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Effects of *Ceratonia siliqua L.* Extract-Loaded Nanoliposomes on a Rat Model of Excisional Wound Healing

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ABSTRACT

Wound healing consists of inflammatory, proliferation, and remodeling phases. The objective of the current study was to investigate the effect of *Ceratonia siliqua L.* extract-loaded liposomes on wound healing. *Ceratonia siliqua L.* commonly called carob, is an evergreen tree that belongs to the Leguminosae family widely cultivated in Mediterranean countries. Twenty-five healthy adult male Wistar rats were randomized into five groups of five animals each: SHAM group with only wound creation. EUCRN group: As a control group with wound creation as well as 5 g eucerin administered topically to the wound bed. NLPSM group: As a group with wound creation, as well as 5 ml nanoliposome (20 mg). EUCRN/NLPSM group: As a group with wound creation as well as 5 g eucerin containing 5 ml nanoliposome (20 mg). TRTMNT group: As a group with wound creation as well as 5 ml eucerin (5 g) containing nanoliposome (20 mg) loaded with hydroalcoholic extract of *Ceratonia siliqua L.* The excisional wound model (8 mm in diameter) was used for biochemical (total antioxidant capacity, total oxidant status, Malondialdehyde levels, and glutathione peroxidase activity), histopathologic, and planimetric assessments. The wound area was significantly reduced in the TREATMENT group compared to other groups ($p < 0.05$). Biochemical and quantitative histopathological analyses revealed a significant difference between TREATMENT and other groups ($p < 0.05$). *Ceratonia siliqua L.* extract-loaded nanoliposomes showed the potential to improve wound healing significantly. This appeared to work by angiogenesis stimulation, fibroblast proliferation, inflammation reduction, and granulation tissue formation during the initial stages of the healing process. This accelerated healing led to earlier wound area reduction of the damaged area due to the reorganization of granulation tissue and collagen fibers. Topical administration of *Ceratonia siliqua L.* extract-loaded nanoliposomes could be recommended for wound healing due to reducing wound healing acceleration.

Introduction

The skin injury is an abrasion of epidermal layer of skin that initiates a dynamic, intricate, and complex process called wound healing to establish the integrity, structure, and function of damaged tissues. Wound recovery is crucial to avoid the penetration of injured tissues by microbes and to partially or completely reform the damaged tissues. This biological cascade began at the time of tissue injury and continue to various periods based on the wounding type.[1]

Ceratonia siliqua L., commonly called carob, is an evergreen tree that belongs to the Leguminosae family widely cultivated in Mediterranean countries.[2] Traditionally, carob has been used to produce animal feed. Nowadays, agricultural and industrial sectors exploit carob fruit and its primary products

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(i.e., flour, powder, and syrup) to develop a variety of foods and beverages.[3] The fruit is a brown pod with an elongated and compressed shape of varying dimensions and a wrinkled surface that becomes leathery when ripe. The pods are mainly made up of sweet edible pulp with a leathery outer layer (pericarp) and a softer inner area (mesocarp), rich in hard seeds.[4] Carob pulp contains a wide range of biologically active compounds.[5] Generally, carob pods have a high sugar content, relatively low content of lipids and protein, and some essential amino acids (aspartic and glutamic acids), as well as oleic, linoleic and linolenic acids. Moreover, the fruit contains a high amount of low-calorie dietary fibers (cellulose, hemicelluloses, and lignin), minerals (calcium, phosphorus, and potassium), and phenolic compounds.[3-6] The phenolic content is mainly represented by gallic acid; the other phenolic compounds are myricetin rhamnocyte, quercetin rhamnocyte, methyl gallate, cinnamic acid, and myricetin glycoside.[4,7,8] Carob pods show significant pharmacological activities including anti-inflammatory, antibacterial, antidiabetic, antihypercholesterolemic, hepatoprotective, neuroprotective, and nephroprotective.[2,5,9-11] Traditional medicine used carob pods for the treatment of human gastrointestinal diseases. Several studies showed that carob pods could be useful for the attenuation of processes related to chronic diseases, such as type 2 diabetes, obesity, and metabolic syndrome.[12] They exert beneficial effects on dyslipidemia and interfere with glucose absorption mechanisms.[13-15] Many of these activities are related to the inhibiting potential of oxidant species.[16] Liposomes have closed vesicular structures as closed and continuous bilayer structures mainly composed of phospholipid and/or lipid molecules. Promising nanocarriers for topical drug delivery are liposomes that enclose the water chamber and enable the transfer of molecules with different properties (lipophilic, hydrophilic, and amphipathic), so keeping the functionality and stability of encapsulated materials. They are biocompatible, biodegradable, and non-toxic able to preserve encapsulated drug and treatment agents. Liposomes have been reported to have the most biological cell characteristics needed for a suitable drug delivery system in general and specifically for growth factors for wound treatment.[17,18]

