Original Article

Evaluation of Anti-Inflammatory Effects of Chitosan Film Loaded Arnebia Euchroma Extract Through In Vitro and In Vivo Studies

Mahjoub, MA[1], Iman, M[1*], Salimi Sabour, E[2], Anbara, H[3]

1. Department of Pharmaceutics, Faculty of Pharmacy, Baqiyatallah University of Medical Sciences, Tehran, Iran.
2. Department of Pharmacognosy, Faculty of Pharmacy, Baqiatallah University of Medical Sciences, Tehran, Iran.
3. *Department of Histology and Embryology, Faculty of Veterinary Medicine, University of Tehran.*

How to cite this article: **Mahjoub MA, Iman M, Salimi Sabour E, Anbara H. Evaluation of Anti-Inflammatory Effects of Chitosan Film Loaded Arnebia Euchroma Extract Through In Vitro and In Vivo Studies. Archives of Razi Institute. 2024;79(6):1271-1280.** DOI: 10.32592/ARI.2024.79.6.1271

Article Info:
Received: 16 December 2023
Accepted: 16 March 2024
Published: 31 December 2024

Corresponding Author's E-Mail:
iman1359@yahoo.com

ABSTRACT

The objective of this study was to assess the anti-inflammatory effects of a chitosan (CHT) film loaded with an Arnebia euchroma extract, both in vitro and in vivo. Arnebia euchroma contains shikonin (SHKN), a naphthoquinone that exhibits notable anti-inflammatory, antimicrobial, and wound-healing properties. A high-quality SHKN extract was standardised and incorporated into CHT films, which were then evaluated in terms of their stability, drug release, antibacterial effectiveness and anti-inflammatory activity. Two concentrations of SHKN were employed the preparation of CHT films. *In vitro* studies showed that the optimal CHT film formulation remained stable for a period of four weeks at 4°C. A biphasic SHKN release profile was observed from the films, indicative of a sustained drug release mechanism. The films exhibited a strong antibacterial effect against Staphylococcus aureus (S. aureus) due to the presence of SHKN, but no such effect against Escherichia coli (E. coli). Furthermore, a synergistic antibacterial effect was noted when CHT was combined with *A. euchroma* extract against S. aureus. In vivo, the CHT film with A. euchroma extract demonstrated anti-inflammatory effects in a mice paw swelling test, comparable to betamethasone. The mice were divided into four groups of six, and the difference was not statistically significant (p-value>0.05). Histological examination substantiated the reduction of immune cell infiltration in the treatment group. The study concluded that CHT films containing A. euchroma extract exhibit promising anti-inflammatory and antimicrobial properties. Furthermore, they are suitable for use as wound dressings, offering high portability, mechanical strength, and non-adhesive properties, which makes them suitable for use in a variety of medical and pharmaceutical applications, as well as potential carriers for antimicrobial agents and antioxidants in various industries. In conclusion, the utilisation of chitosan films embedded with Arnebia euchroma extract may provide an accessible and convenient therapeutic application for a range of wounds and inflammatory conditions.

Keywords: Arnebia euchroma, Chitosan, Shikonin, Anti-Inflammatory, Anti-Bacterial.

1. Introduction

Arnebia euchroma is a plant species that occurs naturally in a variety of geographical regions, including Asia and North Africa. It has a long history of use in traditional medicine, due to its recognised therapeutic properties. The plant contains a variety of phytochemicals, including naphthoquinones, flavonoids, and terpenoids, which have been demonstrated to possess anti-inflammatory, antimicrobial, and wound-healing properties. The phytochemical composition and traditional ethnomedicinal uses of A. euchroma have been the subject of numerous research studies. (1) A number of studies have corroborated the wound-healing, anti-microbial, and anti-bacterial properties of this plant, and it has been demonstrated to be an efficacious treatment for burn wounds when compared to silver sulfadiazine ointment (1-3). The plant has been employed in traditional medicine and the pharmaceutical industry for the treatment of hair problems, chronic diseases, burnt limbs, coughs and colds, and as a vegetable colourant and for dyeing cloths (1). A number of studies have been conducted to investigate the efficacy of this plant in the treatment of second-degree burns. The root of A. euchroma contains naphthoquinone, shikonin (SHKN), alkanin, and isohexenylnaphthazarin derivatives, which have been demonstrated to possess a range of medicinal properties, including anti-inflammatory, antimicrobial, and anti-cancer effects. To date, a number of chemical constituents that can be classified as naphthoquinones have been isolated from A. euchroma. SHKN is the principal active component among these naphthoquinones (4). SHKN is a natural naphthoquinone pigment found in the roots of A. euchroma, a plant species belonging to the Boraginaceae family. Additionally, this substance and its derivatives have been isolated from various species of traditional medicinal plants, including Lithospermum, Alkanna, and Anchusa (5). SHKN has been demonstrated to possess anti-inflammatory properties, which are conferred by its ability to inhibit the production of pro-inflammatory cytokines and chemokines. Furthermore, it has been demonstrated to possess antimicrobial properties against a diverse array of bacterial and fungal species. Furthermore, SHKN has been demonstrated to possess anti-tumour properties, inducing apoptosis and inhibiting cell proliferation in a range of cancer cell lines (6, 7). Chitosan (CHT) is a natural polymer that exhibits distinctive characteristics, including biocompatibility, biodegradability, environmental stability, non-toxicity, high biological activity, cost-effectiveness, the ability to chelate metal ions, and high adsorption properties (8). It has been demonstrated to possess antimicrobial properties against a range of bacteria and fungi, thereby establishing it as a promising candidate for the development of wound healing dressings. Furthermore, it possesses hemostatic properties that facilitate the rapid cessation of bleeding. CHT can be employed to prevent or treat wound and burn infections, not only due to its intrinsic antimicrobial properties, but also by virtue of its capacity to facilitate healing in the context of soft tissue diseases (9). The objective of this study is to examine the impact of Arnebia euchroma extract incorporated into a chitosan film, which exhibits anti-inflammatory and antimicrobial properties, as a potential novel light, portable, and immediate drug delivery system.

