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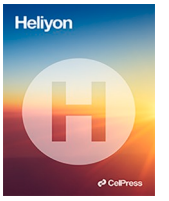

Research article

# Novel garden cress-fish gelatin based ointment: Improvement of skin wound healing in rats through modulation of anti-inflammatory and antioxidant states

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ABSTRACT

This study aimed to investigate the ability of aqueous extract of *Lepidium sativum* seeds (LSE) to improve the wound healing process in rat models. The gelatin, extracted from the skin of smooth-hound shark using citric acid, was used as a support material for ointment. Animals were divided into four groups of six rats each: an untreated control group, a control group treated with Moist Exposed Burn Ointment (MEBO), a treated group with gelatin gel, and a treated group with gelatin gel fortified with 20 mg/mL LSE. Phenolics profile analysis showed that the major compounds in LSE were catechin (125 μg/g) and quinic acid (105 μg/g). *In vitro* antioxidant tests showed that LSE has interesting activities to scavenge ABTS•+ radicals ($IC_{50}$ = 0.22 mg/mL) and inhibit the oxidation of linoleic acid. A significant decline in the antioxidant enzymes activities and an increase in the level of thiobarbituric acid reactive substances (TBARS) and inflammatory markers was observed within the injured tissues of the untreated rats compared to rats treated with MEBO. Interestingly, when the wounded tissue was treated with gelatin gel a remarkable reversal of this trend occurred. Further, by enrichment of gelatin gel with LSE, the levels of CAT, GPx and SOD activities significantly increased by 35, 126, and 212 %, respectively, whereas the TBARS level was reduced by 31 %. These results were consistent with the wound contraction percentage and histological analysis, which suggest the potential effect of LSE-enriched gelatin gels to regenerate damaged tissues.

## 1. Introduction

The skin is a vital organ that belongs to the defense mechanism and constitutes the first line contact with microorganisms and

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pathogens. Thus, the skin is exposed to damage caused by various factors, such as physical and chemical damages, as well as certain diseases. A physical injury is an area of damage to the body, such as a skin laceration or tissue opening, which disrupts its normal functions due to a range of cellular and molecular processes [1,2]. Wound healing is a natural biological process aimed at repairing injury or damage to skin tissue. Hence, the skin possesses inherent self-regeneration capabilities, although associated with a relatively gradual healing process. Many studies investigated the improvement of healing process by adding biological components, such as plant extracts, which can improve wound healing. In fact, the addition of plant extracts was based on their effect to reduce free radicals thanks to the effect of the polyphenol compounds. It's known that free radicals are always produced during the body metabolism. These radicals could affect the mechanism of wound healing and caused the damage wound surrounding cells through the disruption of the transmitted information, which decrease its ability to fight microorganisms. The presence of free radicals resulted in oxidative stress, which in turn led to the depletion of both enzymatic and non-enzymatic antioxidant defenses within the affected cell [3]. Indeed, the oxidative stress could generate lipid peroxidation and DNA breakage, which hampered the healing process. Thus, the scientific researchers are focusing to find the supplementation of these antioxidants by using novel bioactive compounds that helps the wound's recovery and prevent its damage [4,5].

Biological materials constitute an interest issue for researchers and scientists due to their low cost, safety, interesting properties, availability and biodegradability [6,7]. Medicinal plants are frequently used in various applications in the field of natural biomaterials. This is primarily because they contain a wealth of compounds, including phenols and flavonoids, which show intriguing biological activities such as antimicrobial and antioxidant properties. Consequently, they offer a broad spectrum of benefits, with a particular focus on promoting overall human health and, notably, the well-being of skin cells.

Garden cress (*Lepidium sativum* L.) is one of medicinal plants of the cruciferae family. Originating from the Mediterranean region, this plant is now widely distributed across various parts of the world and is commonly cultivated as a culinary vegetable [8]. The L. *sativum* seeds are commonly used as an effective remedy for various diseases, including inflammation, arthritis, hypertension, bronchitis, hepatotoxicity, cancer and diabetes [9,10]. Indeed, the seeds extract contained high amounts of volatile compounds and different antioxidants components, such as phenolic compounds, flavonoids and glucosinolates. In the same context, many works illustrated that seeds and leaves contained volatile oils and polysaccharides that could be used in various therapeutic modalities [7,8,11]. Particularly, these characteristics encourage the scientific researchers to examine the effect of *L. sativum* seeds extract on wound healing treatment.

To accelerate the healing process, wounds can be managed through different treatments, such as ointments, scaffolds, biosynthesis membranes and composite sponges [12,13]. In this context, gelatin, a natural biopolymer derived from collagen in skin or bones, has attracted attention for diverse medicinal applications, including tissue engineering and drug delivery, due to its biocompatibility and gel-forming properties. Besides its hydrophilic and biodegradable characteristics, gelatin was employed to reduce fluid loss in skin tissue, thereby enhancing the wound healing process [12]. On the other hand, the gelatin extraction process should be eco-friendly by using ingredients that are safe for human health, especially when gelatin was intended for pharmaceutical or food applications. One viable alternative was to replace the use of chloride and sodium hydroxide, commonly employed in the extraction of gelatin from various fish processing by-products, with an eco-friendly component like citric acid [14,15].