The aim of the present study was to investigate the effect of *Ceratonia siliqua* L. extract-loaded liposomes on wound healing in an animal model.

Materials and Methods

Ethical Considerations

The procedures of this work were approved by the University Ethical Committee. We followed instructions of National Academy of Sciences Publication with number of 85-23 that was revised in 1985.

Preparation of Ceratonia siliqua L. Extract-Loaded Nanoliposomes

The powder of *Ceratonia siliqua L*. plant (75 g) was added to the soxhlet after adding 300 ml ethanol (50%) and then heated at 90 °C for 10 h. The solvent was evaporated by a rotary evaporator. The extract was stored in the refrigerator at 5 °C.[19] For synthesis of *Ceratonia siliqua L*. liposomes, first, 1.0 g of lecithin was added to the 50 ml of deionized water and stirred to dissolve it. Then extract of *Ceratonia siliqua L*.was added to the above solution. After that, the mixture was sonicated for 30 min. In the next step, it was mixed with a homogenizer. Nano liposome of *Ceratonia siliqua L*. was prepared and then stored in the refrigerator at 10 °C.[20] To prepare the liposomal ointment, first, the nanoliposome (10 ml) was added to the eucerin (20 g). This mixture was stirred strongly for 30 min. The obtained liposomal ointment was stored in the refrigerator at 10 °C.[21] Using the Zetasizer Nano ZS, the particle size (nm), zeta potential (mV), and polydispersity index (PDI) of the liposomes were determined by the DLS method (Malvern, Helix, UK). At a temperature of 25 °C, the analysis was carried out with a He-Ne laser (wavelength of 633 nm) and a detector angle of 90°. Before analysis, samples were diluted in deionized water with a dilution factor of 1:20. The measurements of size and zeta were done in triplicate, and the results were expressed as Mean ± SD (nm and mV, respectively).

Animals

In this study, 25 healthy adult male Wistar rats, between 8 and 10 weeks and weighing within the range of 200 grams, were included in a 21-days study period. The animals were individually housed in cages in a controlled experimental room with a natural light/dark cycle. Environmental conditions were set to a constant room temperature of 23 ± 2 °C. The rats were fed with standard pellets and had continuous access to water and food. Surgical procedures were performed under general anesthesia induced through the intraperitoneal injection (IP) of a combination of xylazine hydrochloride 2% (5 mg/kg, Alfasan International, Woerden, Holland) and ketamine hydrochloride 10% (80 mg/kg, Alfasan International). Animals were randomized into five groups of five animals each: SHAM group with only wound creation. EUCRN group: As a control group with wound creation as well as 5 g eucerin administered topically to the wound bed. NLPSM group: As a group with wound creation as well as 5 ml nanoliposome (20 mg).[5] EUCRN/NLPSM group: As a group with wound creation as well as 5 g eucerin containing 5 ml nanoliposome (20 mg). TRTMNT group: As a group with wound creation as well as 5 ml eucerin (5 g) containing nanoliposome (20 mg)

loaded with hydroalcoholic extract of *Ceratonia siliqua L*. On the final day of the sampling, the rats were euthanized through intraperitoneal administration of a ketamine-xylazine combination. Ketamine (500 mg/kg; IP) and xylazine (50.00 mg/kg; IP) were administered to euthanize the animals.[22]

Excisional Wound Creation

After anesthesia induction, the rat back hair was clipped and the surgical site was prepared by scrubbing. Four excisional wounds were created on the dorsum of the rats by removing an 8 mm in diameter circular, full-thickness section of skin using a size 10 surgical blade. In each rat one wound was allocated for planimetric studies, one for histopathological and biochemical studies on day 7 post operation, one for histopathological and biochemical studies on day 14 post operation and one for histopathological and biochemical studies on day 21 post operation. The wounds for planimetric studies were used on day 21 for hydroxyproline contents estimations.