2. Materials and Methods

The CHT (degree of deacetylation: 85%) was procured from Sigma-Aldrich (USA). The SHKN was procured from Sigma-Aldrich (USA). The analytical grade acetic acid and glycerol were procured from Merck (Germany).

2.1. Preparation of Herbal Extract

In this study, the percolation method, a conventional extraction method in plant studies, was employed to obtain the root extract of the A. euchroma plant. The roots of A. euchroma were collected from mountainous regions in Iran and subsequently authenticated by a botanist. Following the completion of the botanical processes, a herbarium sample was prepared and the samples were subsequently dried. Once the biomass weight of the plant had been stabilised, a powdered root extract was prepared using the percolation method with an ethanol:water (8:2) solution. Following the evaporation of the solvent, the extract was then dried. Subsequently, the concentration of SHKN, the active ingredient, was standardised utilising a standard SHKN and a calibration curve. A calibration curve was constructed by plotting the absorbance of known concentrations of SHKN, and the resulting equation was found to be:

SHKN conc. (mg/ml) = 0.0313×Absorbance - 0.00009; (R^2 = 0.9999).

2.2. Preparation of CHT Film Containing *A. euchroma* Root Extract

The CHT film was prepared based on the moulding process (10). Initially, the CHT polymer with a high molecular weight was added to a solution of 1% (v/v) acetic acid and stirred for a period of 2 hours using a magnetic stirrer in order to facilitate the dissolution of the polymer. Subsequently, the pH of the solution was adjusted to 5.5. At this juncture, the plant extract was also incorporated into the solution and thoroughly stirred. Based on the findings of the conducted studies, a specific quantity of the extract was incorporated into the polymer solution, with the presence of 25 µg and 50 µg of the active substance SHKN. In other studies, various amounts of approximately 10-100 µg (11) or 0.5-10 mg/kg dose in mice or rats (12) have been employed. Subsequently, glycerin was incorporated as a plasticizer, with a concentration of 0.5% (w/w). The incorporation of a plasticizer into the polymer solution can enhance the flexibility and mechanical properties of the resulting film. The resulting solution was then cast onto a glass plate or a Petri dish and left to dry in an oven at 40°C overnight, thus forming a solid film.

2.3. SHKN Release

In order to investigate the release of drugs from a CHT film containing an extract of A. euchroma, the SHKN substance was employed as an indicator. To this end, a defined quantity of the film was introduced into a 5ml solution of

PBS medium containing 1% Tween 80, with a pH of 5.5, a temperature of 37°C and a rotation of 50 rpm within the Franz cell apparatus. At specified intervals (0, 2, 4, 6, 12, and 24 hours), the release medium was sampled and replaced according to the volume of the sample. The amount of the drug released was then quantified using a UV-vis device. The calibration curve was established at a wavelength of 516 nm. It is important to note that due to the low solubility of SHKN in water, 1% Tween was added to the PBS solution.

2.4. Stability

It is imperative that stability studies are conducted in order to guarantee the quality, efficacy and security of drug substances and products. A stability study was conducted on the prepared film, which was stored at 4°C. After one month, the film was examined at scheduled time points (0, 7, 14, 21 and 28 days) for the amount of the effective substance (SHKN) using a UV-vis spectrophotometer (13).

2.5. Antibacterial Effect

In order to ascertain the antibacterial effect, the disc essay method was employed. In this method, agar is inoculated with the microorganism that is undergoing testing. Subsequently, paper discs (with a diameter of approximately 6 mm) containing the test compound at the desired concentration are placed on the agar surface. The Petri dishes are then incubated under suitable conditions. In general, the antimicrobial agent diffuses into the agar and inhibits the growth of the microorganism. The diameter of the inhibited growth zones is then measured. In the present study, the sensitivity of two bacterial strains, namely Staphylococcus aureus (PTCC No. 1826), a Gram-positive bacterium, and Escherichia coli (PTCC No. 1789), a Gram-negative bacterium, to antimicrobial agents was determined using the disk diffusion method. In order to achieve this, a suspension of each of the aforementioned bacterial samples was prepared using McFarland's 0.5 standard and subsequently placed on Mueller Hinton agar. Following a 24-hour incubation period at 35°C, the inhibition zones were measured. The film samples, which contained A. euchroma root extract at a concentration of 5µg/cm² and SHKN at a concentration of 2.5µg/cm², were placed on the culture of bacteria. Each set included ampicillin and vancomycin discs as positive controls, as well as CHT film samples that lacked plant extract, which served as negative controls.

2.6. Paw Swelling Test

A volume of 25 microlitres of a 1% carrageenan solution is administered via a 31-gauge insulin syringe into the subplantar surface of the mouse paw. In the standard method, the volume of the injected paw is measured at the time of injection (T0) and 4 hours later (T4) in order to ascertain the extent of the swelling. One appropriate method for measuring swelling is to use a caliper to determine the thickness of the paw. Additionally, the thickness of the non-injected paw is measured after 4 hours and serves as the control. The change in claw thickness is recorded as AT=T4 – T0. The injection volume results in an immediate increase in paw thickness of approximately 0.8 mm, which largely dissipates within the first hour. In this experiment, mice were used and the mice were divided into 4 groups of 6.

- Group 1: mice treated with CHT film containing Arnebia extract equivalent to 2.5 µg/cm^2 SHKN.
- Group 2: mice treated with CHT film containing Arnebia extract equivalent to 5 µg/cm^2 SHKN.
- Group 3: (negative control): mice treated with CHT film without plant extract.
- Group 4: (positive control): mice treated with betamethasone 0.1% ointment.

2.7. Histology

Following a four-hour period during which carrageenan-induced oedema was permitted to develop, animals from each group were euthanised. Paw samples were obtained for histological examination. The sectioned tissues were subjected to staining with hematoxylin and eosin and subsequently observed under a light microscope (Zeiss, Jena, Germany).

2.8. Statistical Analysis

All data were expressed as mean ± standard deviation. The independent samples t-test was employed to ascertain whether there were any significant differences between the averages of the two distinct groups. Conversely, the ANOVA was utilised when there were more than two groups. In all stages of this research, a significance level of $P<0.05$ was selected. It should be noted that the statistical processing of the results was conducted using the SPSS 18 software.

