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Tunisian *Pistacia lentiscus* Fruit Oil: Biochemical Composition and Wound Healing Activity in a Rat Model

Sameh Ben Khedir[1], Sana Bardaa[2], Dorsaf Moalla[2], Zohra Ghlissi[2], Zouheir Sahnoun[2], Tarek Rebai[1]

[1]Laboratory of Histology, Embryology, and Reproductive Biology, Faculty of Medicine of Sfax, University of Sfax, Sfax, Tunisia
[2]Laboratory of Pharmacology, Faculty of Medicine of Sfax, University of Sfax, Sfax, Tunisia

Email address

samehbkh@yahoo.fr (S. B. Khedir)

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Abstract: The oil extracted from *Pistacia lentiscus* fruit has long been used for wound healing in traditional medicine. The present study aimed to characterize an oil extract from Tunisian *Pistacia L.* fruit and to elucidate on the relationship between its chemical compositions and wound healing properties. The quality indices and composition and content of certain bioactive constituents of the cold pressed oil obtained from *Pistacia lentiscus* fruits were analyzed and studied for their wound healing properties. Uniform full thickness excision wounds were induced on the dorsum of 72 rats, randomly divided into three groups. The wounds were photographed, and topically treated with saline solution (control group), 0.13 mg/mm^2 of a reference drug ("Cicaflora cream®"), and 0.52 µl/mm^2 of *Pistacia lentiscus* fruit oil (PLFO). The results showed an excellent quality of PLFO with High content of monoterpens and sesquiterpens. The fatty acids were dominated by Oleic acid, mono-unsaturated fatty acid, with an amount of 45.66%. The sterol content analysis revealed the prevalence of β-Sitosterol, accounting for 77.94%. The results from morphometric assessment and histological findings revealed that best wound healing activity, with reappearance of skin appendages and well organized collagen fibers without inflammatory cells, was exhibited by PLFO, followed by «CICAFLORA»®. Overall, the findings indicated that *P. lentiscus* fruit oil has a number of promising wound-healing properties that make it a strong candidate for application in human therapy.

Keywords: *Pistacia lentiscus* L., Fruit Oil, Acidity, Peroxide Value, Fatty Acids, Sterols, Wound Healing, Histological Analysis

1. Introduction

Wounds are defined as a disruption or of damage of anatomic or functional continuity of living tissues [1]. Wound healing is a biological process that often starts by trauma and terminates by scar formation [2]. This process involves several phases, including coagulation, epithelization, granulation, collagenation, and tissue remodeling [3]. With the growing concerns over the development of resistance to conventional medicines, researchers have become increasingly interested in the search for novel therapeutic agents from natural origins for use in wound healing. Of particular interest, research in phytotherapy has recently revived the interest in the use of natural products from plant origins for the development of drugs with wound healing properties as educated in popular folk medicine. The literature indicates that several plant species might offer promising sources for the production of effective, safe and cost-effective medicinal agents. In fact, several plants and their parts have traditionally been used for the treatment of several wounds and skin disorders. The Tunisian folk medicine, for instance, includes a wide range of prescriptions for therapeutic purposes, including the healing of wounds, inflammations, skin infections, venereal disease, and ulcers.

Pistacia lentiscus Linn (*P. lentiscus* L.), generally known as the lentisk or mastic tree, is an evergreen shrub of the

Anacardiaceae family that has long been in Mediterranean and Middle Eastern countries as a dietary supplement and herbal remedy [4]. It is well known in for its resin, commonly known as Chios Mastic Gum (CMG), and strong aromatic green leaves. In Tunisia, *P. lentiscus* L. swells in various regions, particularly in the North. This tree is known for its medicinal properties since ancient times [5]. The decoction from its dried roots has been reported to be effective in the treatment of intestinal inflammations and stomach ulcers [6]. The aerial part of *Pistacia lentiscus* has been widely used in traditional medicine for the treatment of high blood pressure due to its diuretic properties [7-10]. The leaves of the plant have also been described to offer a wide array of anti-inflammatory, antibacterial, antifungal, antipyretic, stimulant, astringent, hepatoprotective, and expectorant properties [11-16]. Furthermore, the essential oil of *P. lentiscus* is known for its therapeutic properties against lymph and circulatory problems. The literature also provides evidence that the essential oil from P. lentiscus presents attractive analgesic, antioxidant, anti-inflammatory, and antimicrobial activities [15, 17-24].