This research aimed to test the wound healing activity of *L. sativum* seeds extract. Firstly, the antioxidant potential of *L. sativum* extract was assessed through ABTS•+ and lipid peroxidation tests. Subsequently, for wound treatment the *L. sativum* extract was integrated into a gelatin gel extracted from the skin of smooth-hound shark. The lipid peroxidation and antioxidant enzymes in wound tissue, along with inflammatory markers, wound contraction rate, and histological examination, were studied.

## 2. Materials and methods

### 2.1. Preparation of L. sativum extract (LSE)

In July 2020, seeds of *L. sativum* were purchased from the local market in Gabes, situated in the southeastern region of Tunisia. To prepare the aqueous extract, 6 g of seed powder were immersed in 100 mL of distilled water. The resulting mixture was then incubated at 37 °C for 24 h with continuous stirring. Subsequently, the transparent supernatant was recovered and lyophilized using a freeze-dryer (ModuloyD Freeze dryer, Thermo Fisher, USA). The resulting lyophilized extract was stored at −20 °C until it was ready for use.

### 2.2. Analysis of the phenolic compounds using liquid chromatography coupled with electrospray ionization mass spectrometry

The LSE at a concentration of 20 mg/mL was analyzed using a Shimadzu quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization source and operated in the negative-ionization mode. The identification of phenolic compounds was assessed by comparing their mass spectra with those of commercially available standards of the utmost purity (Sigma Chemical Co., St Louis, MO, USA) as previously reported by Chahbani et al. [16].

### 2.3. Antioxidant activities of LSE

#### 2.3.1. ABTS•+ scavenging activity

The ABTS•+ [2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) radical cation] scavenging activity of the LSE was assessed using the procedure reported by Auddy et al. [17]. A mixture of 7 mM ABTS•+ solution with 2.45 mM ammonium persulfate was incubated in darkness at room temperature for 16 h prior to its utilization. Then, the ABTS•+ solution was diluted with ethanol until an

absorbance reading of 0.8–0.9 at 734 nm was achieved using UV–Vis spectrophotometer (Jenway, Tamil Nadu, India). Subsequently, an aliquot of 950 μL of the diluted ABTS•+ solution was mixed with 50 μL of LSE within a concentration range of 0.1–0.5 mg/mL. The same concentrations were used to determine the activity of the control using the butylated hydroxytoluene (BHT) as a synthetic antioxidant. The absorbance was then measured at 734 nm after a 10-min incubation period at room temperature. The percentage of ABTS•+ scavenging activity of the LSE was calculated using equation (1).

$$\text{ABTS•+ scavenging activity (\%)} = (A_C - As)/Ac \times 100 \quad (1)$$

where Ac is the absorbance of the control reaction and As is the absorbance of LSE sample. Each test was conducted with three separate sample replications, and the results were then averaged.

#### 2.3.2. Lipid peroxidation assay

The potential of LSE against linoleic acid peroxidation was evaluated according to the method of Ghimire et al. [18] with some modifications. Firstly, The LSE, ranging in concentration from 0.1 to 0.5 mg/mL, was dissolved in 2.5 mL of a 50 mM phosphate buffer (pH = 7.0). Subsequently, this solution was combined with 2.5 mL of 50 mM linoleic acid dissolved in 95 % ethanol. The resulting mixtures were then incubated for 8 days at 45 °C.

Linoleic acid oxidation was assessed by measuring the thiobarbituric acid reactive substances (TBARS) formation. Every 2 days, a 250 μL aliquot of the reaction mixture was collected and mixed with 375 μL of a TCA-BHT solution (containing 20 % TCA and 1 % butylated hydroxytoluene) to induce protein precipitation. The resulting mixture was then centrifuged at 10,000×*g* for 15 min at 4 °C. After that, a volume of 400 μL from the supernatant was mixed with 80 μL of 0.6 M HCl and 320 μL of Tris-TBA solution (comprising 26 mM Tris and 120 mM TBA) and the mixture was incubated at 90 °C for 10 min. After cooling, the absorbance was measured using UV–Vis spectrophotometer (Jenway) at 530 nm. The TBARS values were determined referencing a standard curve of malondialdehyde (MDA) and were reported as mmol MDA equivalent/mg of extract (mmol EqMDA/mg extract).

### 2.4. Gelatin extraction and formulation of gel fortified with LSE

The by-product of the smooth-hound shark (*Mustelus mustelus*) was recovered following processing at a fish market located in Sfax, Tunisia. Gelatin was extracted from the fish skin using a process that included citric acid treatment as previously described [19], with minor adjustments made to the procedure. Briefly, the skin was washed with tap water and cut into small pieces measuring 1 × 1 cm. These skin pieces were then immersed in a 50 mM citric acid solution, with a ratio of 500 g of skin per 2.5 L, and the mixture was stirred for 18 h. Subsequently, the blend was subjected to incubation at 50 °C with continuous stirring for 24 h. Following this, the mixture was centrifuged at 6000×*g* for 20 min, resulting in the separation of the supernatant containing gelatin. This gelatin-rich supernatant was then dried using a lyophilizer (Labconco, Kansas City, MO, USA).