3. Results

3.1. Herbal Extract

A total of 198 grams of powdered root from the Arnebia plant was subjected to a final extraction process using 4 litres of 80-degree ethanol. This yielded 7.8 grams of extract, representing a yield of approximately 4% (3.98%). The yield range of the extract obtained from the Arnebia plant has also been demonstrated in other research studies.

3.2. Preparation of CHT Film Containing *A. euchroma* Root Extract

Film formulations containing Arnebia extract with two different concentrations of SHKN were prepared and their morphological characteristics, as well as film thickness, were investigated. The results are presented in Figures 1A and B. The resulting films are transparent, although the dark colour of the Arnebia extract means that they are not completely colourless, with a small amount of dark colour visible (Figure 1B). The thickness of the prepared films was found to be approximately 0.2 to 0.3 mm (Figure 1).

3.3. Stability

The stability of SHKN in the formulation of CHT film was evaluated at a temperature of 4°C for a period of four weeks, and the results are presented in Table 1. The results demonstrated that the optimal formulation was stable for a period of four weeks, with no significant difference observed in the aforementioned characteristics at the conclusion of this period ($P>0.05$).

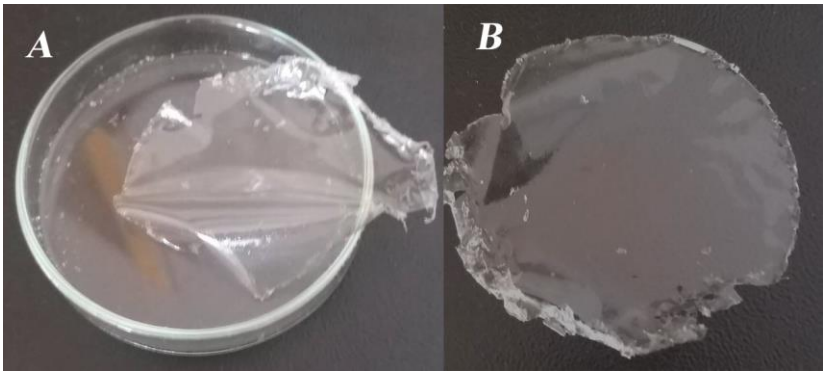

Figure 1: A) chitosan film without plant extract B) chitosan film loaded with plant extract.

Table 1. The stability of the effective ingredient of SHKN during 28 days of storage at 4 °C.

Time (days)	% Remained	
	2.5 µg/ml	5 µg/ml
0	100 ± 0.01	100 ± 0.01
7	99.12 ± 0.01	98.32 ± 0.62
14	98.23 ± 0.02	98.4 ± 0.61
21	97.15 ± 0.02	97.02 ± 0.11
28	97.15 ± 0.02	97.15 ± 0.02

3.4. *In Vitro* Release

To ascertain the release of the drug, the standard curve of SHKN in the release medium was initially constructed and its accuracy and precision were subsequently evaluated. The release profile exhibits two distinct phases, as illustrated in Figure 2. The biphasic release profile comprises a rapid release phase, followed by a sustained release phase. The rapid initial release can be attributed to the hydrophilic swelling of CHT, which facilitates the release of the drug from the CHT film into the release medium. Additionally, drug molecules that are situated on the surface of the film are readily released in this manner. However, at 24 h to 192 h, release may occur due to drug release through CHT corrosion (15). Figure 2 illustrates the drug release diagram.

3.5. Antibacterial effect

Figure 3 illustrates that the root extract of the Arnebia plant demonstrated an antibacterial effect against S. aureus, a gram-positive bacterium, whereas no such effect was observed against the gram-negative bacterium E. coli. Furthermore, the combination of CHT polymer, which has an intrinsic antibacterial effect, with the root extract of the Arnebia plant demonstrates a synergistic effect on S. aureus, as evidenced by a statistically significant p-value ($p<0.05$). Conversely, this synergistic effect is not observed in the case of E. coli.

3.6. Paw Swelling Test

The paw swelling test (Figure 4) demonstrates that both groups 1 and 2, which contained the plant extract, exhibited a comparable statistical effect to the anti-inflammatory drug betamethasone (Figure 5). The second group, which received a higher dose of SHKN, a compound found in the plant, exhibited a superior mean effect compared to the first group and the control group. However, this difference was not statistically significant (p-value>0.05). These findings suggest that the minimum dose employed is efficacious for anti-inflammatory purposes. Conversely, CHT films also demonstrate a pronounced anti-inflammatory effect.

3.7. Histology

The histological analysis of tissue sections revealed a reduction in the infiltration of immune cells in the group treated with CHT films. Microscopic photographs of the control stained with hematoxylin and eosin revealed a considerable degree of infiltration damage resulting from the accumulation of immune cells and the collection of fluid, as indicated by the arrow in Figure 6A. However, the CHT film containing 5µg/cm² SHKN (Figure 6B) exhibited a reduced content of immune cells in comparison to the normal saline group. Conversely, the CHT film containing 5µg/cm² SHKN and betamethasone (Figure 6D & Figure 6C) demonstrated a minimal infiltration of immune cells, suggesting that the 5 µg/cm² treated group exhibited anti-inflammatory effects comparable to those observed in the positive control betamethasone.

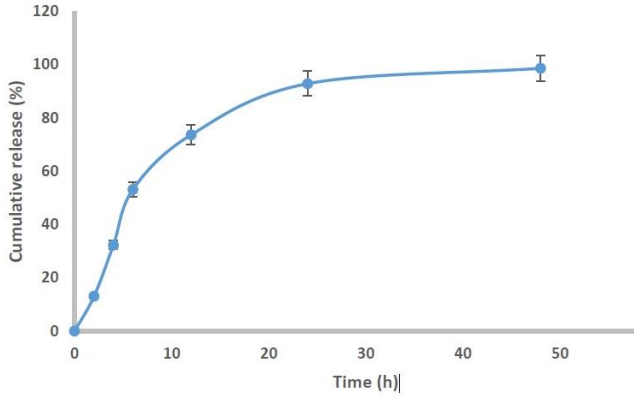

Figure 2. In vitro release profile from chitosan film.