The oil derived from *P. lentiscus* fruit has commonly been used as an agent for the treatment of diabetics and stomach pains and for pain relief after circumcision [25]. It has also been widely used as a remedy for external local application in the form of ointment for burns [26] or backaches [27]. The fixed oil extracted from mature fruits is commonly used in Tunisian folk medicine. Furthermore, the Tunisian population has traditionally used this edible oil in their daily diet in salads and pastries. This oil has frequently been used as an anti-ulcer, wound healing agent, and antiseptic [28, 29]. It has also been used in the treatment of scabies, rheumatism and diarrhea, and served as a condiment in various regions in the North West of Tunisia [30, 31].

The oil extracted from *P. lentiscus* is characterized by a high nutritional value. It contains a significant amount of unsaturated fatty acids (more than 70%) [30, 32] and a high level of phosphatidylinositol [33]. The effects of the growing area on tocopherols, carotenoids and fatty acid content of *Pistacia lentiscus* fixed oil extracted from Fruits harvested from eight different sites located in the north and the centre of Tunisia have previously been investigated [34]. The *Pistacia lentiscus* fruit oil has been reported to contain significant amounts of β -carotene, α -tocopherol and unsaturated fatty acids. These results highlight its nutritional value and endorse its promising potential for application in pharmaceutical and medicinal purposes. The wound healing effects of *Pistacia lentiscus* fruit oil have previously been studied in a rabbit burn model and its ability to increase in re-epithelialization has been demonstrated [35].

The present study aimed to build a direct correlation between the chemical components (fatty acids, sterols and tocopherol) and characteristics (acidity, peroxide value, specific extinction coefficient at 232 nm and 270 nm) of *P. lentiscus* fruit oil and the accelerated wound healing ability of the topical application of *P. lentiscus* fruit oil using a rat wound model. The tissues treated with *Pistacia lentiscus* oil were submitted to morphological and histological investigation and compared to those treated with "CICAFLORA ®" as a reference standard.

The stimulation effects of *P. lentiscus* fruit oil wound healing were assesses by (1) tracing the wound reduction in the wound status over a given period of time, (2) scoring at total period healing of epithelialization, and (3) evaluating histological regeneration and collagen density.

2. Materials and Methods

2.1. Plant Material

P. lentiscus fruits were collected from the region of El Kef (North West Tunisia). The *P. lentiscus* oil was extracted from the freshly harvested fruits according to the traditional procedures used by the women living in the local area. The harvested fruits were initially ground by a stone grinder. The paste was then thoroughly mixed by hands or feet and then left to rest overnight. The following day, cold water was added to the paste, and the upper part was removed and fire-heated till boiling. The liquid phase was separated from the oil meal using a tissue, and then placed reheated until the complete evaporation of water. Finally, the oil was filtered and stored for subsequent assays. The oil had a clear green yellowish color.

2.2. Quality Indices Determinations

The acidity of the oil (free fatty acids) was determined according to the method proposed by ISO660 (1996) [36]. Peroxide content was estimated according to the method described by ISO3960 (2001) [37]. UV constants (K232 and K270) were measured using the analytical method described by IOC.