Wound Area

Assessing wound healing efficacy involved determining the percentage reduction in the wound area and the time for wound closure. To assess the excisional wound contraction and closure rate, photographs were taken immediately after the wound was created and on days 3, 6, 9, 12, 15, 18, and 21 post-operations using a digital camera while a ruler was positioned adjacent to the wounds to provide a scale reference. The Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc., San Jose, CA) was used to measure the wound areas.[23]

Hydroxyproline Determination

Hydroxyproline, a collagen marker, was quantified from day 14 samples of equal weights. Tissues were dried at 60 °C, hydrolyzed in 6 N HCl at 130 °C in sealed tubes, neutralized to pH 7, oxidized with Chloramine T for 20 minutes, terminated with 4 M perchloric acid, and color developed with Ehrlich's reagent at 60 °C. Absorbance at 557 nm was measured via a UV-visible spectrophotometer. Day 21 right-side wounds were used for histopathology-linked assessment.

Biochemical Evaluations

To conduct biochemical assessments, samples were collected from the tissue concurrently with pathological samples on day 21. These samples were promptly frozen at 20 degrees. The tissues were ground in a mortar. One gram of each tissue sample was homogenized on ice with 4.5 ml of the appropriate buffer using an Ultra-Turrax homogenizer (IKA, Werke, Germany) for 15 minutes. Filtered homogenates were subjected to centrifugation using a refrigerated centrifuge at 4 °C. Subsequently, the resulting supernatants were utilized for assessing enzymatic activities. All assays were conducted at room temperature. The supernatant's total antioxidant capacity (TAC), total oxidant status (TOS), and malondialdehyde (MDA) levels were measured by a spectrophotometer using commercial assay kits (Navand Salamat, Tehran, Iran), adhering to the manufacturer's guidelines. Following a previously established protocol, the supernatant derived from samples collected on day 21 was utilized to assess glutathione peroxidase (GPx) activity.[24] The procedure involved the introduction of KH_2PO_4, EDTA, GSH, B-NADPH, NaN_3, and GR into the sample, followed by an incubation period. Upon the addition of H_2O_2, the chronometer was initiated, and the absorbance at 340 nm was monitored at 15-second intervals for 5 minutes. On postoperative day 21, a skin sample was gathered from the fully healed wound area and examined for hydroxyproline content, a fundamental component of collagen. Estimating hydroxyproline levels followed a method outlined by previous researchers.[25] In summary, the tissues were dried in a hot-air oven set at 60 °C to 70 °C until a consistent weight was achieved. Subsequently, they underwent hydrolysis in sealed tubes with 6 N HCl at 130 °C for 4 hours. The resulting hydrolysate was neutralized to pH 7.0 and underwent a 20-minute chloramine-T oxidation process. The reaction concluded with adding 0.4 M perchloric acid, and the color development occurred through applying Ehrlich reagent at 60 °C. The measurement was conducted at 557 nm using a UV-visible spectrophotometer (CamSpec M330).

Histopathological Evaluations

Skin samples were obtained from the wound periphery, along with normal skin, at 7 and 14 days post-excisional wound creation. These samples were then fixed in 10% formalin and embedded in paraffin for subsequent histopathological investigation using a light microscope. Hematoxylin and eosin-stained slides were scrutinized to assess cellular infiltration, encompassing the count of mononuclear cells, polymorphonuclear cells, and fibroblastic aggregation. Additionally, the examination involved quantifying the distribution of blood vessels within one square millimeter of the wound, along with evaluating the thickness of the epithelial tissue in the repair region.

Statistical Analyses

The Kruskal-Wallis variance analysis was adopted for differences among groups. Where the P value (from the Kruskal-Wallis test statistics) was statistically significant, multiple comparison tests were utilized to get the differences. One-way ANOVA test was used for

comparison among days. For retrieving possible multiple comparisons, the Bonferroni correction was utilized. We utilized SPSS 11.5 (SPSS Inc.) for the analyses and considered a *p* value <0.05 as a significant level.