The gelatin gel utilized for treating injured rats was prepared according to the method of Jridi et al. [4] with some modifications. Firstly, 4 g of freeze-dried gelatin were dissolved in 100 mL of distilled water at a temperature of 40 °C, and this mixture was allowed to stand for 30 min. To enhance the gel formation, the solution was subjected to constant stirring for 1 h at 4 °C. Subsequently, an active gelatin solution was prepared by dissolving LSE to achieve a final concentration of 20 mg/mL.

### 2.5. Wound healing treatment

#### 2.5.1. Animals

Adult male Wistar rats, with body weights ranging from 150 to 200 g, were from the Central Pharmacy of Tunisia (SIPHAT, Tunis, Tunisia). The animals were housed in clean polypropylene cages within a standard environment conditions, consisting of a 12 h light-dark cycle with temperatures maintained between 22 and 25 °C and relative humidity between 45 and 55 %. Throughout the experimental period, the rats were *ad libitum* access to food in the form of pellet diet (SICO, Sfax, Tunisia) and water. The handling of the animals was granted approval by the Medical Ethics Committee for the Care and Use of Laboratory Animals of the Pasteur Institute of Tunis (approval number: FST/LNFP/Pro 152012). All experimental procedures were conducted in strict adherence to the principles outlined in the European convention for the protection of living animals used in scientific investigations [20].

#### 2.5.2. Excision wound model

The wound excision was realized following the method described by Jridi et al. [4]. The rats were given an intramuscular injection of ketamine (100 mg/kg body weight) for anesthesia. A circular wound with an area of 150 $mm^2$ was made on the depilated thoracic region of the rats and the day of this operation was considered as day 0.

#### 2.5.3. Treatments

After creating the wounds, the 24 rats were randomly divided into four groups, of six animals each ($n = 6$). In order to prevent other animals from licking or biting the wounded areas, the animals were individually housed and they were subjected to the following treatments during 15 days.

(i) group 1, referred to as "C (−)", consisted of untreated rats where the wounds were cleaned only with physiological serum and served as the negative control;

(ii) group 2, referred to as "C (+)", consisted of treated rats with the reference product MEBO (Julphar Gulf Pharmaceutical Industries, Ras Al Khaimah, UAE) and served as the positive control;
(iii) group 3, referred to as "GEL", consisted of treated rats with a gelatin gel without LSE enrichment;
(iv) group 4, referred to as "GEL-LSE", consisted of treated rats with a gelatin gel fortified with 20 mg/mL LSE.

For wound healing treatment, 1 mL of MEBO or gels were applied manually in a fine layer covering the surface of the wound. Starting from the day 0, the various treatments were applied topically every two days, particularly until complete healing for the group treated with MEBO. At the end of the experimental period, all the rats were anesthetized with ether, euthanized and the tissues were surgically removed from the sacrificed animals.

#### 2.5.4. Blood and skin tissue collection

Blood samples obtained from the sacrificed animals were promptly placed on ice for subsequent analysis. Heparin served as the anticoagulant for the blood. A portion of the skin tissues, in close proximity to the sites of inflammation, was used for biochemical analyses. Initially, the tissue samples were homogenized in a Tris-Buffered Saline (TBS) buffer containing 50 mM Tris-HCl and 150 mM NaCl (pH = 7.4), using an Ultra-Turax homogenizer (IKA Ultra-Turrax T25, Cole-Parmer GmbH, Germany) at a ratio of 1-part skin to 2 parts buffer by weight. Subsequently, the homogenates were centrifuged at 5500×*g* for 15 min at 4 °C, and the resulting supernatants were collected and stored at −80 °C until use.

### 2.6. Parameters for assessing wound healing

#### 2.6.1. Wound contraction rate

The wound contraction rate was measured as previously described by Jridi et al. [4]. The extent of wound contraction was monitored every two days and the results were expressed as a percentage of the healed wound area.

#### 2.6.2. Protein concentration

Protein concentration was measured in the supernatants obtained from tissue homogenates following to the method of Lowry et al. [21], using bovine serum albumin (BSA) with an extinction coefficient of $E^{1\%}_{1cm} = 6.7$ as the reference standard.

#### 2.6.3. Antioxidant enzymes

The supernatants obtained from wound tissues homogenates were used to determine the antioxidant enzymes activities. All absorbances were measured using UV–Vis spectrophotometer (Jenway).

The catalase (CAT) activity was measured by monitoring the reduction in absorbance at 240 nm over a 1-min period. This reduction in absorbance was a consequence of $H_2O_2$ consumption, following the method reported by Aebi [22]. The CAT activity was quantified and expressed as μM $H_2O_2$/mg protein.

The glutathione peroxidase (GPx) activity was measured as previously reported by Flohé and Günzler [23]. This method involves measuring the conversion of glutathione (GSH) oxidation by GPx, which is linked to the conversion of 5,5-dithiobis-(2-nitrobenzoic)-acid (DTNB) into 2-nitro-5-thiobenzoate (TNB), a compound that absorbs at 412 nm. The enzymatic activity was quantified and expressed as mM/mg protein.