2.3. Analysis of *Pistacia lentiscus* Fruit Oil by GC-MS

The chemical composition of the oil was determined by GC analysis using a Perkin–Elmer Auto System XL gas chromatograph equipped with a flame ionisation detector. The column used was an Elite-5 (cross-bond 5.0% diphenyl-95.0% dimethyl polysiloxane) capillary column (30 m x 0.53 mm i.d., 0.50 μm film thicknesses; Supelco, Bellefonte, PA). The oven temperature was initially set at 110°C for 1 min, and then increased to 250°C at 30°C/min for 1.0 min. This was followed by an increase of 25°C/min to 285°C for 2.0 min when all peaks appeared. The injector and detector were held at 285 and 290°C, respectively. Helium was used as carrier gas at 3.0 cm/s linear velocity. The chemical separated components were identified by comparison of retention times with known standards. The chemical components were further identified by gas chromatography–mass spectrometry (GC-MS) analysis was using a single quadrupole mass spectrometer (Varian 1200L, Varian Inc., Palo Alto, CA) under electron impact (EI, ionisation energy 70 eV) conditions with an on-column injector set at 110°C.

2.4. Fatty Acids Composition

After their methylation, FAMEs were determined by gas chromatography capillary column according to a standard protocol (vigorous stirring of the oil solution in n / heptane (0 1 g in 2 mL) with 0.2 ml of 2 N methanolic potassium hydroxide) (*38, 39*). The gas chromatography analysis of FAME was performed on a gas chromatograph equipped with a AutoSystem FID (HP 6890N, Agilent (Palo Alto, CA, USA)). An agilent capillary CP-Sil88 (cyanopropyl polysiloxane) column (length 50 m, id 0.25 mm and 0.20 um thickness movie) was used. The analysis was performed according to the following conditions. The initial temperature of the column was set at 165°C for 25 min and then gradually increased by 5°C/min to until 195°C. The temperatures of the injector and detector were set at 250°C. Helium was used as carrier gas at a flow rate of 1 ml / min and a flow rate of 1:100 split ratio. The injection volume was 1 µl. Fatty acids were quantified based on their percentage area obtained by integrating the peaks. The results were expressed as the percentage of individual fatty acids in the lipid fraction.

2.5. Determination of Sterol Composition

An amount of *Pistacia lentiscus* fruit oil (5 g) was dissolved in 50 ml of 2 N ethanolic potassium hydroxide saponified extract according to the official IOOC (International Olive Oil Council) method [39]. The unsaponifiable fraction was dissolved in chloroform, and approximately 20 mg were charged on a basis of silica TLC plate. The sterol and triterpene fraction diol was separated by elution in hexane-diethylether 65:35 (v / v). After being sprayed with a 2, 7-dichlorofluorescein in 0.2% ethanolic solution, scraped with a spatula, and extracted with chloroform, the corresponding band was visualized under UV light. The sterols and diols were converted to trimethylsilyl ethers by the addition of pyridine-hexamethyldisilazane trimethylchlorosilane (9: 3: 1, v / v / v) and left to rest for 15 min. The extract was evaporated and centrifuged.

The sterols were analyzed by a gas chromatograph Agilent 7890A (Pudong, Shanghai, China) equipped with a flame ionization detector (FID). The column used was an HP-5 capillary (5% phenyl, 95% dimethylpolysiloxane) (length 30 m, id 0.32 mm and 0.25 um film thickness). The furnace isothermal temperature was 260°C, injector temperature was 280°C, and detector temperature was adjusted to 290°C. Helium was used as a gas carrier. The flow rate through the column was 1 ml / min, and a 1:50 split injection volume of 3 µl was used.

The relative retention times (RRT) of the sterols were determined by the analysis of aliquots of the dominant sterol compounds (β-sitosterol), knowing that RRT (β-sitosterol) was equal to 1 as described by the COL statistics, and this was used to identify the individual peaks of sterols [40]..