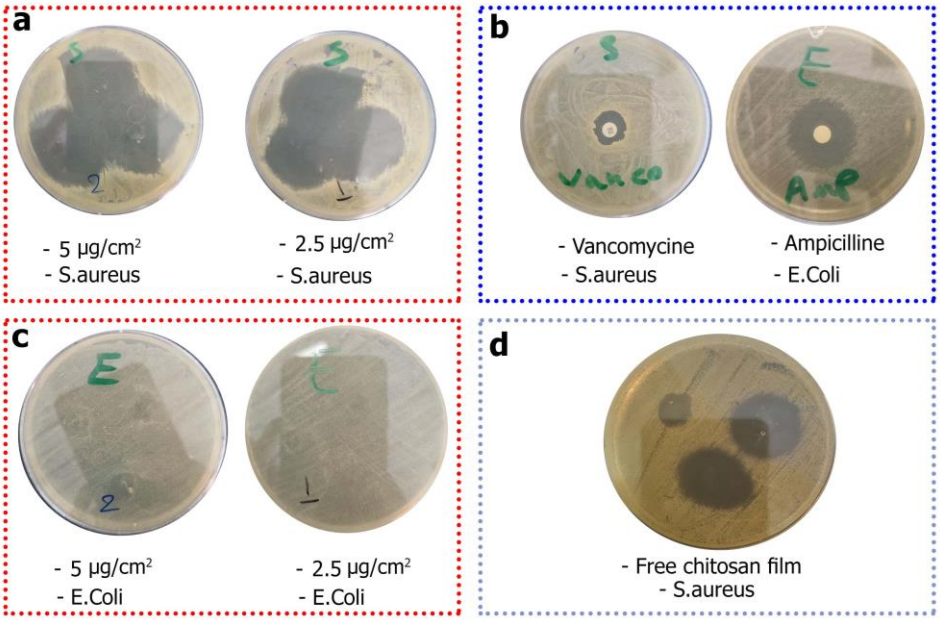

Figure 3. The results of testing the antibacterial effect on two bacteria S.aureus and E.coli. Figure a) Inhibitory effect of chitosan film containing *A. euchroma* extract with a concentration of 5 and 2.5 µg on S.aureus. b) The effect of vecnomycin and ampicillin antibiotics on S.aureus and E.coli bacteria, respectively. c) Inhibitory effect of chitosan film containing *A. euchroma* extract with a concentration of 5 and 2.5 µg on E.coli d). The effect of chitosan film without plant extract on S.aureus bacteria.

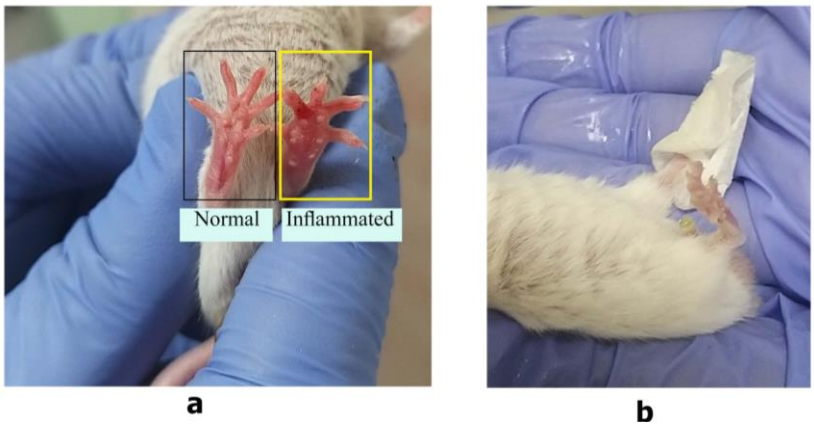

Figure 4. Paw swelling test. a) Comparison of inflammated foot and healthy foot and b) plaster of inflamed foot with CHT film containing *A. euchroma* extract.

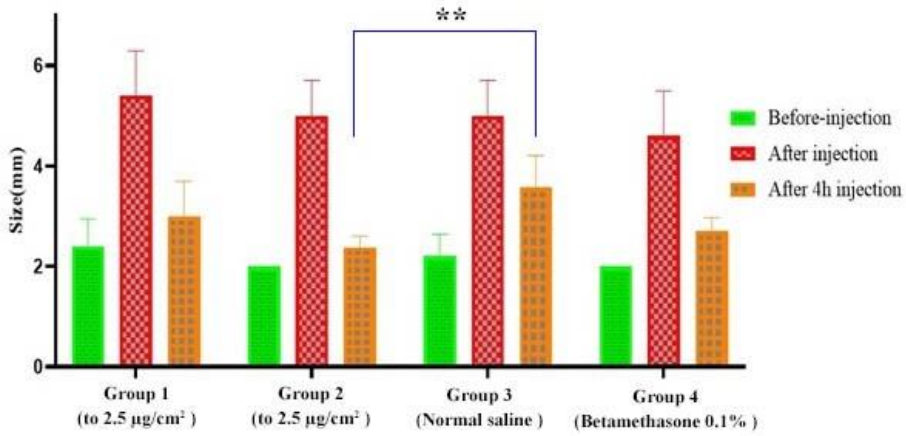

Figure 5. Comparison of 4 different groups in paw swelling test. Group 1: mice treated with CHT film containing *A.euchroma* extract equivalent to 2.5 µg/cm^2 SHKN. Group 2: mice treated with CHT film containing A.euchroma extract equivalent to 5 µg/cm^2 SHKN. Group 3: mice treated with normal saline as control and Group 4: mice treated with betamethasone 0.1% ointment