The superoxide dismutase (SOD) activity was measured by evaluating its ability to inhibit the reduction of nitro-blue tetrazolium (NBT) through a photochemical process, as previously reported by Beauchamp and Fridovich [24]. The absorbance was measured at 580 nm and the enzyme's specific activity was quantified as U/mg protein. A unit of enzyme activity was defined as the quantity of enzyme needed to decrease the oxidation reaction by 50 %.

#### 2.6.4. Lipid peroxidation

The amount of present in various homogenized wound tissue samples was measured using the same method previously employed to measure TBARS formation during the assessment of the protective capacity of LSE against the peroxidation of linoleic acid. The TBARS were expressed as nmol EqMDA/mg protein.

#### 2.6.5. Inflammatory process markers

The inflammatory state was assessed by analyzing C-reactive protein (CRP) and plasma fibrinogen levels in blood samples. All analyses were performed in the Laboratory of Biological Analyses of the regional hospital of Beja (Beja, Tunisia). The CRP and plasma fibrinogen were measured using Selectra Pro XL (Toulon, France) and Concern-Energomash-Start Max (Yerevan, Armenia) automatic biochemistry analyzers, respectively.

#### 2.6.6. Histological study

The histological analysis was conducted following the procedure described by Yadav et al. [1]. Skin tissue samples, in close proximity to the sites of inflammation, were initially fixed in Bouin's solution for 24 h to prepare them for histological examination. Subsequently, these samples were immersed in a 10 % neutral-buffered formalin solution. The tissues were then embedded in paraffin, and sections, each 5 μm thick, were prepared. These sections were stained using hematoxylin and eosin (H&E) and examined under an LCD binocular digital display medical microscope LCD binocular digital display medical microscope (Yuji Optics, Ningbo, China).

### 2.7. Statistical analysis

All tests were carried out in triplicate. Statistical analysis was conducted using SPSS version 18.0, professional edition, employing ANOVA analysis. Statistical significance was determined at $p \leq 0.05$, and the Duncan test was utilized to identify significant differences.

## 3. Results and discussion

### 3.1. Phenolic profile of LSE

Table 1 shows the profile of phenolic compounds in LSE, as determined through the use of liquid chromatography-electrospray ionization coupled with mass spectrometry. The identification of these compounds was accomplished by comparing their mass spectra with a set of 32 authentic standards. It's worth noting that if the LSE contained compounds different from the standards, they could not be identified. The major compound was the catechin (125 μg/g) followed by the quinic acid (105 μg/g). Interestingly, a study investigated by Abd El-Salam et al. [25] reported a different profile, with the gallic acid, ellagic acid, and protocatechuic acid being the major phenolic acids, and the hesperidin being the predominant flavonoid in garden cress seed extract. It's important to consider that the synthesis of phenolic compounds could be influenced by various edapho-climatic factors. Moreover, variations in the methods employed for both extracting and identifying phytochemical compounds, along with the choice of solvents, could result in substantial disparities between different research studies [26].

The catechin is a flavonoid found in various plant-based foods and beverages. It had significant importance in the field of nutrition, health and medicine due to its various properties and potential health benefits. In fact, it was reported that catechin could act as powerful antioxidant, neutralizing harmful free radicals and oxidative stress, and contributing to overall health and well-being [27]. Moreover, it showed anti-inflammatory properties, offering therapeutic benefits in the management of inflammatory conditions [28]. The catechin also exhibited antimicrobial properties, which could help to fight bacterial and viral infections [29]. Interestingly, it was reported that catechin might help to combat oxidative stress, inflammation, and skin damage caused by UV rays, thereby contributing to skin health and potentially slowing the aging process. Thus, the topical application of catechin-rich extract could benefit the skin and improve its elasticity as discussed in previous studies [30,31]. It's important to note that while catechin offered numerous potential health benefits, its effectiveness could vary depending on factors like dosage, individual response and the specific source of catechin.

### 3.2. LSE antioxidant properties

The antioxidant potential of LSE was assessed in terms of ABTS•+ scavenging ability and inhibition of linoleic acid peroxidation. The ABTS•+ scavenging activity showed a dose-dependent antioxidant effect, with an $IC_{50}$ value of 0.22 mg/mL (Fig. 1A). LSE was also tested to inhibit the linoleic acid oxidation in terms of EqMDA content during 8 days (Fig. 1B). The reduction in EqMDA content showed a significant decrease ($p \leq 0.05$) as the concentration of LSE increased, indicating a highly effective inhibition of linoleic acid oxidation. At a concentration of 0.1 mg/mL of LSE, the EqMDA content on day 8 was notably elevated compared to the level observed on day 2. The LSE, when tested at a concentration of 0.5 mg/mL, consistently demonstrated the lowest EqMDA content over the course of 8 days, never exceeding 0.92 mmol EqMDA/mg. These results align with other antioxidant assays, highlighting *L. sativum*'s promising potential in terms of DPPH•-radical scavenging activity, β-carotene bleaching activity, ferric ($Fe^{3+}$) reducing power and ferrous ($Fe^{2+}$) chelating activity [32]. Thus, LSE, being a polyphenol-rich extract, has been incorporated to enhance the gelatin gel for wound healing, potentially offering various benefits due to its antioxidant and anti-inflammatory properties.