2.6. Animals

A total of ninety healthy male Wistar rats weighing 182-187g were used in all the in vivo assays of the present study. The animals were housed in individual clean polyethylene cages under standardized environmental conditions: 23–25°C temperature, 12-h light-dark cycle, and free access to food and water. They were maintained under standard conditions for 2 weeks to be acclimatized prior to the investigation. The experimental protocols were conducted in accordance with the guide for the care and use of laboratory animals issued by the University of Sfax, Tunisia, and approved by the Committee of Animal Ethics (Protocol no. 94-1939).

2.7. Excision Wound Model

The wound contraction was evaluated using a circular excision wound model. Before wound excision, the rats were anesthetized by intramuscularly injecting 50 mg/kg of ketamine, along with 5 mg/kg Midazolan, and the hair at their backs was shaved with a sterilized clipper. A circular wound (having an area of 1. 2 cm^2) was created on the dorsal region of each animal. The excision wound was inflicted by cutting away a full thickness of skin from a predetermined area and left open.

2.8. Wound Healing Activity

After wound formation, the animals were randomly divided into three groups of thirty rats each. Group I consisted of rats that did not receive any treatment (just cleaning the wounds with a physiologic serum) and served as a control group. Group II contained rats that were treated with CICAFLORA® (0.13 mg/mm^2) and was served as a reference group. Group III consisted of rats treated with the *P. lentiscus* fruit oil (0.52 µl /mm^2) and was designated as the experimental group.

The reference product "CICAFLORA ®" was used to carry out a comparative study with the *P. lentiscus* oil. It is a restorative emulsion that promotes the repair of altered epidermis: open or closed wounds (cuts, burn). The product contained 10% of *Mimosa Tenuiflora Sophora Japonica* extract, allantoine, Sorbitol, and Glycerine. The *Mimosa Tenuiflora* extract was obtained from the powdered bark of a Mexican tree known for its action on cell stimulation, which promotes the repair of weakened epidermis, and bacteriostatic nature due to its richness in tannins, trace elements, and bioflavonoids [41, 42].

All the rats were weighed before and after treatments. The wounds were treated with the different preparations, with 24-h intervals for 21 days. The first application was performed directly after wound formation. The duration of epithelialization was measured from the initial day. The rats were individually housed, maintained on normal food and water ad labium, and those which showed infection signs were separated and excluded from the study.

2.9. Wound-healing Evaluation Parameters

Wound healing activity was evaluated based on three criteria, quantitative, microscopic and Histopathology

Quantitative analysis relied on data derived from the study of the wound surfaces. Microscopic analysis involved data

from histological evaluation.

The quantitative study involved the measurement of wound dimension, which was daily traced on a transparent paper. Wound surface area was monitored by applying the following mathematical formula, which was adapted to its general elliptical form according to Mayrovitz:

Wound surface=L*w*0.785 (L=length, w=width).

For Histopathology**,** Six animals from each group were sacrificed on the 3rd, 7th, 14th and 21st day, and cross sections through the longitudinal aspect of the scarred areas were made. Skin fragments were prepared for microscopic observation with an optical microscope following the procedures described by Gabe (1968). In brief, samples were fixed in 10% buffered formalin, processed, and then blocked with paraffin. Three- micrometer sections were stained with Hematoxylin Eosin (HE) and Van Gieson (VG).

2.10. Statistical Analysis

The data were analyzed using the Statistical Package for the Social Sciences (SPSS, Inc) Software. The results were expressed with their standard deviations. The comparison between groups was performed using the Student's t-test. The difference was considered significant at $p<0.05$.

3. Results

3.1. Free fatty Acids Percentage (Acidity)

Free fatty acids have often been used as a marker of oil quality. Table 1 shows the free fatty acid (FFA) content (% C18:1) of the *P. lentiscus* fruit oil. The results revealed that it was present at a value lower than 3.02%.

3.2. Peroxide Value

The peroxide value (PV) of oil is an important indicator of oxidation level, which is a measure of primary oxidation. Table 2 presents the PV (meq O_2/kg) of *Pistacia lentiscus* fruit oil. The findings indicated that the oil exhibited a PV of 7.21meq O_2/kg.

