Development and Evaluation of Antiinflammatory Potential of Chondroitin Sulphate-Carboxyl Methyl Cellulose Hydrogel of Martynia Annua L. In Rheumatoid Arthritis

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Abstract
The present study focuses on the development and evaluation of a Chondroitin Sulphate–Carboxymethyl Cellulose (CS-CMC) hydrogel loaded with the methanolic leaf extract of *Martynia annua* for its potential application in the treatment of rheumatoid arthritis. The research involved systematic investigation of extraction, phytochemical screening, formulation, characterization, and pharmacological evaluation of the developed hydrogel system. Among various solvents, methanol showed the highest extraction yield (15%), indicating the abundance of polar bioactive compounds such as flavonoids and phenolics. Phytochemical analysis confirmed the presence of multiple therapeutic constituents responsible for anti-inflammatory activity. The prepared hydrogel formulations exhibited desirable physicochemical properties, including appropriate pH, good spreadability, homogeneity, and viscosity suitable for topical application. Among all formulations, F3 was optimized based on superior characteristics such as highest yield (91.6%) and swelling index. Morphological studies (AFM and SEM) revealed uniform nanosized particles, supporting enhanced drug delivery. The CS-CMC hydrogel demonstrated higher entrapment efficiency (91.34%) and reduced particle size compared to plain CMC hydrogel, indicating improved drug incorporation and stability. In vitro drug release studies showed a sustained release pattern for CS-CMC hydrogel, whereas conventional formulations exhibited rapid release. Pharmacological studies confirmed significant anti-inflammatory activity through inhibition of protein denaturation, lipoxygenase, and other markers. In vivo studies further demonstrated enhanced permeation, increased bioavailability, reduced granuloma weight, and significant suppression of proinflammatory cytokines (TNF-α, IL-6, and PGE2). Overall, the developed CS-CMC hydrogel of *Martynia annua* showed promising results as an effective and biocompatible system for controlled drug delivery in inflammatory disorders.
Keywords: Martynia annua, CS-CMC Hydrogel, Anti-inflammatory, Rheumatoid Arthritis, Controlled Drug Delivery, Phytochemicals

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1. Introduction

Medicinal plants have been an integral part of healthcare systems since ancient times and continue to play a significant role in the prevention and treatment of numerous diseases. Traditional systems of medicine, including Ayurveda, Traditional Chinese Medicine (TCM), and Unani, have extensively utilized plant-derived formulations for the management of inflammatory disorders, infectious diseases, metabolic abnormalities, and several chronic illnesses. Among these, Ayurveda, which originated in India more than 5,000 years ago, is one of the oldest documented healthcare systems and describes numerous medicinal plants possessing anti-inflammatory, analgesic, antioxidant, and immunomodulatory properties. Likewise, Traditional Chinese Medicine has relied on herbal remedies for over 2,000 years, while the Unani system has also emphasized the therapeutic value of medicinal plants for maintaining health and treating disease. The long history of herbal medicine has significantly contributed to the discovery of many modern therapeutic agents and continues to provide a valuable source of bioactive compounds for pharmaceutical research.

Despite remarkable advances in modern medicine, herbal drugs remain an important component of global healthcare. According to the World Health Organization (WHO), nearly 80% of the population in developing countries depends on traditional herbal medicines for primary healthcare. Furthermore, several clinically important drugs, including morphine, quinine, aspirin, and paclitaxel, have been developed from natural plant sources, demonstrating the immense therapeutic potential of medicinal plants. The increasing prevalence of chronic diseases, coupled with the

adverse effects, high treatment costs, and long-term toxicity associated with synthetic drugs, has renewed scientific interest in herbal medicines as safer, cost-effective, and sustainable therapeutic alternatives. Consequently, medicinal plants are increasingly being investigated as potential sources of novel anti-inflammatory, antioxidant, antimicrobial, and anticancer agents for the management of chronic diseases [1,2,3].

According to the WHO, herbal medicines are defined as plant-derived materials or preparations containing raw or processed ingredients obtained from one or more medicinal plants that provide therapeutic or other health benefits. Herbal formulations may contain different plant parts, including leaves, roots, stems, bark, flowers, fruits, and seeds, which are rich in biologically active phytoconstituents such as flavonoids, phenolic compounds, alkaloids, glycosides, terpenoids, and tannins. These phytochemicals exhibit diverse pharmacological activities and have attracted considerable attention for their ability to modulate multiple biological pathways involved in inflammation, oxidative stress, and immune regulation.

Inflammation is a highly coordinated biological response initiated by the immune system against harmful stimuli, including pathogens, toxins, damaged tissues, allergens, or environmental irritants. Under normal physiological conditions, inflammation serves as a protective mechanism that eliminates injurious agents, removes damaged cells, and promotes tissue repair and regeneration. However, prolonged or dysregulated inflammatory responses contribute to the development of numerous chronic diseases, including rheumatoid arthritis, osteoarthritis, cardiovascular disorders, diabetes mellitus, inflammatory bowel disease, and cancer. Persistent inflammation is characterized by continuous production of inflammatory mediators, oxidative stress, and immune cell infiltration, ultimately resulting in progressive tissue damage and organ dysfunction. Therefore, effective regulation of inflammatory pathways has become an important therapeutic strategy for preventing chronic inflammatory diseases and improving patient outcomes [4,5].

The inflammatory response is regulated through several intracellular signaling pathways that coordinate the expression of pro-inflammatory mediators and cytokines. Among these, the nuclear factor-kappa B (NF-κB) signaling pathway plays a central role in initiating and maintaining inflammation by regulating the transcription of genes encoding tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). Similarly, the mitogen-activated protein kinase (MAPK) pathway regulates cellular proliferation, differentiation, apoptosis, and inflammatory cytokine production through activation of transcription factors such as AP-1. Another important signaling cascade is the Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway, which mediates cytokine signaling and immune responses involved in chronic inflammatory disorders. Dysregulation of these molecular pathways contributes significantly to the initiation and progression of inflammatory diseases, making them attractive therapeutic targets for the development of novel anti-inflammatory agents [6,7].

Rheumatoid arthritis (RA) is a chronic, progressive, and systemic autoimmune disorder characterized by persistent inflammation of the synovial joints, ultimately leading to cartilage degradation, bone erosion, joint deformity, and functional disability. It is one of the most prevalent inflammatory rheumatic diseases worldwide and affects approximately 0.5–1.0% of the global population, with a higher incidence among women than men. Although RA can occur at any age, its onset is most common between 30 and 60 years. In addition to joint destruction, RA is frequently associated with several extra-articular manifestations, including cardiovascular complications, pulmonary disorders, osteoporosis, and fatigue, significantly impairing patients' quality of life and increasing healthcare burden [8,9].

The exact etiology of rheumatoid arthritis remains incompletely understood; however, genetic predisposition, environmental factors, smoking, hormonal influences, and microbial infections have been implicated in disease initiation. The disease is characterized by the loss of immune tolerance, resulting in activation of autoreactive T lymphocytes, B lymphocytes, macrophages, and dendritic cells. These immune cells infiltrate the synovial membrane and initiate a chronic inflammatory cascade, leading to excessive production of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Persistent cytokine release stimulates synovial hyperplasia, angiogenesis, and recruitment of additional inflammatory cells, thereby perpetuating joint inflammation and tissue destruction [10].

The pathogenesis of RA involves a complex sequence of immunological events. Initially, environmental or genetic triggers activate antigen-presenting cells and autoreactive T cells within the synovial tissue. Activated T cells subsequently release inflammatory cytokines that stimulate macrophages, fibroblast-like synoviocytes, and B cells. The activated synovial cells proliferate abnormally, resulting in synovial hyperplasia and formation of an invasive granulation tissue known as the pannus. This pannus aggressively invades

adjacent cartilage and subchondral bone while secreting matrix metalloproteinases (MMPs), collagenases, and osteoclast-activating factors that accelerate joint destruction. Continuous inflammation eventually causes irreversible cartilage degradation, bone erosion, joint deformity, stiffness, chronic pain, and progressive loss of joint function [10].