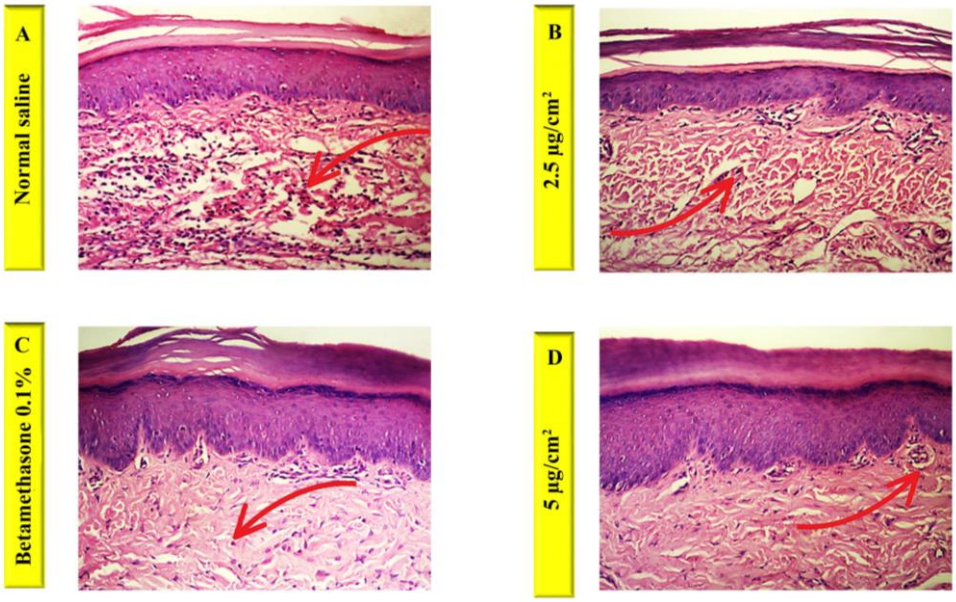

Figure 6. Histological examination of paw tissue sections 4 h after carrageenan injection. Red arrows indicate infiltrated neutrophils.

4. Discussion

Extensive research has been conducted into the medical and pharmaceutical applications of CHT and its derivatives. Furthermore, given the plethora of advantages associated with CHT and its derivatives, there is a growing interest in their potential applications across a range of industries. For example, CHT films have been employed in the context of wound dressings, drug delivery systems, tissue engineering systems, and bandages. Furthermore, CHT films can be utilised as drug carriers, for instance, to deliver antimicrobial and antioxidant agents, in the food industry (21-23). In the field of medicine and pharmacy, CHT and its derivatives have been extensively researched due to their competitive biological properties, including biocompatibility, biodegradability, and non-toxicity. Additionally, they possess a range of medicinal properties, such as anti-inflammatory, anti-tumour, hemostatic, antimicrobial, and antioxidant effects (24, 25). The extraction yield, defined as the quantity of the desired extract obtained from the plant material, is a critical factor in the development of herbal products derived from the herbal extract. The yield range of the extract has also been demonstrated in other research studies. For example, Sing et al. (14) reported an extraction efficiency of 1.98% for this plant. In the study conducted by Shokrzadeh et al. (2017), the yield percentage of Arnebia root extract was reported as 6.34%. These properties are of significant utility in the fields of tissue engineering, wound healing, and drug

delivery (27-29). A variety of CHT-based products are available, including powders, films, nanoparticles, suspensions, and hydrogels (30). The initial response to infection or injury in the body is inflammation, which is carried out by a specific set of immune and inflammatory cells in a tissue compartment with the aim of restoring its structural and functional integrity after exposure to an undesirable stimulus (30). A number of studies have been conducted on the anti-inflammatory and pre-inflammatory properties of CHT and its derivatives in the context of medical and pharmaceutical applications. Davydova and colleagues evaluated the anti-inflammatory efficacy of CHT with high (MW: 115 kDa) and low (MW: 5.2 kDa) molecular weights. Their findings revealed that both CHT samples induced a robust anti-inflammatory cytokine, interleukin-10, in the blood of animals and effectively mitigated the progression of colitis. The principal contribution to the anti-inflammatory activity of CHT is attributed to its structural elements and is not dependent on its molecular weight. Friedman and colleagues have reported the inhibitory capacity of CHT-alginate nanoparticles against inflammatory cytokines and chemokines induced by Propionibacterium acnes (P. acnes). The results demonstrated that CHT-alginate nanoparticles effectively suppress the production of cytokines induced by P. acnes in human monocytes and keratinocytes. In addition to its anti-inflammatory capacity, CHT also exhibits a high potential for controlled and localised drug delivery. Oliviera et al. investigated the anti-inflammatory and pre-inflammatory cytokine activities of CHT films. The findings revealed a reduction in TNF-α (tumour necrosis factor) (pre-inflammatory cytokines) within three to ten days of cultured cells on CHT films, accompanied by a notable elevation in anti-inflammatory cytokines IL-10 and TGF-β1 (transforming growth factor). The anti-inflammatory activities of CHT on cyclooxygenase (COX) have been demonstrated by numerous scientists, although the precise mechanism remains unclear. Chang et al. investigated the anti-inflammatory capacity of two types of CHT with high (70 kDa) and low (MW <1 kDa) molecular weight on COS. In the case of low molecular weight, a notable inhibitory impact on IL-4, IL-13, and TNF-α cytokines was observed, suggesting the possibility of reducing allergic inflammation within the body. Li et al. put forth a mechanism by which lipopolysaccharide-induced NF-κB-dependent inflammatory gene expression in COS is associated with a reduction in NF-κB nuclear translocation (36). The precise mechanism of the antimicrobial activity of CHT remains unclear, despite the fact that several studies have been conducted in this area. The antimicrobial effect of CHT is significantly greater than that of chitin, due to the amino groups that are responsible for the cationic property of CHT. The positive charge of CHT may facilitate interactions with the negative residues of carbohydrates, lipids, and proteins located on the surface of bacterial cells, which subsequently inhibit bacterial growth (37, 38). It can be concluded, therefore, that the electric charge of CHT plays a pivotal role in the mechanism of microbial inhibition. The high density of positive charge on the CHT structure or its derivatives gives rise to a robust electrostatic interaction, which is contingent upon the extent of CHT deacetylation. This theory posits that CHT is a more promising inhibitor of gram-negative bacteria than gram-positive bacteria, given that the surfaces of gram-negative cells interact more with CHT with a positive charge (24, 37, 39, 40). Nevertheless, numerous studies have demonstrated that CHT is a more potent inhibitor of gram-positive bacteria than gram-negative bacteria (40, 41). An alternative hypothesis has been put forth regarding the mechanism of CHT inhibition, wherein the molecular weight (MW) is identified as the primary determinant of antimicrobial activity (42). This theory posits that CHT inhibits RNA and protein synthesis by penetrating the cell nucleus and ultimately disrupting and leaking intracellular components. It has been demonstrated that CHT with a low MW is readily able to penetrate the bacterial cell wall, combining with DNA and thereby inhibiting mRNA synthesis and DNA transcription. As the molecular weight (MW) increases, the capacity to penetrate the cell nucleus is diminished. In particular, in the case of CHT with high MW, binding to negatively charged components on the bacterial cell wall results in the formation of an impermeable layer around the cell, which alters cell permeability and blocks the transfer into the cell (28, 43). In a study conducted by Liu et al., the impact of MW on the inhibitory capacity against E. coli was examined when the deacetylation remained consistent (~80%) (44). The authors tested MWs ranging from 55 to 155 KD, and the results demonstrated that lower MWs exhibited higher inhibitory activity against E. coli. In addition to the molecular weight and degree of deacetylation, other factors, including solubility, pH, and environmental temperature, also influence the antimicrobial activity of CHT. At lower pH values, the positive ion charge increases, resulting in enhanced absorption of CHT by bacterial cells (37, 42). Despite the numerous studies that have been conducted thus far, a definitive conclusion regarding the precise relationship between the antimicrobial effect of CHT, its MW, and deacetylation remains elusive. This is due to the fact that a multitude of additional factors influence the inhibitory effect, including the specific bacterial strains and the particular biological test conditions employed (45). The objective of this study was to examine the effects of A. euchroma peel extract with two different concentrations of the active ingredient SHKN on CHT film. The morphology and thickness of the layers were examined, and the stability of SHKN in the CHT film was evaluated at 4 degrees Celsius for a period of four weeks. The results demonstrated that the optimal formulation remained stable for a period of four weeks, and that there was no significant difference in the concentration of the active ingredient at the conclusion of this period. The release profile of the A. euchroma extract from the CHT film exhibited a two-phase