Results

Wound Area Reduction

The effect *Ceratonia siliqua L.* extract-loaded nanoliposomes on circular excision wound contraction area is shown in Table 1. Local application of *Ceratonia siliqua L.* extract-loaded nanoliposomes in TRTMNT group significantly reduced the wound area and enhanced the healing rate compared to other groups (p <0.05).

Hydroxyproline Contents

Contents of hydroxyproline in the SHAM, EUCRN, NLPSM, EUCRN/NLPSM, and TRTMNT groups were recorded as 53.31 ± 4.79, 63.12 ± 3.55, 72.20 ± 3.11, 81.10 ± 3.22, and 93.54 ± 3.82 mg/g, respectively. The concentrations were significantly bigger in the TRTMNT group implying further deposition of collagen in comparison to other groups (p <0.05).

Biochemical Evaluations

The levels of antioxidant enzymes (GPx), the natural product of lipid peroxidation (MDA), TAC, and TOS were estimated in this study to evaluate the effect of cultured macrophage in combination with mesenchymal stem cell/ macrophage culture supernatants on wound-healing progress (Table 2). Biochemical analyses revealed that TAC and GPx activity values in the TRTMNT group were significantly higher than those observed in the other experimental groups. In contrast, MDA and TOS levels significantly decreased in the TRTMNT group compared to the other groups (p <0.05).

Histopathological Evaluations

The histopathology of the wound tissues was evaluated on the 7th, 14th, and 21st day after wound creation. The number of PMN cells and mononuclear cells in the TRTMNT group was significantly less compared to those in the other experimental groups at all-time points (p <0.05). The number of blood vessels and fibroblast cells was significantly higher in the TRTMNT group on days 7 and 14 compared to the other groups (p <0.05). The thickness of the granulation tissue on day 7 was significantly higher in the TRTMNT group than the other groups, while on day 14, the granulation tissue was thicker in the TRTMNT groups than in the other groups (Figures 1 and 2) (p <0.05).

Discussion

The advancement in wound healing technology has enabled the use of cells to address the limitations of conventional methods. Assessing wound contraction is an indicator of understanding the progress of skin wound healing. Wound contraction accelerates the healing rate by limiting the amount of granulation tissue that needs to be produced.[1]

It has been suggested that, the methanolic extract of *Ceratonia siliqua* has a significant anti-inflammatory activity and may be related with inhibition of inflammatory mediators like serotonin, histamine, kinin, cyclooxygenase, prostaglandin and cytokine.[26] During inflammation, the leukocytes and macrophages migrating to the site of injury are known to produce the superoxide radicals (O_2-), which in turn mediates the generation of hydrogen peroxide.[27] Furthermore, in the presence of suitable transitional elements, hydrogen peroxide may be transformed to the highly reactive hydroxyl radicals. These radicals can also act as secondary messengers,

Table 1. Effect of *Ceratonia siliqua L,* extract-loaded nanoliposomes on circular excision wound contraction area (mm^2). Values are given as mean ± SEM.

Groups	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
SAHM	56.77 ± 3.17	42.35 ± 3.75	33.25 ± 3.80	24.54 ± 3.12	11.11 ± 0.75	8.25 ± 1.03	5.09 ± 0.15
EUCRN	52.18 ± 8.15*	39.70 ± 4.15*	26.30 ± 2.37*	21.12 ± 3.20*	9.15 ± 0.76*	6.19 ± 1.11*	4.70 ± 0.66*
NLPSM	48.10 ± 3.55**	38.80 ± 3.71**	24.65 ± 2.15**	16.17 ± 0.23**	7.07 ± 0.09**	4.05 ± 02.05**	1.07 ± 0.06**
EUCRN/NLPSM	45.12 ± 2.45**	34.11 ± 3.71**	22.60 ± 3.10**	12.42 ± 0.20**	6.05 ± 0.05**	3.03 ± 02.07**	0.00 ± 0.00**
TRTMNT	41.10 ± 3.55**	32.21 ± 3.71**	19.68 ± 3.15**	10.55 ± 0.23**	4.02 ± 0.03**	0.00 ± 00.00**	0.00 ± 0.00**

*,**: The mean difference is significant at the 0.05 level vs other groups.

Table 2. Comparison of the activities of TAC, TOS, MDA, and GPx in the tissue samples taken from experimental groups on day 21. Data are expressed as Mean ± SD.