Although conventional pharmacological therapies, including non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, disease-modifying antirheumatic drugs (DMARDs), and biological agents, have substantially improved disease management, long-term treatment remains associated with several limitations. Chronic administration of NSAIDs and corticosteroids may cause gastrointestinal irritation, renal impairment, cardiovascular complications, and immunosuppression, whereas biological therapies are expensive and may increase susceptibility to opportunistic infections. Furthermore, these treatments primarily control disease symptoms rather than completely preventing disease progression. Consequently, there is increasing interest in identifying safer and more effective therapeutic alternatives, particularly plant-derived bioactive compounds with anti-inflammatory and immunomodulatory properties, for long-term management of rheumatoid arthritis [8,9].

Martynia annua L. (Family: Martyniaceae), commonly known as Devil's Claw, Tiger's Claw, or Bichhu, is an annual herbaceous medicinal plant widely distributed throughout tropical and subtropical regions, including India. The plant has been extensively used in traditional systems of medicine for the treatment of inflammation, rheumatism, wounds, skin disorders, epilepsy, sore throat, and microbial infections. Different parts of the plant, including the leaves, fruits, roots, and seeds, have been reported to possess significant pharmacological activities owing to the presence of diverse phytoconstituents such as flavonoids, phenolic acids, iridoid glycosides, tannins, alkaloids, saponins, and terpenoids.

Among these bioactive constituents, flavonoids and phenolic compounds have attracted considerable attention because of their potent anti-inflammatory, antioxidant, analgesic, antimicrobial, and immunomodulatory activities. Experimental studies have demonstrated that *Martynia annua* extracts inhibit the production of inflammatory mediators, including TNF-α, IL-1β, prostaglandins, cyclooxygenase (COX), and nitric oxide, thereby reducing inflammatory responses and oxidative stress. These findings suggest that *Martynia annua* possesses significant therapeutic potential for the management of chronic inflammatory disorders such as rheumatoid arthritis. However, despite its promising pharmacological properties, its clinical application remains limited due to poor aqueous solubility, instability of phytoconstituents, and inadequate bioavailability following conventional administration. Therefore, the development of advanced drug delivery systems capable of enhancing stability, controlled release, and localized delivery is essential to maximize its therapeutic efficacy.

Hydrogels are three-dimensional, hydrophilic polymeric networks capable of absorbing and retaining large quantities of water or biological fluids while maintaining their structural integrity. Owing to their high water content, flexibility, biocompatibility, and tissue-like properties, hydrogels have emerged as promising biomaterials for pharmaceutical and biomedical applications. Their porous architecture enables the encapsulation of therapeutic agents and facilitates controlled and sustained drug release, thereby improving drug bioavailability and therapeutic efficacy. Furthermore, hydrogels can be fabricated from natural polymers, synthetic polymers, or hybrid polymeric systems, allowing the optimization of their mechanical strength, swelling behavior, biodegradability, and drug release characteristics according to the intended therapeutic application [11,12].

In the management of rheumatoid arthritis, topical and transdermal hydrogel systems have gained considerable attention as alternatives to conventional oral drug delivery. These systems provide localized drug delivery directly to inflamed joints, resulting in higher drug concentrations at the site of inflammation while minimizing systemic exposure and adverse effects. Sustained drug release from hydrogel matrices also reduces dosing frequency and improves patient compliance, making hydrogels particularly suitable for chronic inflammatory diseases requiring prolonged therapy. Consequently, hydrogel-based formulations represent an effective platform for delivering anti-inflammatory phytoconstituents with enhanced therapeutic performance [11,12].

Among various natural biomaterials, chondroitin sulphate (CS) has attracted significant interest because of its intrinsic biological functions and excellent biocompatibility. Chondroitin sulphate is a naturally occurring sulphated glycosaminoglycan abundantly present in cartilage, connective tissues, tendons, and synovial fluid, where it contributes to maintaining cartilage structure and joint homeostasis. Besides its structural role, chondroitin sulphate possesses anti-inflammatory and chondroprotective properties by suppressing inflammatory mediators, reducing cartilage degradation, and improving joint lubrication. Incorporation of chondroitin sulphate into hydrogel formulations not only enhances the biological activity of the delivery system but also promotes cartilage protection, improves joint mobility, and prolongs drug retention at the site of inflammation.

These characteristics make chondroitin sulphate an attractive biomaterial for localized treatment of rheumatoid arthritis [13].

Carboxymethyl cellulose (CMC) is another widely used hydrophilic polymer in hydrogel formulations because of its excellent biocompatibility, biodegradability, non-toxicity, and superior film-forming ability. The abundant hydroxyl and carboxymethyl functional groups of CMC facilitate hydrogel formation with desirable swelling capacity and mechanical strength. Furthermore, CMC improves viscosity, moisture retention, and bioadhesion, enabling prolonged residence time of the formulation at the site of application. The combination of chondroitin sulphate and carboxymethyl cellulose provides a stable polymeric matrix capable of sustained drug release while maintaining favorable physicochemical properties required for topical drug delivery. Such hybrid hydrogels can effectively protect encapsulated phytoconstituents from degradation and improve their penetration into inflamed tissues, thereby enhancing therapeutic efficacy.

The incorporation of medicinal plant extracts into hydrogel systems further expands their therapeutic potential. Martynia annua possesses diverse phytoconstituents with well-documented anti-inflammatory, antioxidant, and analgesic activities. Delivery of Martynia annua extract through a chondroitin sulphate–CMC hydrogel may enhance the local concentration of bioactive compounds at inflamed joints while minimizing systemic toxicity. The phytochemicals present in Martynia annua have been reported to suppress the production of key inflammatory mediators, including TNF-α, IL-1β, IL-6, cyclooxygenase-2 (COX-2), and prostaglandins, thereby reducing synovial inflammation and slowing the progression of rheumatoid arthritis. Simultaneously, chondroitin sulphate contributes to cartilage preservation and joint lubrication, producing a synergistic therapeutic effect [14,15].

Recent advances in biomaterials and drug delivery have further improved hydrogel technology through the development of multifunctional and stimuli-responsive systems. Modern hydrogel formulations can be designed to respond to physiological stimuli such as pH, temperature, enzymes, and oxidative stress, enabling site-specific and controlled drug release. Integration of nanotechnology with hydrogel systems has also enhanced drug loading efficiency, stability, tissue penetration, and therapeutic performance. These hybrid nano-hydrogel systems combine the advantages of polymeric hydrogels with nanoscale drug carriers, providing prolonged drug release, improved bioavailability, and enhanced anti-inflammatory efficacy. Such innovative approaches represent a promising strategy for the treatment of chronic inflammatory diseases, including rheumatoid arthritis [16].

Based on these considerations, the present study was undertaken to develop and evaluate a chondroitin sulphate–carboxymethyl cellulose hydrogel incorporating Martynia annua Linn. extract for the treatment of rheumatoid arthritis. The developed hydrogel was designed to provide localized and sustained delivery of phytoconstituents, enhance anti-inflammatory activity, improve cartilage protection, and minimize the systemic adverse effects associated with conventional anti-inflammatory therapies.

2. Materials & Methods

The materials used in the formulation of the hydrogel include Chondroitin Sulphate (CS) and Sodium Carboxymethyl Cellulose (CMC), both of which were sourced from Sigma-Aldrich, USA. Preservatives, and other excipients such as Propylene Glycol (PG) (5%), Methyl Paraben (0.5%), and Propyl Paraben (0.2%) were also sourced from Sigma-Aldrich, USA. Triethanolamine (TEA), used for pH adjustment, was procured from Merck, Germany, and Distilled Water was obtained from Fisher Scientific, USA. The extraction solvents, Methanol and Ethanol, required for preparing the Martynia annua leaf extract, were also sourced from Sigma-Aldrich, USA. These materials were carefully selected to ensure the biocompatibility, stability, and effectiveness of the hydrogel formulation for the intended anti-inflammatory activity.

2.1. Methodology

Extraction and Phytochemical Screening

The dried leaves of *Martynia annua* Linn. were extracted using the Soxhlet extraction method, an efficient technique for isolating bioactive phytoconstituents from plant materials. Approximately 50 g of dried leaf powder was extracted separately with 300 mL of petroleum ether, methanol, chloroform, and distilled water for 6–8 h using a Soxhlet apparatus. Petroleum ether was used to extract non-polar constituents such as lipids and waxes, methanol for flavonoids, phenolics, glycosides, and alkaloids, chloroform for moderately polar compounds including terpenes, steroids, and saponins, while distilled water was employed for extracting water-soluble phytoconstituents. After completion of extraction, the solvents were removed under reduced pressure using a rotary evaporator, and the concentrated extracts were stored under appropriate conditions until further investigation [17–20].

