 7th DPW; (e) control, (f) CS and (g) QI-CS at 14th DPW

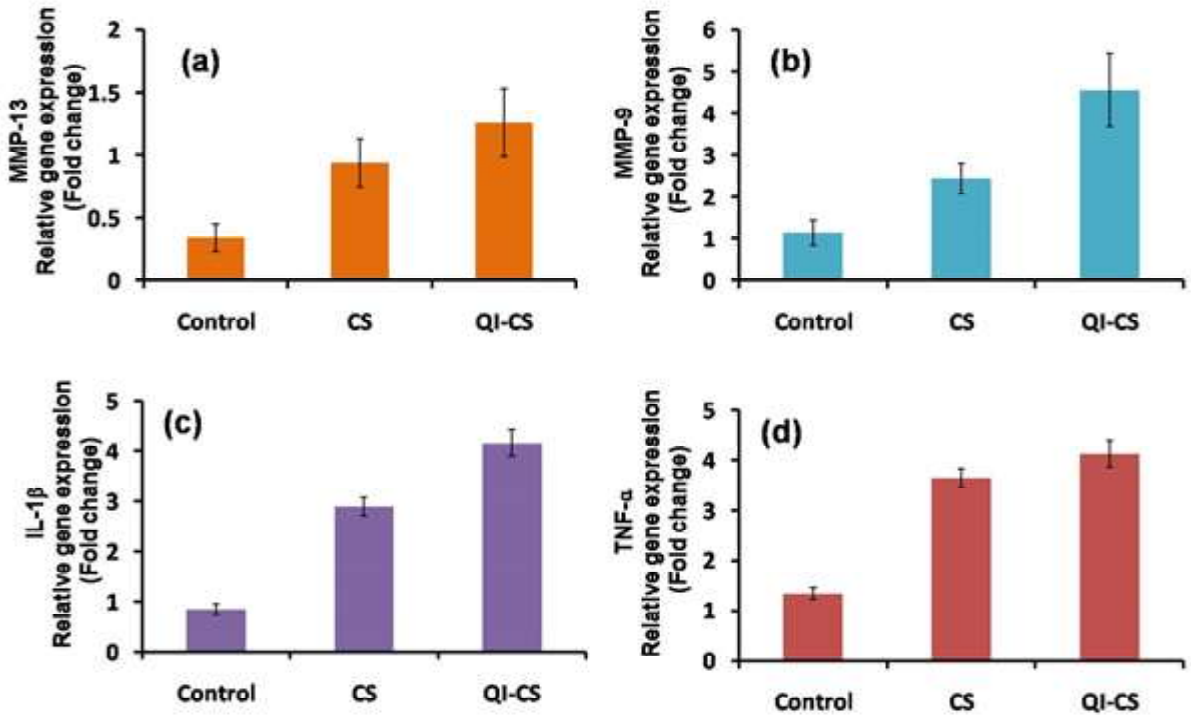

Figure 6: Wound closure percentage in zebrafish at different time points; H & E staining images of (b) control, (c) CS and (d) QI-CS at 7th DPW; (e) control, (f) CS and (g) QI-CS at 14th DPW

could be attributed to the bioactive phenolic compounds present in QI, which are known for their ability to modulate inflammatory pathways, including the inhibition of pro-inflammatory cytokines and enzymes such as COX-2. This synergistic effect between chitosan and QI may also result from the improved bioavailability and sustained release of the active compounds, which are facilitated by the chitosan matrix [16]. These findings suggest that QI-chitosan could serve as a more potent anti-inflammatory agent compared to pure chitosan, making it a promising candidate for therapeutic applications in inflammation-related conditions.

Wound healing analysis in Zebrafish models

Figure 5(a) shows a clear trend in zebrafish wound closure over time in the untreated control, CS, and QI-CS treatments. At Day 3, the control group shows the lowest wound closure, while both CS and QI-CS treatments significantly enhance the closure process. By Day 7, CS and QI-CS continue to show substantial improvement, with QI-CS reaching the highest closure rate at ~ 59%, compared to ~ 45% for CS and ~ 24% for the control. At Day 14, both treatments, particularly QI-CS, show strong wound closure, surpassing the untreated control by a significant margin. These findings suggest that both CS and QI-CS treatments accelerate wound closure in zebrafish, with QI-CS showing the most pronounced effect, indicating its potential as a more effective treatment for enhancing tissue repair and regeneration in wound healing [17,18].

The histopathological analysis of zebrafish wound healing reveals distinct differences between the control (untreated) and sample (treated) groups and displayed in figure 5(b-g). In the control group, tissue structure appears disrupted with loose and irregular collagen fibers, indicating incomplete healing. Additionally, a visible inflammatory response is evident due to immune cell infiltration at the wound site. The presence of tissue gaps and poorly organized fibroblasts further supports delayed wound recovery. In contrast, the treated sample group exhibits more structured tissue architecture, with collagen fibers aligned in a parallel manner. A reduction in immune cell infiltration suggests lower inflammation levels, while enhanced fibroblast proliferation and extracellular matrix deposition indicate advanced tissue regeneration. These findings suggest that the treatment promotes improved wound healing and tissue repair.

Gene expression analysis

The gene expression data (figure 6) for MMP-13, MMP-9, IL-1β, and TNF-α indicate that both QI-CS treatments significantly up-regulate the expression of these genes compared to the untreated control group, suggesting that these treatments are promoting tissue remodelling and inflammation, which are essential for wound healing in zebrafish. MMP-13 is involved in ECM degradation and tissue remodelling. The data show that MMP13 expression increases from ~0.34 in the control group to ~0.94 and ~1.26 in the CS and QI-CS treatments, respectively. This suggests that both treatments enhance ECM degradation and matrix remodelling, which is crucial for wound closure. Similarly, MMP9 expression also increases significantly, from 1.14 in the control to 2.44 in the CS and 4.56 in the QI-CS treatments. The elevated expression of MMP9 supports ongoing matrix remodelling and immune cell recruitment, which are essential for wound healing. The higher expression of IL-1 beta (0.84 in control, 2.89 in CS, and 4.16 in QI-CS) and TNF-α (1.34 in control, 3.64 in CS, and 4.13 in QI-CS) indicates a stronger inflammatory response in both treatment groups. These cytokines

play key roles in immune cell recruitment, inflammation, and tissue repair. The increase in these pro-inflammatory cytokines is consistent with the early inflammatory phase of wound healing, where inflammation is necessary for clearing damaged tissue and initiating repair processes [13].

Conclusion

The development of the herbal-chitosan cream, incorporating QI and CS, presents a promising natural alternative for managing skin allergies and promoting wound healing. The formulation demonstrated excellent anti-inflammatory properties, enhanced wettability, and improved wound healing potential in zebrafish models. FTIR and SEM analyses confirmed the successful integration of QI with chitosan, contributing to its bioactive properties. Histopathological and gene expression analyses further corroborate the potential of QI-CS to accelerate wound healing by enhancing ECM degradation, inflammatory response modulation, and fibroblast proliferation. Overall, this study highlights the potential of herbal-chitosan-based formulations as effective, biocompatible solutions for dermatological applications, paving the way for further research and potential commercialization.

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