structure. A rapid release phase was followed by a stable release phase. The A. euchroma extract demonstrated antibacterial activity against S. aureus, but no such effect was observed against E. coli. Furthermore, the combination of CHT and A. euchroma extract exhibited a synergistic effect against S. aureus. Furthermore, the films demonstrated anti-inflammatory efficacy in the paw edema test, and the optimal dose of chiconin exhibited anti-inflammatory activity. In the study conducted by Yan et al., it was observed that CHT may facilitate wound healing by stimulating the growth of human natural keratinocytes and dermal fibroblasts. Furthermore, CHT has been demonstrated to reduce nuclear translocation of NF-kB p65 in human dermal fibroblasts. Epithelial-mesenchymal transition (EMT) represents a crucial process in the context of wound healing. CHT has been shown to effectively enhance wound healing in damaged skin tissues, and local treatment with CHT has been observed to increase the expression of EMT-regulating molecules, including E-cadherin repressors (46). The results presented in Figure 3 demonstrate that the A. euchroma extract is effective against gram-positive bacteria but not against gram-negative bacteria. Other studies have demonstrated that SHKN and its derivatives are effective against Gram-positive bacteria, including S. aureus, Enterococcus faecium, and Bacillus subtilis, as well as various species of lactic acid bacteria at MICs between 0.3 and 6.25 μg/ml. In contrast, the extract is inactive against Gram-negative bacteria, including Escherichia coli, Pseudomonas aeruginosa, and Micrococcus luteus (17). However, a study published in 2011 by Ding et al. (18) demonstrated that at a dose of 200 μM SHKN inhibits biofilm formation by P. aeruginosa and Stenotrophomonas maltophilia. Initially, the antibacterial activity of SHKN was hypothesised to be due to a bacteriostatic effect. However, subsequent studies demonstrated that this compound has bactericidal properties. Additionally, the same study posited that SHKN may serve as a valuable therapeutic agent against methicillin-resistant S. aureus (19). In 2002, Shen et al. (20) conducted an analysis of the activity of SHKN and some derivatives against methicillin-resistant S. aureus and vancomycin-resistant E. faecium and E. faecalis. In this experiment, the minimum inhibitory concentration (MIC) of SHKN was observed to be 6.25 μg/ml against S. aureus (both methicillin-resistant and non-resistant) and 50 and 25 μg/ml against E. faecicum and E. faecalis, respectively. Nevertheless, the impact of CHT on Gram-positive or Gram-negative bacteria remains a topic of contention in the scientific community. Some authors have asserted that CHT exerts a more pronounced effect on Gram-positive bacteria (such as Listeria monocytogenes, Bacillus megaterium, B. cereus, S. aureus, Lactobacillus plantarum, L. brevis, L. bulgaris, etc.) than on Gram-negative bacteria. For instance, E. coli, Pseudomonas fluorescens, Salmonella typhymurium, Vibrio parahaemolyticus, etc. (20, 21). The antibacterial activity of SHKN and its associated mechanisms have been the subject of investigation in a number of studies, including that conducted by Ozaki et al. (47). In combination with membrane-permeable agents and ATPase inhibitors, CHT has been demonstrated to significantly inhibit the growth of methicillin-resistant S. aureus (MRSA23), causing disruption of the cytoplasmic membrane in MRSA and leading to cellular breakdown and destruction. The antiviral activity of CHT against the H1N1 influenza virus has been investigated by Zhang et al. Their findings indicate that CHT inhibits neuraminidase and reduces viral nucleocapsids in infected cells, as well as inhibiting apoptosis of infected cells. Furthermore, the antiviral activity of the CHT ester derivative against human enterovirus 71 (EV71) has been demonstrated by Zhang et al. (48). This resulted in a notable reduction in the mRNA and protein levels of EV71/VP1, as well as a decrease in the expression of inflammatory cytokines, including IL-1β, IL-6, IL-8, and TNF-α, in rhabdomyosarcoma cells. The histological assessment of the carrageenan-induced paw oedema model in mice yielded significant findings. It is noteworthy that the control samples exhibited a high density of immune cells, indicative of an active inflammatory response. However, the number of immune cells was notably reduced in tissues treated with the CHT film containing Arnebia euchroma extract. This reduction indicates a diminished inflammatory response. This observation is consistent with other evidence presented in the article, which collectively supports the overarching inference that CHT films loaded with Arnebia euchroma extract have significant anti-inflammatory potential. The collective data serve to reinforce the therapeutic promise of this formulation, which may be considered a viable candidate for the treatment of inflammatory conditions. The findings of this study indicate that a CHT film containing an A. euchroma extract has demonstrated efficacy in addressing inflammatory and antimicrobial concerns. Furthermore, due to its minimal weight and lack of adhesive properties, it is highly portable and may be considered an appropriate option for use as a companion dressing. CHT films possess mechanical properties, transparency, and suitable protective characteristics that make them suitable for food packaging, wound dressings, drug delivery systems, and tissue engineering structures. Additionally, CHT films can be utilized as carriers of effective substances such as antimicrobial agents and antioxidants.