Phytochemical Screening

The petroleum ether, methanol, chloroform, and aqueous extracts of *Martynia annua* Linn. were

subjected to preliminary phytochemical screening to identify major classes of secondary metabolites responsible for its pharmacological activity. Standard qualitative chemical tests were performed for the detection of alkaloids, flavonoids, phenolic compounds, saponins, glycosides, steroids, terpenes, tannins, carbohydrates, and proteins using established procedures [21,22].

Chromatographic Studies

Chromatography is an essential analytical technique used in chemistry for the separation and identification of compounds in mixtures. In this study, Thin Layer Chromatography (TLC) was employed to separate and identify the various phytoconstituents present in the ethanol extract of Martynia annua. This method is widely used for its simplicity, speed, and effectiveness in separating complex mixtures [23].

TLC is a technique where a sample is applied to a stationary phase, typically a thin layer of silica gel or alumina on a flat, inert surface (such as a glass plate). The sample, dissolved in a small volume of solvent, is moved up the plate by capillary action. This process is driven by the solvent (mobile phase) which interacts differently with the components of the sample based on their chemical properties (such as polarity, solubility, and molecular size). As the solvent moves up the plate, the components of the sample travel at different rates and separate from each other. The separated components can be detected visually by various means such as UV light, iodine vapor, or other chemical reagents that form distinct colored spots on the TLC plate.

The distance traveled by each component (known as the Retention factor, Rf) is a key feature of TLC. The Rf value is calculated as the ratio of the distance traveled by the compound to the distance traveled by the solvent front. This helps in identifying and comparing compounds based on their affinity for the solvent and the stationary phase [24].

Total Flavonoid Content Determination

Flavonoids are a group of plant metabolites known for their antioxidant, anti-inflammatory, and anticancer properties. The total flavonoid content of the ethanol extract was determined using a colorimetric assay involving aluminum chloride. The aluminum chloride forms a complex with flavonoids, and this complex can be detected by measuring absorbance at 355 nm [25].

Preparation and Characterization of Hydrogel

Herbal extracts are rich in bioactive phytoconstituents, including flavonoids, phenolics, glycosides, carbohydrates, and amino acids, which contribute to their diverse pharmacological activities. *Martynia annua* Linn. has been traditionally used for the treatment of inflammation, wounds, skin disorders, tuberculosis, and sore throat. Owing to its reported anti-inflammatory and wound-healing properties, the methanolic leaf extract of *Martynia annua* (MAME) was selected for the development of a topical hydrogel and its anti-inflammatory potential was evaluated through in vitro studies [26].

Selection of Dosage Form

Hydrogels are three-dimensional polymeric networks widely employed for topical drug delivery because of their high water content, biocompatibility, and ability to provide sustained drug release. Natural polymer-based hydrogels are preferred due to their biodegradability, safety, and excellent tissue compatibility. In the present study, chondroitin sulphate (CS) was selected as the primary gelling polymer because of its superior swelling capacity, moisture-retaining ability, and biocompatibility. Sodium carboxymethyl cellulose (Na-CMC) was incorporated to improve gel consistency, mechanical strength, and stability. The optimized CS–Na-CMC hydrogel containing *Martynia annua* methanolic extract was developed for topical application with the aim of enhancing anti-inflammatory efficacy and promoting skin healing [27,28].

Method of Preparation of Chondroitin Sulphate–CMC Hydrogel Containing Methanolic Leaf Extract of *Martynia annua* L.

The Chondroitin Sulphate (CS)-modified Carboxymethyl Cellulose (CMC) hydrogel containing methanolic leaf extract of *Martynia annua* L. was prepared by a modified method of Chirayath et al. (2019) [29]. Five formulations, including one control (without extract), were developed using different polymer concentrations.

Accurately weighed CS and Sodium CMC were separately dispersed in distilled water under continuous magnetic stirring until homogeneous solutions were obtained. The polymer solutions were mixed and the pH was adjusted to 5.0–5.5. EDC and NHS were then added to activate the carboxyl groups of the polymers, followed by the slow addition of adipic acid dihydrazide (ADH) to promote amide bond formation and crosslinking. The reaction mixture was stirred for 2–4 h at room temperature to obtain a stable crosslinked polymeric network.

Separately, methyl paraben and propyl paraben were dissolved in distilled water with gentle heating and mixed with glycerin, which served as a humectant and improved the consistency of the hydrogel. Subsequently, 1% (w/w) methanolic leaf extract of *Martynia annua* was incorporated into the polymeric dispersion under continuous stirring at 700 rpm until a uniform gel was formed.

Finally, the pH of the formulation was adjusted to 6.8–7.0 using triethanolamine (TEA) to ensure skin

compatibility. The prepared hydrogels were mixed thoroughly, transferred into airtight containers, and allowed to stabilize at room temperature before further physicochemical characterization and in vitro anti-inflammatory evaluation. This method ensured uniform drug incorporation, stable crosslinking, and suitable rheological properties for topical drug delivery [29].

Table 1. Formulations of Hydrogel Containing Methanol Extract of Martynia annua Leaves

Ingredients	F1	F2	F3	F4	F5
Chondroitin Sulphate (g)	0.25	0.5	1	1.5	2
Sodium CMC (g)	2	1.5	1	0.5	0.25
Extract (%w/w)	1	1	1	1	1
Propylene Glycol 400 (5%) (ml)	2.5	2.5	2.5	2.5	2.5
Methyl Paraben (0.5%) (ml)	0.1	0.1	0.1	0.1	0.1
Propyl Paraben (0.2%) (ml)	2.5	2.5	2.5	2.5	2.5
Triethanolamine (ml)	q.s.	q.s.	q.s.	q.s.	q.s.
Distilled Water (ml)	50	50	50	50	50

Characterization of Hydrogel

Physical Evaluation

The prepared hydrogel formulations were visually evaluated for appearance, color, odor, homogeneity, and consistency to ensure uniformity and suitability for topical application.

pH Determination

The pH of the hydrogels was measured using a digital pH meter at room temperature. The pH was maintained between 6.8 and 7.0 to ensure skin compatibility and minimize irritation.

Spreadability

Spreadability was determined by placing the hydrogel between two glass slides under an 80 g weight. The time required for the upper slide to move 7.5 cm was recorded and spreadability was calculated using:

$$Spreadibility = \frac{M \times L}{T}$$

where S = Spreadability, M = Weight (80 g), L = Length moved (7.5 cm), and T = Time (s).

Homogeneity

Hydrogels were examined visually for uniformity, smooth texture, and absence of lumps or phase separation.

Viscosity

Viscosity was measured using a Brookfield Viscometer to evaluate the flow behavior and suitability of the hydrogel for topical application [122].

Percentage Yield

The percentage yield of the prepared hydrogel was calculated by comparing the weight of the dried hydrogel with the total weight of the initial formulation ingredients using the following equation:

$$\%\ Yield = \frac{Weight\ of\ Dried\ Hydrogel\ (W\_final)}{Total\ Initial\ Weight\ of\ Components\ (W\_initial)} \times 100$$

where Wfinal is the weight of dried hydrogel and Winitial is the total weight of ingredients used.

Swelling Index

The swelling behavior of the hydrogel was evaluated by immersing the dried hydrogel in simulated gastric fluid (pH 1.2) at 37°C. The swollen hydrogel was weighed at predetermined intervals, and the swelling index was calculated as:

$$Swelling\ Index = \frac{Weight\ of\ Swellon\ Bead\ (W_{\_Swellon}) - Initial\ Weight\ of\ Dried\ Bead\ (W\_Dry)}{Initial\ Weight\ of\ Dried\ Bead\ (W\_Dry)} \times 100$$

where Wswollen is the weight after swelling and Wdry is the initial dry weight.