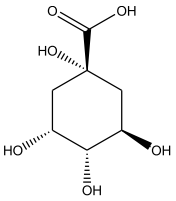
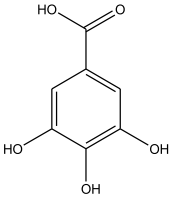
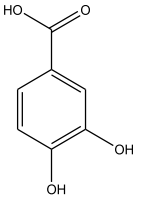
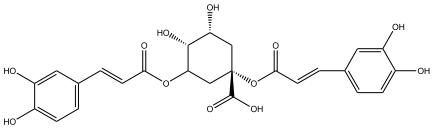
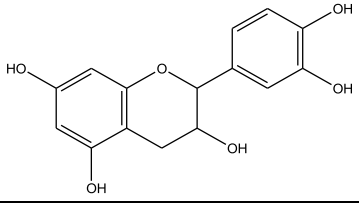
### 3.3. Study of wound healing

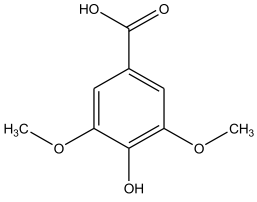
#### 3.3.1. Antioxidant status

When a tissue was injured, complex biochemical processes were triggered in the body to fight against this aggression. It was reported that the presence of an injury lead to an elevated level of reactive oxygen species, hindering the progression of the healing process [33]. Thus, TBARS and key antioxidant enzymes (CAT, GPx and SOD) were measured on wound tissues homogenates (Table 2). A notable decline in the antioxidant enzyme activities was observed within the injured tissues of the untreated rats (C (−)) compared to rats treated with MEBO (C (+)). The reduction in enzymatic antioxidant activities coincided with an increase in the level of oxidative stress and the elevation of TBARS content, reaching 101.66 nM MDA eq/mg (Table 2). Interestingly, when the wounded tissue was treated with GEL and GEL-LSE, a marked correction of TBARS level was noted, where the treated tissues presented the lowest contents (Table 2). Moreover, by the application of the LSE-enriched gelatin gel, the activities of the antioxidant enzymatic defense were significantly increased, and the extent of oxidation was substantially reduced. In fact, the activities of CAT, GPx and SOD in GEL-LSE-treated skin were corrected to better values, reaching respectively 58.15 μM $H_2O_2$/mg, 0.43 μM/mg and 18.73 U/mg, compared to 43.17 μM $H_2O_2$/mg, 0.19 μM/mg and 6.01 U/mg in the untreated rats (C (−)), respectively.

These findings could be attributed to gelatin's barrier properties, limiting oxygen permeation into the injury site. Additionally, the presence of antioxidant peptides could play a role in breaking down oxidative damage reactions, enhancing cell viability and promoting tissue regeneration [4]. In addition, LSE contained a wide range of bioactive substances characterized by interesting antioxidant potential, which strengthen cellular protection against oxidative damage and improve their viability [34]. Similar results

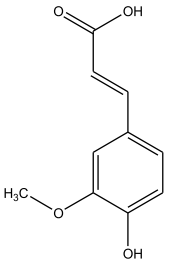
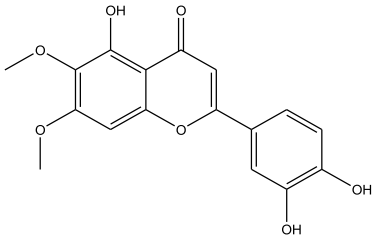
**Table 1**
Phenolic profile of the aqueous extract of *L. sativum* seeds.

| Compounds[a] | | Molecular mass | $[M-H]^-$ *m/z* | Content (μg/g extract) |
|---|---|---|---|---|
| Quinic acid |  | 192 | 191 | 105 |
| Gallic acid |  | 170 | 169 | 5.5 |
| Protocatechuic acid |  | 154 | 153 | 6.2 |
| 1,3-di-*O*-Caffeoylquinic acid |  | 516 | 515 | 3.6 |
| Catechin |  | 290 | 289 | 125 |

| Compounds[a] | | Molecular mass | $[M-H]^-$ *m/z* | Content (μg/g extract) |
|---|---|---|---|---|
| Syringic acid |  | 198 | 197 | 7.3 |

(*continued on next page*)

**Table 1** (*continued*)

| Compounds[a] | | Molecular mass | $[M-H]^-$ *m/z* | Content (μg/g extract) |
|---|---|---|---|---|
| *trans*-Ferulic acid |  | 194 | 193 | 1.0 |
| Cirsiliol |  | 330 | 329 | 4.0 |

[a] Identification was confirmed using 32 authentic commercial standards.

reported that grape seed extract helps to treat many skin pathologies and accelerate the wound healing [35]. These authors suggest that grape seed extract is rich in antioxidants such as tannins, which lead to free radicals scavenging and increase the regeneration of capillary vessels and fibroblasts. The obtained results highlight the potential of antioxidant-enriched gelatin in not only bolstering antioxidant defenses but also in mitigating oxidative stress, thereby holding promise for enhancing the wound healing process and managing inflammation at the cellular level.