Acknowledgment

The authors would like to express their gratitude to all those who provided invaluable assistance in the successful completion of this research project, including professors, teachers, and technicians.

Authors' Contribution

Study concept and design: M. I, E. S.-S., M.A.M
Acquisition of data: M.A.M,
Analysis and interpretation of data: M.A.M, H.A.

Drafting of the manuscript: M.A.M
Critical revision of the manuscript for important intellectual content: M. I, E. S.-S.
Statistical analysis: M.A.M
Administrative, technical, and material support: M. I, E. S.-S.
Histological study: H.A.

Ethics
The animal studies were approved by the ethics committee of Baqiyatallah University of Medical Sciences in Tehran, Iran.

Conflict of Interest
The authors certify that they have no conflicts of interest.

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

References

1. Kumar A, Shashni S, Kumar P, Pant D, Singh A, Verma RKJJoE. Phytochemical constituents, distributions and traditional usages of Arnebia euchroma: A review. 2021;271:113896.
2. Ashkani-Esfahani S, Imanieh M, Khoshneviszadeh M, Meshksar A, Noorafshan A, Geramizadeh B, et al. The healing effect of arnebia euchroma in second degree burn wounds in rat as an animal model. 2012;14(2):70.
3. Nasiri E, Hosseinimehr SJ, Hosseinzadeh AZ, Azadbakht M, Akbari J, Azadbakht MJJoe. The effects of Arnebia euchroma ointment on second-degree burn wounds: a randomized clinical trial. 2016;189:107-16.
4. Liu T, Ma C, Yang L, Wang W, Sui X, Zhao C, et al. Optimization of shikonin homogenate extraction from Arnebia euchroma using response surface methodology. 2013;18(1):466-81.
5. Huang X-Y, Fu H-L, Tang H-Q, Yin Z-Q, Zhang W, Shu G, et al. Optimization extraction of shikonin using ultrasound-assisted response surface methodology and antibacterial studies. 2020;2020:1-10.
6. Yadav S, Sharma A, Nayik GA, Cooper R, Bhardwaj G, Sohal HS, et al. Review of shikonin and derivatives: isolation, chemistry, biosynthesis, pharmacology and toxicology. 2022;13:905755.
7. Wang B, Wu Z, Wang J, Li W, Liu G, Zhang B, et al. Insights into the mechanism of Arnebia euchroma on leukemia via network pharmacology approach. 2020;20(1):1-15.
8. Jiménez-Gómez CP, Cecilia JAJM. Chitosan: a natural biopolymer with a wide and varied range of applications. 2020;25(17):3981.
9. Gupta RK, Guha P, Srivastav PPJFCA. Natural polymers in bio-degradable/edible film: A review on environmental concerns, cold plasma technology and nanotechnology application on food packaging-A recent trends. 2022;1:100135.
10. Kienzle-Sterzer CA, Rodriguez-Sanchez D, Rha CJDMCMC, Physics. Mechanical properties of chitosan films: effect of solvent acid. 1982;183(5):1353-9.
11. Tanaka S, Tajima M, Tsukada M, Tabata MJJoNP. A comparative study on anti-inflammatory activities of the enantiomers, shikonin and alkannin. 1986;49(3):466-9.
12. Liao P-L, Lin C-H, Li C-H, Tsai C-H, Ho J-D, Chiou GC, et al. Anti-inflammatory properties of shikonin contribute to improved early-stage diabetic retinopathy. 2017;7(1):44985.
13. Selvamani V. Stability studies on nanomaterials used in drugs. In Staphylococcus aureus Characterization and biology of nanomaterials for drug delivery 2019 Jan 1 (pp. 425-444). Elsevier.
14. Singh B, Sharma M, Meghwal P, Sahu P, Singh SJP. Anti-inflammatory activity of shikonin derivatives from Arnebia hispidissima. 2003;10(5):375-80.
15. Shokrzadeh M, Azadbakht M, Shakibamanesh HJP, Research B. The hepatoprotective effect of Arnebia euchroma hydro-alcoholic extract against liver toxicity induced by CCl4 in mice. 2017;3(4):23-9.
16. Feng H, Zhang L, Zhu CJC, Biointerfaces SB. Genipin crosslinked ethyl cellulose–chitosan complex microspheres for anti-tuberculosis delivery. 2013;103:530-7.
17. Andújar I, Ríos JL, Giner RM, Recio MCJPm. Pharmacological properties of shikonin–a review of literature since 2002. 2013:1685-97.
18. Ding X, Yin B, Qian L, Zeng Z, Yang Z, Li H, et al. Screening for novel quorum-sensing inhibitors to interfere with the formation of Pseudomonas aeruginosa biofilm. 2011;60(12):1827-34.
19. Papageorgiou VP, Assimopoulou AN, Couladouros EA, Hepworth D, Nicolaou KJACIE. The chemistry and biology of alkannin, shikonin, and related naphthazarin natural products. 1999;38(3):270-301.
20. Shen C-C, Syu W-J, Li S-Y, Lin C-H, Lee G-H, Sun C-MJJoNP. Antimicrobial activities of naphthazarins from arnebia e uchroma. 2002;65(12):1857-62.
21. Coma V, Deschamps A, Martial-Gros AJJofs. Bioactive packaging materials from edible chitosan polymer—antimicrobial activity assessment on dairy-related contaminants. 2003;68(9):2788-92.
22. De Masi A, Tonazzini I, Masciullo C, Mezzena R, Chiellini F, Puppi D, et al. Chitosan films for regenerative medicine: Fabrication methods and mechanical characterization of nanostructured chitosan films. 2019;11:807-15.
23. Melro E, Antunes FE, da Silva GJ, Cruz I, Ramos PE, Carvalho F, et al. Chitosan films in food applications. Tuning film properties by changing acidic dissolution conditions. 2020;13(1):1.
24. Kim S, Nakamatsu J, Maurtua D, Oliveira FJJoAPS. Formation, antimicrobial activity, and controlled release from cotton fibers with deposited functional polymers. 2016;133(8).
25. M. Ways TM, Lau WM, Khutoryanskiy VVJP. Chitosan and its derivatives for application in mucoadhesive drug delivery systems. 2018;10(3):267.