Surface Morphology

The surface morphology of the optimized hydrogel was characterized using Atomic Force Microscopy (AFM) (AIST-NT Smart SPM 1000, USA) and Scanning Electron Microscopy (SEM) (JEOL JSM-5400, Japan). Samples were gold-coated before SEM analysis to obtain high-resolution surface images [30].

Entrapment Efficiency

Entrapment efficiency was determined using HPLC (Agilent 1220 Infinity LC) equipped with a C18 column. The amount of entrapped *Martynia annua* extract was estimated at 355 nm, and encapsulation efficiency was calculated using [31]:

$$Percentage\ Encapsulation\ Efficiency = \frac{Total\ Drug - Unentrapped\ Drug}{Total\ Drug} \times 100$$

Particle Size and Zeta Potential

The particle size of the hydrogel was measured using a laser diffraction particle size analyzer (Malvern Instruments, UK). Zeta potential was determined using a Malvern Zetasizer based on laser Doppler anemometry to evaluate particle size

distribution, surface charge, and formulation stability [32].

Percentage Cumulative Leaf Extract Release

The in vitro drug release study was performed to evaluate the sustained release behavior of *Martynia annua* leaf extract from the CS-CMC hydrogel formulation [30]. Drug-loaded hydrogels were placed in 50 mL of physiological medium maintained at 37 ± 0.5°C. Samples were withdrawn at predetermined intervals (1, 6, 8, 10, 12, and 24 h) and replaced with an equal volume of fresh medium to maintain sink conditions. The amount of extract released was quantified by HPLC using a previously prepared calibration curve, and cumulative drug release was calculated using the following equation [30,33]:

$$\%\ \textbf{Cumulative Drug Release} = \frac{\textbf{Amount of Drug Released at Time } (t)}{\textbf{Total Amount of Drug Loaded in Hydrogel}} \times 100$$

Pharmacological Studies

The anti-inflammatory potential of *Martynia annua* methanolic leaf extract-loaded CS-CMC hydrogel was evaluated using standard in vitro, ex vivo, and in vivo models. These studies assessed its ability to inhibit inflammatory mediators, stabilize biological membranes, improve skin permeation, and suppress chronic inflammation.

Anti-Lipoxygenase Activity

Anti-lipoxygenase activity was evaluated using the linoleic acid oxidation assay, as lipoxygenase catalyzes leukotriene formation during inflammation [34]. Different concentrations of the hydrogel (100–500 µg/mL) and Indomethacin (standard) were incubated with lipoxygenase enzyme in borate buffer (pH 9.0). The reaction was initiated with linoleic acid, and absorbance was measured at 234 nm. Reduced absorbance indicated inhibition of lipoxygenase activity.

Protein Denaturation Inhibition

The anti-inflammatory effect was assessed by the BSA protein denaturation assay [35]. Hydrogel samples (100–500 µg/mL) were mixed with bovine serum albumin, incubated at 37°C, followed by heating at 70°C. Absorbance was recorded at 660 nm, and the percentage inhibition of protein denaturation was calculated. Lower turbidity indicated better anti-inflammatory activity.

$$\textbf{Percentage Inhibition} = 100 \times \left(1 - \frac{A_2}{A_1}\right)$$

Proteinase Inhibition

Proteinase inhibitory activity was determined using standard in vitro protease inhibition assay [36]. Various concentrations (100–500 µg/mL) of the hydrogel were tested, and absorbance was measured at 660 nm. Higher inhibition indicated protection against proteinase-mediated tissue damage associated with inflammation.

Membrane Stabilization

Membrane stabilizing activity was evaluated using the human RBC membrane stabilization method [37]. Erythrocytes were exposed to hypotonic solution in the presence of hydrogel formulations (100–500 µg/mL). Hemoglobin release was measured at 540 nm, and reduced hemolysis indicated effective membrane stabilization.

Hypotonicity-Induced Hemolysis

The membrane protective effect was further confirmed using the hypotonicity-induced hemolysis assay [38]. Human RBCs were treated with different concentrations of hydrogel under hypotonic conditions. The percentage inhibition of hemolysis was determined spectrophotometrically at 540 nm, with higher inhibition indicating stronger membrane protection.

In Vitro Permeation Study

The Franz diffusion cell method was used to evaluate transdermal permeation of the hydrogel through excised rat skin [30]. Approximately 250 mg of hydrogel was applied to the donor compartment, while physiological saline containing 1% Tween 80 served as receptor medium maintained at 32 ± 0.5°C. Samples were collected at predetermined intervals, analyzed by HPLC, and permeation flux was calculated.

In Vivo Permeation Study

The in vivo skin permeation study was carried out in male albino rats [30]. The hydrogel was applied topically, and blood samples were collected at specified intervals. After completion of the study, plasma, epidermis, and dermis samples were analyzed by HPLC to determine drug permeation and skin retention.

Cotton Pellet Granuloma Model

The chronic anti-inflammatory activity was evaluated using the cotton pellet granuloma model [39]. Sterile cotton pellets were implanted subcutaneously in rats, followed by daily treatment for 7 days. On day 8, pellets were removed, dried, and weighed. Reduction in granuloma weight indicated anti-inflammatory activity.

$$\%\ \textbf{inhibition} : \frac{\textbf{Granuloma Weight (Control)} - \textbf{Granuloma Weight (tre}}{\textbf{Granuloma Weight (Control)}} \times 100$$

Assessment of Pro-inflammatory Markers

Granuloma tissue collected on day 8 was processed for estimation of TNF-α, IL-6, and PGE_2 levels using ELISA. Tissue homogenates were centrifuged, and the supernatants were analyzed according to the manufacturer's protocol. Absorbance was measured at 450 nm, and cytokine concentrations were expressed as pg/mL. The results were statistically analyzed to determine the

anti-inflammatory efficacy of the developed hydrogel.

Results and Discussion

Extraction Yield and Phytochemical Content

The extraction of *Martynia annua* leaves using solvents of different polarities showed considerable variation in extract yield, indicating the presence of diverse phytoconstituents. Petroleum ether, chloroform, methanol, and aqueous extracts yielded 8.4%, 10.0%, 15.0%, and 7.2%, respectively. The methanolic extract exhibited the highest yield (15%), demonstrating its superior ability to extract polar phytochemicals such as flavonoids, phenolics, glycosides, and alkaloids. In contrast, petroleum ether extracted non-polar constituents, chloroform recovered moderately polar compounds, while the aqueous extract showed the lowest recovery of water-soluble constituents.

The total flavonoid content of the methanolic extract was found to be 136.24 ± 4.08 mg luteolin equivalent/100 g extract, confirming the abundance of bioactive phenolic compounds. These flavonoids are well recognized for their antioxidant and anti-inflammatory properties and contribute significantly to the therapeutic potential of *Martynia annua*. Overall, the extraction profile indicates that methanol is the most suitable solvent for obtaining maximum bioactive constituents, supporting the selection of the methanolic extract for hydrogel formulation and subsequent anti-inflammatory evaluation.

Phytochemical Screening

The results of the phytochemical screening for alkaloids, flavonoids, phenols, glycosides, saponins, terpenes, steroids, tannins, carbohydrates, and proteins in the petroleum ether, methanol, chloroform, and aqueous extracts are summarized in Table 2.

Table 2. Phytochemical Screening of Martynia annua Linn. Extracts

Phytochemical	Petroleum Ether	Aqueous	Methanol	Chloroform
Alkaloids	+++	---	+++	+++
Flavonoids	---	+++	+++	---
Phenols	---	+++	+++	+++
Saponins	---	+++	+++	+++
Glycosides	---	+++	+++	---
Steroids	+++	---	+++	+++
Terpenes	+++	---	+++	+++
Tannins	---	+++	+++	---
Carbohydrates	---	+++	---	---
Proteins	---	---	+++	---

TLC of Different Extracts

The study presents the results of TLC performed on the methanol extract of Martynia annua leaves, using a variety of solvent mixtures. The objective was to evaluate the separation and number of distinct spots (indicating different phytoconstituents) for each solvent system. The observations are summarized in the table 3, mentioned below:

Table 3. TLC observation with different solvent system

Solvent System	Ratio (mL)	Observation (No. of Spots)
Benzene: Chloroform	70:30:00	3
Benzene: Chloroform: Ethyl Acetate	70:25:05	5
Benzene: Chloroform: Ethyl Acetate: Acetic Acid	70:20:5:5	6
Benzene: Chloroform: Ethyl Acetate: Acetic Acid	80:15:3:2	6
Chloroform: Ethyl Acetate: Acetic Acid: Water	85:11:2:2	6

The number of spots observed after development indicates the number of distinct compounds that have been separated from the mixture. The presence of multiple spots in all the solvent systems suggests that the methanol extract contains a complex mixture of compounds, which vary in polarity and affinity for the stationary and mobile phases.