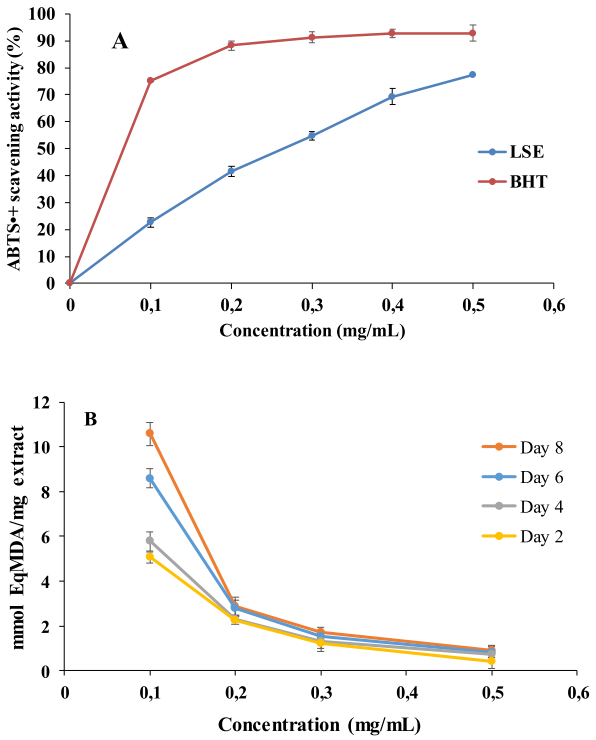



**Fig. 1.** Antioxidant activities of the aqueous extract of *L. sativum* seeds. (A) ABTS•+ scavenging activity; (B) Lipid peroxidation assay.

**Table 2**
Lipid peroxidation, antioxidant enzymes and inflammatory markers.

| | Treatments | | | |
|---|---|---|---|---|
| | C (−) | C (+) | GEL | GEL-LSE |
| **TBARS (nmol EqMDA/mg protein)** | $101.66 \pm 1.11^{a}$ | $80.87 \pm 1.78^{b}$ | $84.37 \pm 1.22^{b}$ | $70.45 \pm 0.22^{c}$ |
| **CAT (µM $H_2O_2$/mg protein)** | $43.17 \pm 1.62^{c}$ | $57.32 \pm 1.20^{b}$ | $54.79 \pm 1.45^{b}$ | $58.15 \pm 1.78^{a}$ |
| **GPx (µM/mg protein)** | $0.19 \pm 0.07^{c}$ | $0.39 \pm 0.02^{b}$ | $0.32 \pm 0.01^{b}$ | $0.43 \pm 0.02^{a}$ |
| **SOD (U/mg protein)** | $6.01 \pm 0.08^{c}$ | $17.92 \pm 1.02^{b}$ | $15.91 \pm 1.32^{b}$ | $18.73 \pm 1.45^{a}$ |
| **CRP (mg/mL)** | $2.75 \pm 0.35^{a}$ | $2.07 \pm 0.02^{c}$ | $2.18 \pm 0.04^{b}$ | $2.02 \pm 0.04^{c}$ |
| **Fibrinogen (g/L)** | $5.22 \pm 0.47^{a}$ | $3.34 \pm 0.06^{b}$ | $3.85 \pm 0.08^{b}$ | $3.01 \pm 0.06^{c}$ |

C (−), C (+), GEL, and GEL-LSE groups designed the untreated rats, rats treated with MEBO, rats treated with gelatin gel, and rats treated with gelatin gel fortified with 20 mg/mL LSE, respectively. $^{a,b.c}$ Different lower case letters within the same row indicate significant differences between groups ($p \leq 0.05$).

### *3.3.2. Inflammation assessment: marker analysis*

Table 2 also shows that the untreated wounded rats (C (−)) showed the highest content of C-reactive protein (CRP), an acute-phase protein synthesized by the liver in response to inflammation or tissue injury. Similarly, the highest content of fibrinogen, a protein crucial for blood clotting, was noted in the same group. This observation further indicated the body's response to tissue damage and inflammation. A decrease of inflammatory markers was observed for rats treated with gelatin gel (GEL) compared to the C (−) group, which suggest that the extracted gelatin used as an ointment support could decrease the inflammatory damage. Interestingly, the treatment with LSE-enriched ointment (GEL-LSE) led to a further reduction in the levels of CRP and fibrinogen in the rats' plasma (Table 2). This result suggested that the antioxidants treatment may have anti-inflammatory properties and help to mitigate the inflammatory response associated with the skin wounds. In the same context, Yahla et al. [7] studied the anti-inflammatory activity of *L. sativum* seeds in mice. They suggest that *L. sativum* extract improved the initiation of the immune cells by stimulating cytokines, which accelerate the regeneration of the damaged tissue. Vazifeh et al. [34] also reported that *L. sativum* extract stopped lipid peroxidation and ROS generation and increased GSH content, which improved cell viability and minimized the inflammatory status of the injured wounds.