Indices	SAHM	EUCRN	NLPSM	EUCRN/NLPSM	TRTMNT
TAC	0.53 ± 0.10	0.82 ± 0.60	0.85 ± 0.60	0.89 ± 0.35	1.29 ± 0.20*
TOS	105.43 ± 15.28	83.30 ± 6.20	58.35 ± 6.25	48.30 ± 3.23	37.60 ± 18.20*
MDA	0. 95 ± 0.27	0.78 ± 0.51	0.68 ± 0.80	0.55 ± 0.80	0.35 ± 0.99*
GPx	0.12 ± 0.14	0.20 ± 0.11	0.39 ± 0.18	0.45 ± 0.10	0.54 ± 0.15*

TAC: Total antioxidant capacity, TOS: Total oxidant status, MDA: Malondialdehyde, GPx: Glutathione peroxidase dismutase. *: p < 0.05 *vs.* TRTMNT groups.

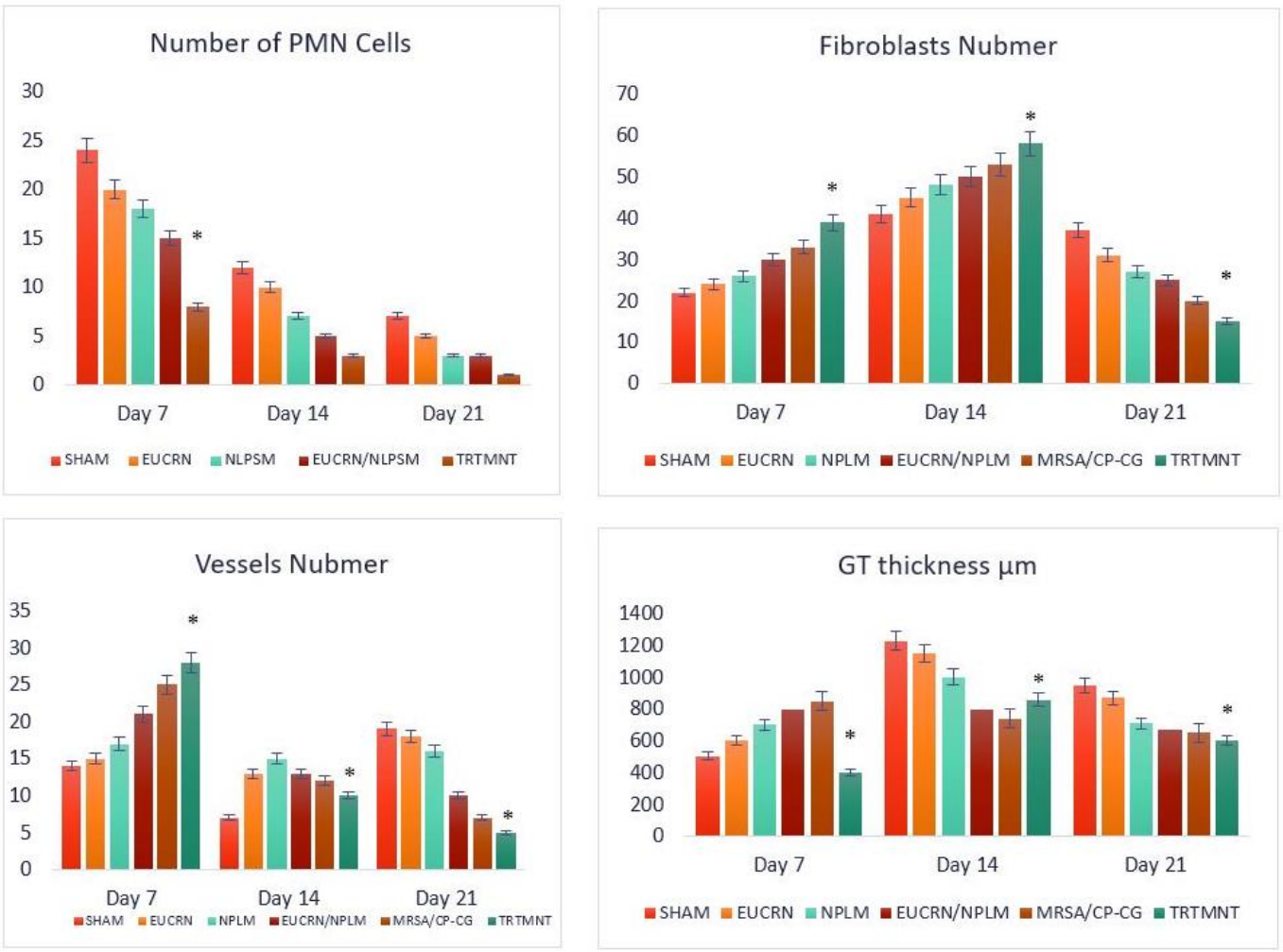

Figure 1. Bar graphs indicating the number of PMN cells (top left), Fibroblast (top right), number of vessels (down left) and granulation tissue thickness (down right) in excisional wound models of the skin in experimental groups. Results are expressed as mean ± SEM. * $p < 0.05$ versus other experimental groups.

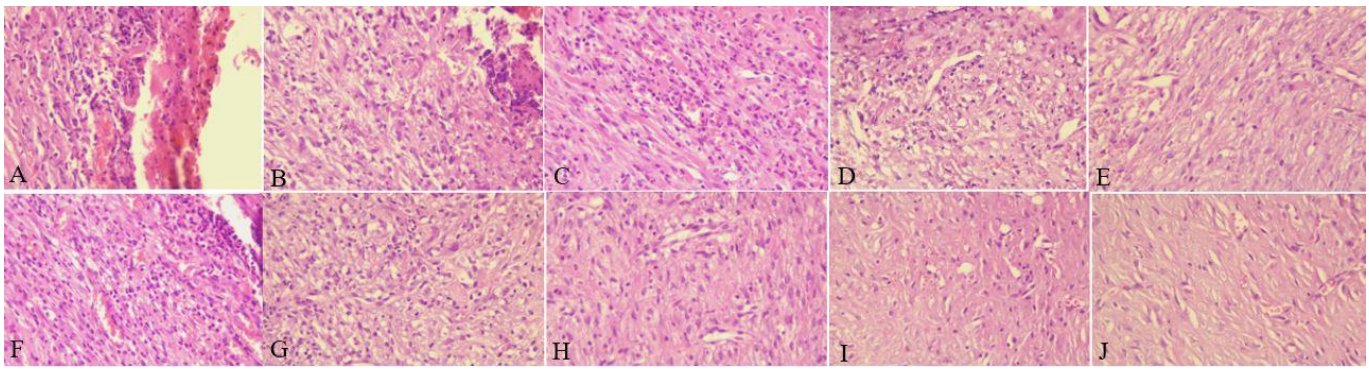

Figure 2. Histological characteristics of the skin on the days 7 (A-E) and14 (F-J) after wound creation in excisional wound model in SHAM (A and F), EUCRN (B and G), NPLM (C and H), EUCRN/NPLM (D and I) and TRTMNT (E and J) Groups (H&E staining, ×400).

thereby activating the production of other inflammatory mediators.