Total Flavonoid Content Determination

The methanolic extract of *Martynia annua* showed a higher extraction yield (15%) than the petroleum ether extract (8.4%), indicating better recovery of polar bioactive compounds. Phytochemical screening confirmed the presence of steroids, terpenoids, and fatty oils in the petroleum ether extract, while the methanolic extract contained glycosides, phenolics, flavonoids, and amino acids. TLC analysis revealed multiple phytoconstituents with Rf values ranging from 0.36 to 0.74. Quantitative estimation showed total phenolic content of 107.25 ± 3.66 mg GAE/g and total flavonoid content of 136.24 ± 4.08 mg luteolin equivalents/100 g, confirming the richness of

antioxidant and anti-inflammatory compounds. The methanol-soluble fraction exhibited higher yield and was selected for further formulation studies, highlighting *Martynia annua* as a promising natural source of therapeutic phytoconstituents.

Hydrogel Characterization Parameters

Physical Evaluation

All hydrogel formulations exhibited a smooth texture, brownish color, and were odorless, indicating acceptable physical characteristics suitable for topical application.

pH

The pH of all formulations ranged from 6.95 to 7.01, which is close to the physiological skin pH. This confirms good biocompatibility and minimizes the risk of skin irritation during topical use.

Spreadability

The spreadability values ranged from 14.87 ± 0.23 to 16.54 ± 0.87 g·cm/s, indicating good ease of application. Formulation F3 exhibited the highest spreadability, suggesting better coverage and patient compliance.

Homogeneity

All formulations were uniform, smooth, and free from phase separation, confirming homogeneous distribution of the extract and good formulation stability.

Viscosity

The viscosity of the hydrogels ranged from 194,300 to 196,800 cps, demonstrating suitable gel consistency for topical application and sustained drug release. Minor variations were attributed to differences in polymer concentration without affecting overall formulation performance.

Table 4. Physical evaluation of CS-CMC hydrogel formulations containing *Martynia annua* leaf extract.

Parameters	F1	F2	F3	F4	F5
Physical Evaluation	brownish color, odorless	brownish color, odorless	brownish color, odorless	brownish color, odorless	brownish color, odorless
pH	6.95	7.01	6.97	6.96	6.98
Spreadability (g.cm/s)	15.13 ± 0.15	16.03 ± 0.87	16.54 ± 0.56	14.87 ± 0.23	15.98 ± 0.64
Homogeneity	Homogeneous smooth	Homogeneous smooth	Homogeneous smooth	Homogeneous smooth	Homogeneous, smooth
Viscosity (cps)	1,94,300	1,94,700	1,96,800	1,94,500	1,96,600

*F represents the Formulation.

Percentage Yield

The percentage yield was determined to evaluate the efficiency of hydrogel preparation. All formulations showed satisfactory yields ranging from 83.3% to 91.6%. Among them, Formulation F3 exhibited the highest yield (91.6%), indicating superior encapsulation efficiency and minimal material loss during preparation. The high yield suggests that F3 possesses an optimal polymer composition, making it the most suitable formulation for further studies.

Swelling Index

The swelling index was evaluated in simulated gastric fluid (pH 1.2) at 37°C to determine the water absorption capacity of the hydrogels. All formulations exhibited progressive swelling with time; however, Formulation F3 showed the highest swelling index throughout the study, increasing from 20.22 ± 0.30% (1 h) to 75.14 ± 0.52% (8 h). The enhanced swelling behavior of F3 indicates superior water uptake, gel expansion, and sustained drug retention, which are desirable for controlled drug release.

Table 5. Swelling Index of CS-CMC hydrogel formulations containing *Martynia annua* leaf extract.

Time Interval (hrs)	F1 (Swelling Index %)	F2 (Swelling Index %)	F3 (Swelling Index %)	F4 (Swelling Index %)	F5 (Swelling Index %)
1	17.34 ± 0.21	18.15 ± 0.27	20.22 ± 0.30	19.12 ± 0.25	18.60 ± 0.28
2	25.67 ± 0.32	27.42 ± 0.35	30.15 ± 0.33	28.90 ± 0.37	26.70 ± 0.33
4	40.82 ± 0.40	45.29 ± 0.41	50.50 ± 0.45	47.60 ± 0.42	44.29 ± 0.39
6	52.17 ± 0.46	57.60 ± 0.48	62.85 ± 0.49	59.80 ± 0.46	55.52 ± 0.43
8	63.45 ± 0.51	68.35 ± 0.50	75.14 ± 0.52	71.63 ± 0.48	66.45 ± 0.44

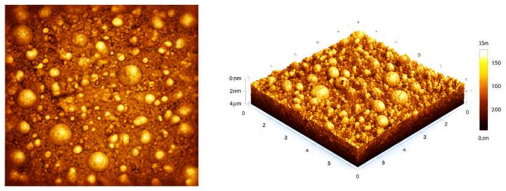
Overall, Formulation F3 demonstrated the best performance with an optimum pH (6.97), highest spreadability (16.54 ± 0.56 g·cm/s), maximum percentage yield (91.6%), highest swelling index (75.14 ± 0.52%), and excellent homogeneity without phase separation. These findings identify F3 as the optimized hydrogel formulation for further in vitro and in vivo anti-inflammatory evaluation.

Morphology

The morphology of the optimized CS-CMC hydrogel was evaluated using Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM). AFM analysis revealed a uniform, smooth surface with nanoscale features, indicating low surface roughness and a well-formed hydrogel network suitable for efficient drug loading and sustained release. SEM micrographs confirmed the presence of uniform, spherical, and well-dispersed nanoparticles without significant aggregation. The nanostructured morphology and high surface area of the hydrogel are expected to enhance drug encapsulation, improve skin permeation, and support controlled release, making the formulation suitable for topical anti-inflammatory therapy.

Figure 1. (a) AFM and (b) SEM image of CS-CMC hydrogel formulations containing *Martynia annua* leaf extract.

(a)



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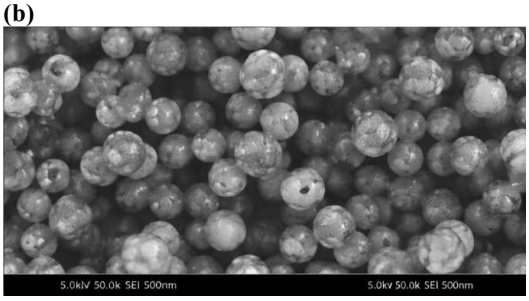


Entrapment Efficiency

Entrapment efficiency (EE) of Martynia annua leaf extract in CS-CMC hydrogel was determined by HPLC using a C18 column and detection at 355 nm. The CMC hydrogel exhibited an EE of 76.98 ± 0.75%, whereas the CS-CMC hydrogel showed a significantly higher EE of 91.34 ± 1.15%. The improved entrapment efficiency indicates superior encapsulation and retention of the bioactive constituents within the modified hydrogel matrix. This enhanced drug loading is expected to improve sustained drug release and therapeutic efficacy, making the CS-CMC formulation more suitable for topical anti-inflammatory applications.

Particle Size Analysis

Particle size analysis was performed using a laser diffraction particle size analyzer. The CMC hydrogel exhibited an average particle size of 167.43 ± 1.15 nm, while the CS-CMC hydrogel showed a significantly smaller particle size of 125.87 ± 0.75 nm. The reduced particle size of the CS-CMC hydrogel provides a larger surface area, leading to enhanced drug encapsulation, improved skin permeation, and better bioavailability. The higher entrapment efficiency (91.34%) observed in the CS-CMC formulation further confirms the positive influence of smaller particle size on hydrogel performance.