### *3.3.3. Assessment of wound healing progress*

The percentage of the wound area closure generated by the different groups was measured and the results was presented in Table 3. Gelatin treatment as ointment (GEL) resulted in a wound area closure significantly higher than the untreated wound. The wound closure percentage showed by the LSE-enriched gelatin ointment was comparable to that obtained with the standard MEBO ointment. Indeed, after 15 days, the wound closure percentages were 53.12 % for untreated rats, 80.61 % for rats treated with gelatin gel, 95.31 % for rats treated with LSE-enriched gelatin gel and 97.61 % for MEBO-treated rats. These results suggested the effectiveness of gelatin-based ointment particularly that enriched with LSE, in promoting effective wound healing in the rat model. Similarly, Al-Warhi et al. [37] reported that *Vitis vinifera* seed extract showed a rapid wound contraction (95 %), compared to the control group treated with MEBO. The rapid contraction rate of wounds treated with *L. sativum* seeds extract could be in relation with its antioxidant and anti-inflammatory properties, which accelerate the epithelialization rate [1,36]. Salem et al. [32] also reported that *L. sativum* seeds showed an interesting antibacterial activity, which could protect the injury from the negative effect of bacterial infection. In addition, it has been reported that a decrease in ROS levels is associated with improved activity of certain macrophages, which strengthens the body's defense mechanism against bacteria [38]. Altogether, this could help to stimulate the regeneration of wound tissue, leading to a higher percentage of wound closure.

### *3.3.4. Histological study*

The microscopic observations of each skin wound tissues collected from the different groups were presented in Fig. 2. The C (−) control group showed severe damaged tissue, proved by an increased inflammatory cells (I) indicating a high inflammatory state in the dermis area. On the other hand, group C (+) serving as a standard group, presented the best healing state and marked by an excellent wound re-epithelization with a regeneration of collagen fibers with an increased number of blood vessels. The wound tissue sections of

**Table 3**
Effect of different treatments on the percentage of wound closure.

| Treatments | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | 8 | 10 | 12 | 15 |
| **C (−)** | 0.00 | $7.07 \pm 0.57^{b}$ | $13.11 \pm 1.19^{b}$ | $27.18 \pm 1.83^{b}$ | $41.56 \pm 1.41^{b}$ | $44.46 \pm 0.88^{b}$ | $49.75 \pm 1.79^{b}$ | $53.12 \pm 1.45^{c}$ |
| **C (+)** | 0.00 | $13.02 \pm 0.37^{a}$ | $18.16 \pm 1.13^{a}$ | $36.47 \pm 1.75^{a}$ | $65.56 \pm 0.96^{b}$ | $67.46 \pm 1.80^{a}$ | $70.92 \pm 1.01^{a}$ | $97.61 \pm 2.23^{a}$ |
| **GEL** | 0.00 | $11.33 \pm 1.23^{a}$ | $16.64 \pm 1.14^{a}$ | $35.15 \pm 2.54^{a}$ | $60.79 \pm 1.55^{a}$ | $64.65 \pm 2.63^{a}$ | $70.89 \pm 2.78^{a}$ | $80.61 \pm 2.49^{b}$ |
| **GEL-LSE** | 0.00 | $12.04 \pm 1.00^{a}$ | $17.08 \pm 1.72^{a}$ | $37.50 \pm 0.27^{a}$ | $62.50 \pm 2.11^{a}$ | $69.44 \pm 2.74^{a}$ | $75.69 \pm 1.54^{a}$ | $95.31 \pm 1.98^{a}$ |

C (−), C (+), GEL, and GEL-LSE groups designed the untreated rats, rats treated with MEBO, rats treated with gelatin gel, and rats treated with gelatin gel fortified with 20 mg/mL LSE, respectively. $^{a,b.c}$ Different lower case letters within the same row indicate significant differences between groups ($p \leq 0.05$).