26. Yuan Y, Chesnutt BM, Haggard WO, Bumgardner JDJM. Deacetylation of chitosan: Material characterization and in vitro evaluation via albumin adsorption and pre-osteoblastic cell cultures. 2011;4(8):1399-416.
27. Cheung RCF, Ng TB, Wong JH, Chan WYJMd. Chitosan: an update on potential biomedical and pharmaceutical applications. 2015;13(8):5156-86.
28. Zhang M, Li X, Gong Y, Zhao N, Zhang XJB. Properties and biocompatibility of chitosan films modified by blending with PEG. 2002;23(13):2641-8.
29. Ahmed S, Ikram SJAitls. Chitosan based scaffolds and their applications in wound healing. 2016;10(1):27-37.
30. Kumirska J, Weinhold MX, Thöming J, Stepnowski PJP. Biomedical activity of chitin/chitosan based materials—influence of physicochemical properties apart from molecular weight and degree of N-acetylation. 2011;3(4):1875-901.
31. Gallo J, Raska M, Kriegova E, Goodman SBJJoot. Inflammation and its resolution and the musculoskeletal system. 2017;10:52-67.
32. Davydova V, Kalitnik A, Markov P, Volod'Ko A, Popov S, Ermak IJAB, et al. Cytokine-inducing and anti-inflammatory activity of chitosan and its low-molecular derivative. 2016;52:476-82.
33. Friedman AJ, Phan J, Schairer DO, Champer J, Qin M, Pirouz A, et al. Antimicrobial and anti-inflammatory activity of chitosan–alginate nanoparticles: a targeted therapy for cutaneous pathogens. 2013;133(5):1231-9.
34. Oliveira MI, Santos SG, Oliveira MJ, Torres AL, Barbosa MAJECM. Chitosan drives anti-inflammatory macrophage polarisation and pro-inflammatory dendritic cell stimulation. 2012;24(136):133-6.
35. Chung MJ, Park JK, Park YIJIi. Anti-inflammatory effects of low-molecular weight chitosan oligosaccharides in IgE–antigen complex-stimulated RBL-2H3 cells and asthma model mice. 2012;12(2):453-9.
36. Li Y, Liu H, Xu Q-S, Du Y-G, Xu JJCP. Chitosan oligosaccharides block LPS-induced O-GlcNAcylation of NF-κB and endothelial inflammatory response. 2014;99:568-78.
37. Younes I, Rinaudo MJMd. Chitin and chitosan preparation from marine sources. Structure, properties and applications. 2015;13(3):1133-74.
38. Aranaz I, Mengíbar M, Harris R, Paños I, Miralles B, Acosta N, et al. Functional characterization of chitin and chitosan. 2009;3(2):203-30.
39. Bessa LJ, Fazii P, Di Giulio M, Cellini LJIwj. Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection. 2015;12(1):47-52.
40. Kim S, Fernandes MM, Matamá T, Loureiro A, Gomes AC, Cavaco-Paulo AJC, et al. Chitosan–lignosulfonates sono-chemically prepared nanoparticles: characterisation and potential applications. 2013;103:1-8.
41. Silva C, Matamá T, Kim S, Padrão J, Prasetyo EN, Kudanga T, et al. Antimicrobial and antioxidant linen via laccase-assisted grafting. 2011;71(7):713-20.
42. Park BK, Kim M-MJIjoms. Applications of chitin and its derivatives in biological medicine. 2010;11(12):5152-64.
43. Zheng L-Y, Zhu J-FJCp. Study on antimicrobial activity of chitosan with different molecular weights. 2003;54(4):527-30.
44. Liu N, Chen X-G, Park H-J, Liu C-G, Liu C-S, Meng X-H, et al. Effect of MW and concentration of chitosan on antibacterial activity of Escherichia coli. 2006;64(1):60-5.
45. Kumar-Krishnan S, Prokhorov E, Hernández-Iturriaga M, Mota-Morales JD, Vázquez-Lepe M, Kovalenko Y, et al. Chitosan/silver nanocomposites: Synergistic antibacterial action of silver nanoparticles and silver ions. 2015;67:242-51.
46. Yin S-Y, Peng A-P, Huang L-T, Wang Y-T, Lan C-W, Yang N-SJE-BC, et al. The phytochemical shikonin stimulates epithelial-mesenchymal transition (EMT) in skin wound healing. 2013;2013.
47. Ozaki Y, Ohno A, Saito Y, Satake MJB, Bulletin P. Accelerative effect of shikonin, alkannin and acetylshikonin on the proliferation of granulation tissue in rats. 1994;17(8):1075-7.
48. Zhang Y, Han H, Sun L, Qiu H, Lin H, Yu L, et al. Antiviral activity of shikonin ester derivative PMM-034 against enterovirus 71 in vitro. 2017;50.