Drug Release

The in vitro drug release study demonstrated sustained release of Martynia annua leaf extract from both hydrogel formulations. The CMC hydrogel showed an initial burst release, reaching 65.62 ± 0.98% drug release after 24 hours. In contrast, the CS-CMC hydrogel exhibited a slower initial release followed by a controlled and sustained release, achieving 85.37 ± 1.10% cumulative drug release after 24 hours. The improved release profile of the CS-CMC hydrogel indicates its superior ability to provide prolonged drug delivery, making it a promising formulation for topical anti-inflammatory therapy.

The CMC-based hydrogel showed a rapid release in the initial hours, but the drug release slowed significantly after the first 12 hours, indicating a fast initial release phase. On the other hand, the CS-CMC-based hydrogel exhibited a more prolonged release, with a gradual increase in drug release over 24 hours, which is advantageous for controlled drug delivery. The slower and more sustained release observed in the CS-CMC hydrogel formulation indicates its potential for applications that require long-term therapeutic effects, such as topical treatments for inflammation or wound healing, where drug exposure extended is needed.

Overall, the CS-CMC-based hydrogel offers a more consistent and prolonged drug release profile, making it a promising candidate for controlled drug delivery systems, providing sustained therapeutic effects over time.

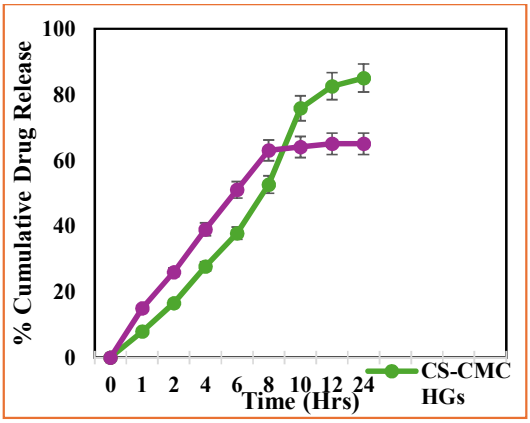
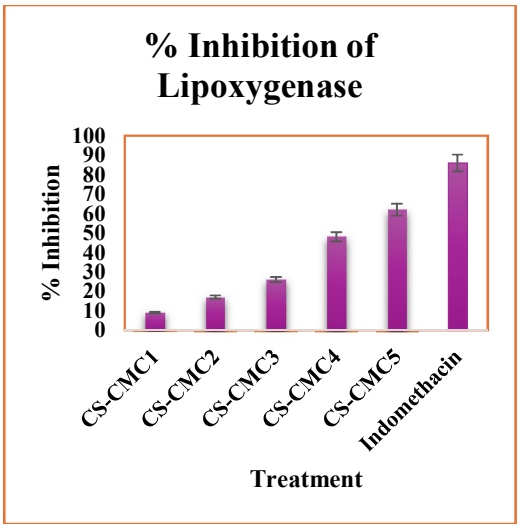


Figure 2. Percentage Drug Release of Formulated Hydrogels Containing Methanol Extract of Martynia annua Leaves

Anti-Lipoxygenase Activity

The anti-lipoxygenase activity of the Martynia annua extract-loaded CS-CMC hydrogel was evaluated at concentrations ranging from 100–500 µg/ml. The formulation exhibited a clear concentration-dependent inhibition of lipoxygenase, with inhibition increasing from 9% at 100 µg/ml to 62% at 500 µg/ml. Correspondingly, absorbance values decreased from 0.36 ± 0.03 to 0.15 ± 0.02, confirming effective suppression of enzyme activity. The inhibitory effect is attributed to flavonoids and phenolic compounds present in the methanolic extract, which may inhibit LOX by scavenging free radicals or chelating the enzyme's iron cofactor. Although Indomethacin produced higher inhibition (86%) at 100 µg/ml, the CS-CMC hydrogel demonstrated promising natural anti-inflammatory activity with the added advantage of potentially fewer adverse effects. These findings indicate that the formulation can effectively suppress leukotriene synthesis and oxidative stress, supporting its potential as a sustained topical anti-inflammatory agent.

Figure 3. Anti-Lipoxygenase Activity of CS-CMC



Protein Denaturation Inhibition

The protein denaturation inhibition assay demonstrated that the Martynia annua extract-loaded CS-CMC hydrogel possesses significant concentration-dependent anti-inflammatory activity. The percentage inhibition increased from 32% at 100 µg/ml to 71% at 500 µg/ml, while absorbance decreased from 0.38 ± 0.05 (control) to 0.11 ± 0.07, indicating effective prevention of heat-induced protein denaturation. The activity is mainly attributed to flavonoids, phenolics, tannins, and alkaloids, which stabilize protein structure through antioxidant and protein-binding mechanisms. Although Indomethacin showed 68% inhibition at 100 µg/ml, the CS-CMC hydrogel achieved a slightly higher inhibition (71%) at its highest concentration, highlighting its strong anti-inflammatory potential. These findings suggest that the formulation can effectively prevent protein denaturation, reduce inflammatory responses, and serve as a promising natural alternative for topical anti-inflammatory therapy.

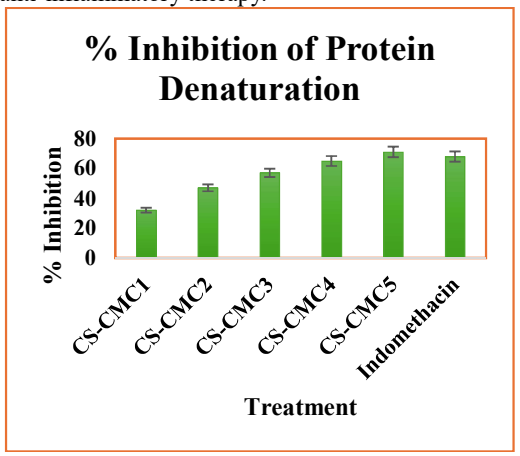


Figure 4. Effect of CS-CMC on Heat-Induced Protein Denaturation

Proteinase Inhibition

The proteinase inhibition assay demonstrated that the Martynia annua extract-loaded CS-CMC hydrogel exhibited significant concentration-dependent inhibitory activity. The percentage inhibition increased from 21% at 100 µg/ml to 53% at 500 µg/ml, while absorbance decreased from 0.38 ± 0.09 (control) to 0.18 ± 0.03, confirming effective suppression of proteinase activity. The inhibitory effect is attributed to bioactive phytochemicals such as flavonoids, phenolics, tannins, and alkaloids, which interfere with proteolytic enzymes and protect tissues from inflammatory damage. Indomethacin showed 55% inhibition at 100 µg/ml, indicating slightly higher potency; however, the CS-CMC hydrogel exhibited comparable efficacy at higher concentrations. These findings suggest that the formulation effectively inhibits proteinase-mediated tissue degradation and supports its potential as a natural anti-inflammatory agent for topical drug delivery.

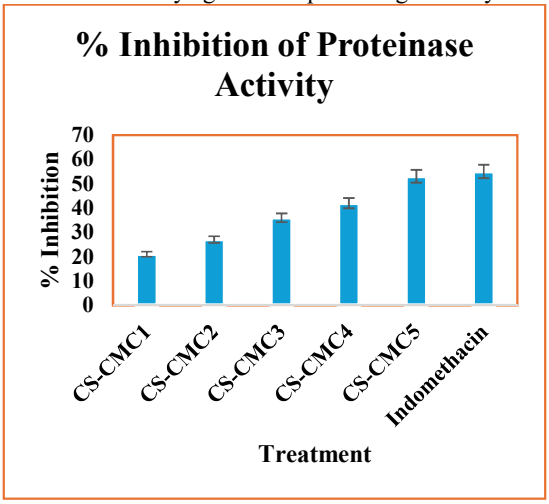


Figure 5. Effect of CS-CMC on Proteinase Inhibition

Membrane Stabilization

The membrane stabilization assay demonstrated that the Martynia annua extract-loaded CS-CMC hydrogel exhibited concentration-dependent protection against hypotonicity-induced hemolysis. The percentage inhibition increased from 21% at 100 µg/ml to 51% at 500 µg/ml, while absorbance decreased from 0.30 ± 0.03 (control) to 0.15 ± 0.03, indicating effective stabilization of red blood cell membranes. The activity is mainly attributed to flavonoids, tannins, phenolics, and alkaloids, which protect membrane integrity through antioxidant activity and inhibition of lipid peroxidation. Indomethacin exhibited higher inhibition (71%) at 100 µg/ml; however, the CS-CMC hydrogel showed considerable membrane-protective activity at higher concentrations. These findings suggest that the formulation can reduce membrane damage, prevent the release of inflammatory mediators, and serve as a promising natural anti-inflammatory agent for topical drug delivery.