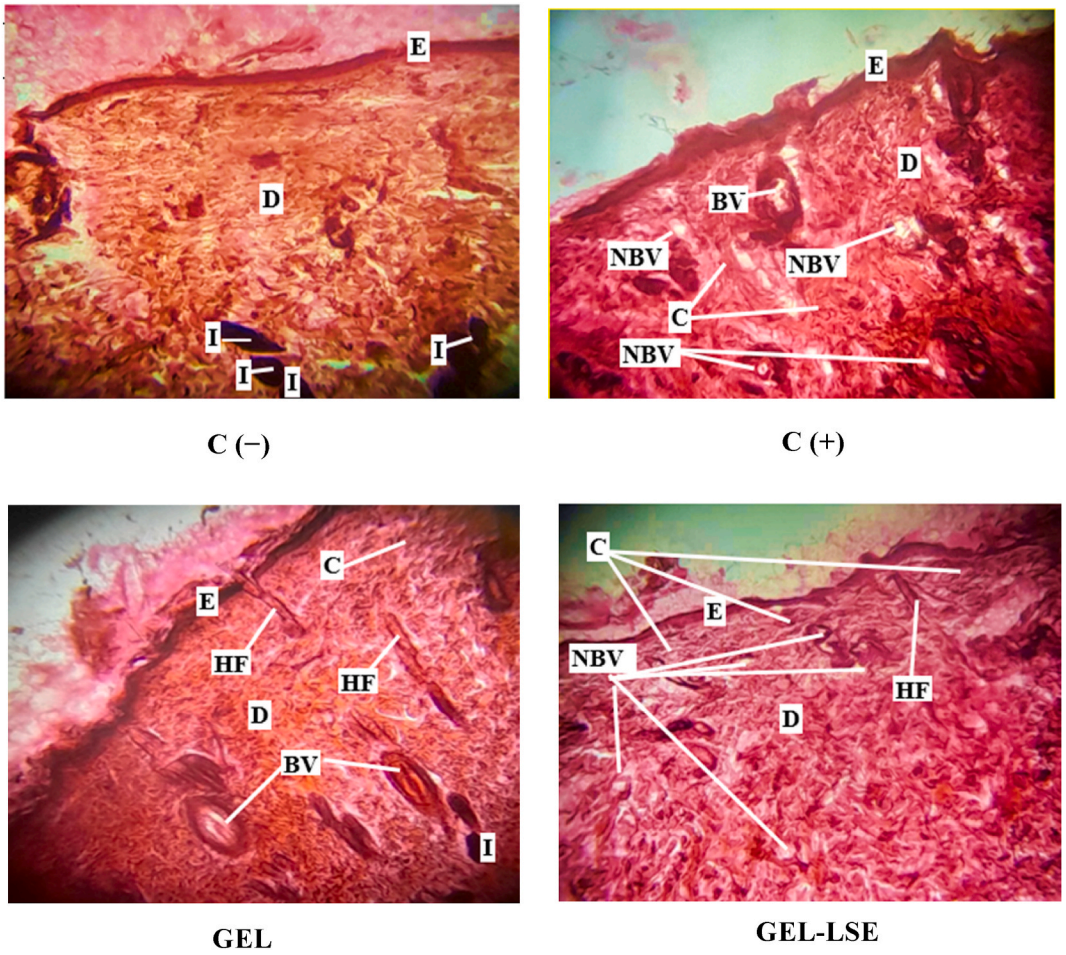



**Fig. 2.** Microscopic observations of the wounds architecture on the 15th day (40 × ). C (−), C (+), GEL, and GEL-LSE groups designed the untreated rats, rats treated with MEBO, rats treated with gelatin gel, and rats treated with gelatin gel fortified with 20 mg/mL LSE, respectively. E: epidermis; D: dermis; I: inflammatory cell; BV: blood vessel; NBV: new blood vessel; HF: hair follicle; C: collagen fibers.

GEL group revealed better tissue-healing statue compared to C (−) control group. Microscopic tissue observation of skin tissues in the GEL group revealed the regeneration of blood vessels (BV) and hair follicles (HF) surrounded by collagen fibers (C). Therefore, the extracted gelatin could be considered as an efficient healing agent, when used as an ointment material. In addition, GEL-LSE group showed better improvement in epidermis section regeneration with the development of collagen fibers (C), which enhance the vascularization of the damaged tissue, as proved by the increased new blood vessels (NBV), and improved re-epithelization rate. The obtained results suggested the positive effect of LSE to enhance the mechanisms required for the healing process. In the same context, similar results were reported by Elzayat et al. [2] who used extracts from henna, pomegranate and myrrh, blended with paraffin and wax, and test their wound healing capacity. The authors reported that the observed fast wound healing effect could be associated with anti-inflammatory effect of the flavonoids and polyphenols, resulting in a complete regeneration of skin structure. In addition, Attia et al. [39] studied the effect of *L. sativum* seeds' extracts on alloxan-induced diabetic rats, and reported a significant decrease of the inflammatory cells number. The positive effect of *L. sativum* extract in wound healing process could be explained by the anti-inflammatory and antioxidant properties of the phenolic compounds, able to reduce ROS and inflammation state at the wound site. Moreover, Kaparekar and Znandasadagopan [40] reported that phenolic compounds could stimulate blood circulation, which is essential for oxygen and nutrients delivery to support collagen synthesis and wound contraction.

## 4. Conclusion

The present study investigated the potential use of the aqueous extract from *L. sativum* seeds (LSE) incorporated into fish gelatin matrix as ointment to improve the wound healing process in injured rat models. The applied smooth-hound shark skin gelatin was found effective on regenerating collagen fibers and accelerating the healing rate thanks to its oxygen and bacteria barrier properties, showing similar results to the standard MEBO ointment. This effect was particularly pronounced after the addition of LSE as anti-oxidant and anti-inflammatory agent. In fact, marked enhancements on TBARS, CRP and fibrinogen levels, as well as antioxidant

enzyme activities, were noted, emphasizing the potential use of LSE-enriched fish gelatin gels as healing ointment for damaged tissues.

## Ethics statement

The experimental animals were granted by the approval by the Medical Ethics Committee for the Care and Use of Laboratory Animals of the Pasteur Institute of Tunis (approval number: FST/LNFP/Pro 152012). All experimental procedures were conducted in strict adherence to the principles outlined in the European convention for the protection of living animals used in scientific investigations.

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## Data availability statement

All relevant data are within this published paper.

## CRediT authorship contribution statement

**Ali Salem:** Writing – original draft, Investigation. **Ola Abdelhedi:** Data curation. **Fadia Ben Taheur:** Methodology. **Chalbia Mansour:** Investigation. **Sihem Safta Skhiri:** Methodology. **Hichem Sebai:** Conceptualization. **Mourad Jridi:** Data curation, Conceptualization. **Nacim Zouari:** Writing – review & editing, Data curation. **Nahed Fakhfakh:** Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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