[28] Many plant extracts having antioxidant properties have been shown to scavenge free radicals and thereby act as anti-inflammatory agents. Polyphenols (including phenolic compounds and flavonoids) are plant compounds that can exert significant antioxidant activity, mainly due to their redox properties,[29] which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides. The results of phytochemical screening of methanolic extract of a previous study on *Ceratonia siliqua* barks showed the presence of polyphenols, flavonoids, tannins, and sterols and have antioxidant effect comparable to the BHT. These compounds have been shown to possess anti-inflammatory, and antioxidant effects, and are known to inhibit some molecular targets of pro-inflammatory mediators in inflammatory responses. They also act as antioxidants by scavenging radicals and thereby attenuate the inflammatory process.[29]

In the last few decades, researchers have used herbal nanoliposome to treat and manage a variety of diseases

like wound healing. Presently, several liposomal formulations are on the market for the treatment of diseases.[30] In this light, many researchers focused on the encapsulation of phytochemicals in liposomes to increase their bioavailability and wound healing effectiveness. For example, Takahashi *et al.* showed liposomes encapsulating *Aloe vera* leaf gel extract significantly enhance proliferation and collagen synthesis in human skin cell lines. Hence, *Aloe vera* leaf gel should have great potential as an effective skincare formulation.[31]