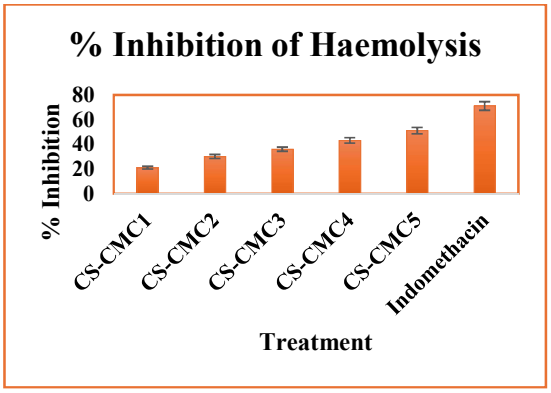


Figure 6. Effect of CS-CMC on Heat-Induced Haemolysis

Hypotonicity-Induced Hemolysis

The hypotonicity-induced hemolysis assay demonstrated that the Martynia annua extract-loaded CS-CMC hydrogel exhibited significant concentration-dependent membrane protective activity. The percentage inhibition increased from 30% at 100 µg/ml to 75% at 500 µg/ml, while absorbance decreased from 0.31 ± 0.02 (control) to 0.07 ± 0.02, indicating effective prevention of RBC hemolysis. The membrane stabilizing effect is attributed to the presence of flavonoids, phenolics, and other bioactive phytochemicals that protect cell membranes and reduce the release of inflammatory mediators. Although Indomethacin showed 51% inhibition at 100 µg/ml, the CS-CMC hydrogel produced superior protection (75%) at the highest concentration. These findings confirm the formulation's strong cytoprotective and anti-inflammatory potential, supporting its suitability for topical drug delivery and warranting further in vivo and clinical investigations.

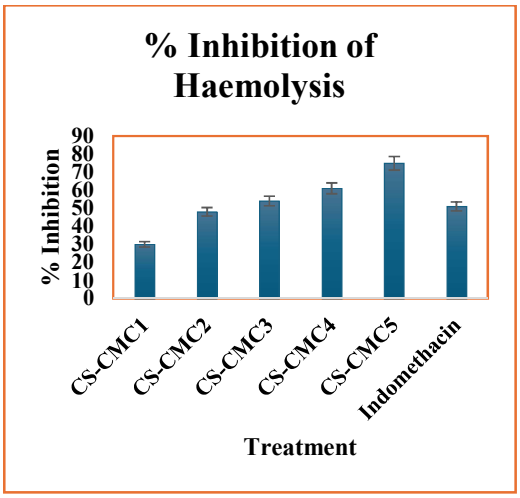


Figure 7. Effect of CS-CMC on Hypotonicity Induced Haemolysis

In Vitro Skin Permeation Study

The in vitro skin permeation study using Franz diffusion cells demonstrated that the Martynia annua extract-loaded CS-CMC hydrogel significantly enhanced transdermal drug delivery compared to the plain formulation. The plain formulation exhibited a flux of 4.75 mg cm^{-2} h^{-1}, whereas the CMC and CS-CMC hydrogels showed higher flux values of 5.12 and 7.89 mg cm^{-2} h^{-1}, respectively. Unlike the plain formulation, which showed a 1-hour lag time, the CS-CMC hydrogel exhibited no lag time, indicating immediate drug diffusion and controlled zero-order release. The improved permeation is attributed to the hydrogel's hydrophilic, swelling, and film-forming properties, which enhance hydration of the stratum corneum and facilitate drug penetration. These findings confirm that the CS-CMC hydrogel is an efficient carrier for sustained topical delivery of Martynia annua extract with enhanced anti-inflammatory potential.

Table 6. In Vitro Skin Permeation Parameters of Hydrogel Formulations

Formulation	Flux (Js) (mg cm^{-2} h^{-1})	Permeation Coefficient	Release Kinetics
Plain Formulation (Indomethacin)	4.75	4.75×10^{-4}	Non-zero order (with lag)
Extract-loaded CMC Hydrogel	5.12	5.12×10^{-4}	Zero-order
Extract-loaded CS-CMC Hydrogel	7.89	7.89×10^{-4}	Zero-order

In Vivo Permeation Study

The in vivo permeation study demonstrated that the Martynia annua extract-loaded CS-CMC hydrogel significantly enhanced transdermal drug delivery compared to the plain CMC hydrogel. The CS-CMC hydrogel showed a higher Cmax (7.28 mg/L) than the plain hydrogel (2.91 mg/L), indicating improved systemic absorption. Similarly, the AUC_{0}–t was significantly greater (20.43 h·mg/L) than the plain formulation (9.23 h·mg/L, $p < 0.05$), confirming enhanced bioavailability.

The improved permeation is attributed to the hydrogel's ability to maintain close contact with the skin, hydrate the stratum corneum, and facilitate deeper drug penetration. Drug distribution studies showed that the plain hydrogel accumulated mainly in the epidermis (14.62 ± 1.15 mg/mL), whereas the CS-CMC hydrogel achieved higher drug levels in the dermis (26.97 ± 0.7 mg/mL) with lower epidermal retention (4.98 ± 1.10 mg/mL).

Overall, the CS-CMC hydrogel exhibited superior skin permeation, deeper tissue penetration, and enhanced bioavailability, supporting its potential as an effective topical and transdermal anti-inflammatory drug delivery system.

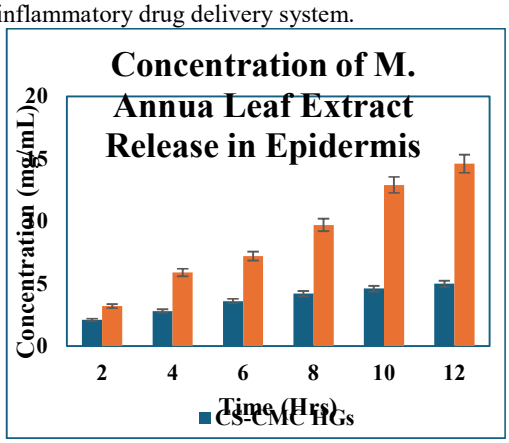


Figure 8. Concentration of Methanolic Extract of M. Annua in Epidermis from CS-CMC HGs and CMC HGs System.

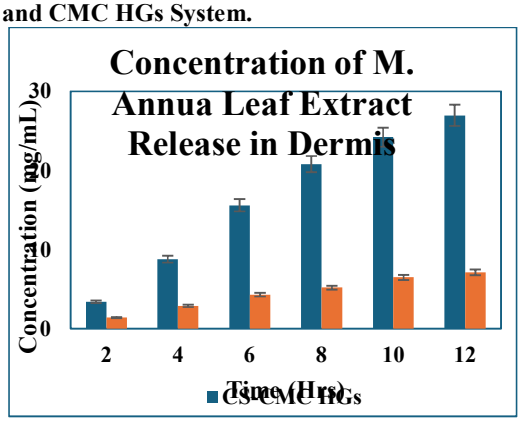


Figure 9. Concentration of Methanolic Extract of M. Annua in Dermis from CS-CMC HGs and CMC HGs System.

The enhanced permeation may also be attributed to the reduced particle size and efficient cross-linked polymeric network of the CS-CMC hydrogel, which improves interaction with the skin surface. The small particle size increases the surface area, thereby enhancing penetration efficiency when the formulation is in close contact with the skin.

Overall, the results clearly indicate that the extract-loaded CS-CMC hydrogel significantly enhances transdermal delivery of *Martynia annua* leaf extract compared to the plain formulation. The higher Cmax, increased AUC, deeper dermal penetration, and improved bioavailability confirm the superiority of the hydrogel system. These findings suggest that the developed hydrogel formulation is a promising carrier for transdermal drug delivery, particularly for achieving enhanced therapeutic efficacy in anti-inflammatory applications.