The better antioxidant effects at the wound site in TRTMNT group in this study also contributed to improved granulation and healed tissue quality in this group as supported by histopathological findings. Hydroxyproline, a main component of collagen, enables the sharp twisting of the collagen helix. It provides firmness to the collagen triple-helical structure by forming hydrogen bonds. Hydroxyproline has been used as a reliable indicator for evaluating collagen content because it is present in a few proteins apart from collagen.[32]

The higher concentration of hydroxyproline in TRTMNT group, as a direct measure of collagen synthesis, demonstrated an increase in collagen deposition. Collagen provides the tensile strength of wounds. Therefore, the β-cryptoxanthin treated group in the incisional skin wound model exhibited statistically significant improvements in biomechanical parameters compared to other groups. Angiogenesis, the development of new blood vessels, is one of the necessities of the wound healing process, which can increase blood supply and subsequently lead to faster healing.[33]

In the current study, the highest amount of angiogenesis was observed in the TRTMNT group. Angiogenesis provides a framework for the creation of connective tissue in the early days of healing. The formed micro vessel enables the transfer of fluid, oxygen, nutrients, and immune-competent cells to the wound area.[34] Increased formation of new vessels following wound creation indicated that β-cryptoxanthin treatment promoted the healing process due to facilitating cellular infiltration. On day 21 after injury, the number of blood vessels in the *Ceratonia siliqua L*. extract-loaded nanoliposomes treated group was lower than in other groups. This acceleration in blood vessel reduction indicated the positive effect of *Ceratonia siliqua L*. extract-loaded nanoliposomes in healing.

The higher production of fibroblasts in the *Ceratonia siliqua L*. extract-loaded nanoliposomes treated group on days 7 and 14 showed that this treatment could induce the growth of fibroblasts. Fibroblast number is a widely recognized index in the assessment of connective tissue healing quality. The primary role of fibroblasts is collagen production.[35] Fibroblasts synthesize essential components of the primary extracellular matrix in the wound bed, providing an optimal condition for cell migration and proliferation. Subsequently, fibroblasts produce collagen, which is crucial for imparting tensile strength to the wound bed.[36] Collagen contributes to the strength and integrity of the tissue matrix and plays a key role in homeostasis and epithelialization during the later stages of healing.[37]

Granulation tissue acts as the base, creating a matrix for proper healing. Formation of granulation tissue in the early days of the healing process is regarded as a crucial factor in wound healing acceleration[38] Superior granulation tissue is characterized by the presence of well-developed blood vessels in perpendicular directions, along with a dominance of fibroblasts and a well-organized extracellular matrix formation. In the initial stage, immature-type granulation tissue consists of inflammatory cells, angioblasts, new blood vessels, fibroblasts, and collagen fibers. In the later stages, this immature granulation tissue matures, becoming more permanent, which is essential for effective wound healing. In the current study, the maximum thickness of the granulation tissue was observed in all groups at 7 days after wounding. Moreover, this thickness was significantly greater in the *Ceratonia siliqua L*. extract-loaded nanoliposomes treated group than in the other groups. On days 14 post-injury, the thickness of the granulation tissue was decreased due to maturation. The thickness of the granulation tissue in the *Ceratonia siliqua L*. extract-loaded nanoliposomes treated group was significantly lower compared to other treatment groups. The initial stage of wound healing begins with an inflammatory phase, characterized by inflammatory cells. This phase is critical in wound healing because of its association with cellular events, contraction, and wound closure.[39] The lowest polymorphonuclear and mononuclear cell count on all time points was observed in the *Ceratonia siliqua L*. extract-loaded nanoliposomes treated group in our study. Our findings suggested that this treatment improved the wound-healing process by reducing the duration of the inflammatory phase and rapid transition to the proliferative phase, during which increased wound contraction was observed. We did not perform molecular studies to track cellular events supporting anti-inflammatory properties of *Ceratonia siliqua L*. extract-loaded nanoliposomes in the wound during healing process that could be considered as limitation of our study.

In conclusion, our study suggested that *Ceratonia siliqua L*. extract-loaded nanoliposomes had potential advantages in wound healing via its antioxidant properties, induction of angiogenesis, number of fibroblast, early formation of granulation tissue, reduction in the number of inflammatory cells, improving

collagen deposition, and accelerating wound closure.

Conflict of Interest

The authors declare that they have no competing interests.

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