Table 7. Pharmacokinetic Parameters of Hydrogel Formulations

Pharmacokinetic Parameter	Extract-loaded CS-CMC HGs	CMC HGs (Plain)
Cmax (mg/L)	7.28	2.91
Tmax (h)	6	8
AUC_{0-12} (h·mg/L)	20.43	9.23
$AUC_{0-\infty}$ (h·mg/L)	24.85	11.67

Effect of Methanolic Extract of M. annua Loaded CS-CMC Hydrogels on Granuloma Weight

The anti-inflammatory activity of the methanolic extract-loaded CS-CMC hydrogel was evaluated using the cotton pellet-induced granuloma model. The control group showed the highest granuloma weight (70.87 ± 2.50 mg). All treated groups exhibited a dose-dependent reduction in granuloma weight, confirming significant anti-inflammatory activity.

CS-CMC at 100 mg/kg produced 8.63% inhibition, which increased progressively to 20.65%, 32.05%, 38.66%, and 45.64% at doses of 200, 300, 400, and 500 mg/kg, respectively. The highest dose (500 mg/kg) reduced granuloma weight to 38.52 ± 1.28 mg, showing activity comparable to Indomethacin (100 mg/kg), which produced 48.15% inhibition with a granuloma weight of 36.74 ± 1.15 mg.

Overall, the results demonstrate a dose-dependent reduction in granuloma formation, indicating that the Martynia annua-loaded CS-CMC hydrogel possesses significant anti-inflammatory activity. The highest dose showed efficacy comparable to the standard drug, supporting its potential as a promising topical anti-inflammatory formulation for further pharmacological and clinical studies.

Table 8. Effect of Methanolic Extract of M. Annua loaded CS-CMC HGs on granuloma weight in cotton pellet induced granuloma model

Animal Treatment Groups	Granuloma Weight (mg)	Percentage Inhibition (%)
Control (Normal Saline 10 ml/kg)	70.87 ± 2.50	-
CS-CMC 1 (100mg/kg)	**64.75 ± 2.38**	**8.63**
CS-CMC 2 (200mg/kg)	**56.23 ± 2.25**	20.65
CS-CMC 3 (300mg/kg)	**48.15 ± 1.98**	32.05
CS-CMC 4 (400mg/kg)	**43.47 ± 1.76**	38.66
CS-CMC 5 (500mg/kg)	**38.52 ± 1.28**	45.64
Standard (Indomethacin 100 mg/kg)	**36.74 ± 1.15**	48.15

Effect of Methanolic Extract of M. annua Loaded CS-CMC Hydrogels on Proinflammatory Markers

The effect of the methanolic extract-loaded CS-CMC hydrogel on proinflammatory markers (TNF-α, IL-6, and PGE_2) was evaluated using the cotton pellet-induced granuloma model. The control group showed the highest cytokine levels, indicating severe inflammation. Treatment with CS-CMC hydrogels produced a dose-dependent reduction in all inflammatory markers.

At 100 mg/kg, only a slight decrease in TNF-α, IL-6, and PGE_2 levels was observed. Increasing the dose progressively reduced cytokine levels, with the 500 mg/kg formulation showing the greatest effect (TNF-α: 21.65 ± 0.96 pg/mL; IL-6: 16.86 ± 1.05 pg/mL; PGE_2: 32.76 ± 1.15 pg/mL). These values were comparable to those of the standard drug Indomethacin (100 mg/kg). Overall, the results demonstrate that the Martynia annua-loaded CS-CMC hydrogel effectively suppresses proinflammatory cytokines in a dose-dependent manner. The highest dose exhibited anti-inflammatory activity comparable to Indomethacin, highlighting its potential as a promising natural topical anti-inflammatory formulation.

Table 9. Observation of proinflammatory markers level after treatment with Methanolic Extract of M. Annua loaded CS-CMC HGs in cotton pellet induced granuloma model in rats

Drug /Extract	TNF- α (pg/mL)	IL-6 (pg/mL)	PGE2 (pg/mL)
Control (Normal Saline) (10 ml/Kg	40.87±1.98	33.41±1.48	54.56±2.45
CS-CMC 1 (100mg/kg)	36.98 ±1.85	29.24±1.32	52.12±2.25
CS-CMC 2 (200mg/kg)	32.87± 1.50	27.12±1.29	48.34±1.98
CS-CMC 3 (300mg/kg)	29.24±1.35	24.45±1.20	42.68±1.65
CS-CMC 4 (400mg/kg)	25.46±1.29	21.23±1.12	37.23±1.50
CS-CMC 5	21.65±0	16.86±1	32.76±1

(500mg/kg)	.96	.05	.15
Standard (Indomethacin 100 mg/kg)	21.98±1.15	17.21±1.10	33.15±1.25

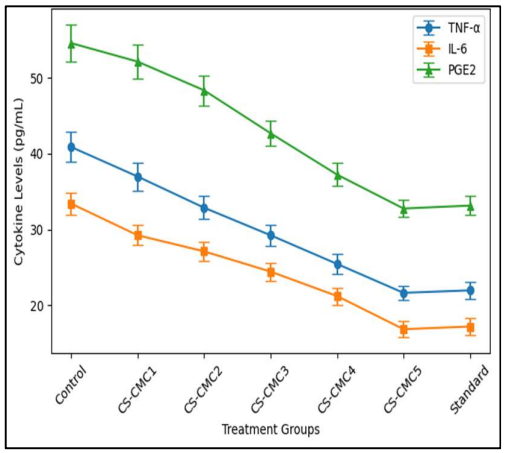


Figure 10. Proinflammatory markers level after treatment with Methanolic Extract of M. Annua loaded CS-CMC HGs in cotton pellet induced granuloma model in rats

The reduction in TNF-α, IL-6, and PGE_2 levels confirms the strong anti-inflammatory activity of the methanolic extract of *Martynia annua* delivered through the CS-CMC hydrogel. The dose-dependent suppression of these cytokines indicates enhanced therapeutic efficacy at higher doses, likely due to the presence of flavonoids and phenolic compounds. The CS-CMC hydrogel further improves bioavailability and provides sustained drug release, ensuring prolonged anti-inflammatory action. The highest dose formulation (CS-CMC 5) produced results comparable to Indomethacin, suggesting that the developed hydrogel is a promising natural alternative for topical anti-inflammatory therapy with potential for fewer side effects.

Conclusion

The present study successfully developed and evaluated a Chondroitin Sulphate–Carboxymethyl Cellulose (CS-CMC) hydrogel loaded with the methanolic extract of Martynia annua for the management of rheumatoid arthritis. Methanol proved to be the most suitable extraction solvent, yielding the highest amount of bioactive compounds, particularly flavonoids and phenolics, which are responsible for the plant's antioxidant and anti-inflammatory activities.

The prepared hydrogel exhibited desirable physicochemical properties, including suitable pH, good spreadability, homogeneity, high swelling capacity, and excellent stability for topical application. Among all formulations, F3 was identified as the optimized formulation due to its highest percentage yield, superior swelling index, and overall performance. AFM and SEM analyses confirmed a uniform nanostructured morphology, while particle size analysis and high entrapment efficiency demonstrated effective drug encapsulation. The CS-CMC hydrogel also provided sustained drug release and enhanced skin permeation compared with the plain CMC hydrogel.

Pharmacological studies confirmed significant dose-dependent anti-inflammatory activity. The formulation effectively inhibited lipoxygenase, protein denaturation, proteinase activity, membrane destabilization, and hypotonicity-induced hemolysis. In vitro and in vivo permeation studies demonstrated improved drug penetration, higher bioavailability, and controlled transdermal delivery. In the cotton pellet granuloma model, the hydrogel significantly reduced granuloma weight and markedly decreased pro-inflammatory cytokines (TNF-α, IL-6, and PGE_2), with results comparable to Indomethacin at the highest dose.

Overall, the Martynia annua-loaded CS-CMC hydrogel demonstrated excellent physicochemical characteristics, sustained drug release, enhanced permeation, and potent anti-inflammatory activity. These findings suggest that the developed hydrogel is a promising natural topical drug delivery system for the treatment of rheumatoid arthritis and warrants further preclinical and clinical evaluation.

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