3.3. Specific Extinction Coefficient at 232 nm and 270 nm

The determination of UV-specific extinction values allows for the estimation of the oxidation process in unsaturated oils [43]. The K232 and K270 values for *P. lentiscus* fruit oil were 6.856 and 0.458, respectively (Table 1).

Table 1. *Acidity, PV and Fatty acid composition of P. lentiscus fruit oil.*

Paramètre	*Pistacia lentiscus* fruit oil
Acidity (%)	3.02
PV (meq O_2/Kg)	7.21
K_{232}	6.856
K_{270}	0.458
FA	Amount (% of TFA)
C14:0	0.05
C16:0	24.93
C16:1	2.76
C17:0	0.04
C17:1	0.11
C18:0	1.37
C18:1	45.66
C18:2	24.21
C18:3	0.55
C20:0	0.17
1	0.15
SFA	26.56
MUFA	48.68
PUFA	24.76

3.4. GC–MS Composition of *P. lentiscus* Fruit Oil

The *P. lentiscus* fruit oil was analyzed by GC–MS. Table 2 presents the individual components, identified in the oil extract with their relative percentages. Overall, forty-one components were identified, representing 100% of the total oil. The results revealed that the oil contained a complex mixture of several components, predominately hydrocarbons, monoterpens and sesquiterpens. The major components identified were α-Pinene (13.35%), β-Phellendrene (10.45%), α–Phellendrene (10.12%) Sabinene (7.01%), Germacrene-D (6.68%), 2-β-Pinene (5.57%), β-Caryophyllene (4.58%), and Myrcene (4.33%).

Table 2. *Composition of P. lentiscus fruit oil obtained by GC–MS.*

No	Rt (min)	Compound	(%)	No	Rt (min)	Compound	(%)
1	6.264	Tricyclene	0.92	24	15.404	Alpha -cubebene	0.27
2	6.386	Alpha phellandrene	10.12	25	15.938	Alpha-copaene	1.10
3	6.592	Alpha.-pinene	13.35	26	16.224	Beta.-Elemene	1.01
4	6.878	Camphene	2.88	27	16.807	Beta.-caryophyllene	4.58
5	7.461	Sabinene	7.01	28	17.331	Alpha –copaene	0.38
6	7.535	2-Beta-pinene	5.57	29	17.416	Alpha.- Humulene	1.27
7	7.821	Myrcene	4.33	30	17.538	Neoalloocimene	0.61
8	8.393	Alpha -terpipene	1.59	31	17.739	Epi-bicyclosesquiphellendrene	0.36
9	8.599	p-Cymene	2.16	32	17.808	Gamma-muurolene	1.86
10	8.726	Beta-phellandrene	10.45	33	17.935	Germacrene-D	6.86
11	9.086	Trans-. beta.-ocimene	0.78	34	18.205	Alpha-muurolene	1.87
12	9.240	Butanoic acid	0.48	35	18.475	Alpha –amorphene	0.76
13	9.346	Gamma.-terpinene	2.33	36	18.613	Delta.-cadinene	3.55

No	Rt (min)	Compound	(%)	No	Rt (min)	Compound	(%)
14	9.997	Terpinolene	1.7	37	18.782	Naphthalene, 1, 2, 3, 4, 4a, 7-hexahydro -1-6-dimethyl-4-(1-methylethyl)	0.2
15	10.039	2-Nonanone	0.64	38	19.703	Nopinone	0.48
16	10.807	Chrysanthenone	0.24	40	23.484	Neophytadiene	0.21
17	11.252	Camphor	0.55	41	32.936	1, 2-benzenedicarboxylic acid, bis (2-ethylhexyl)	3.87
18	11.978	1-4-terpineol	1.24				
19	12.269	Alpha. terpineol	0.20				
20	13.354	Hexanoic acid	0.18				

3.5. Fatty Acid Compositions in *Pistacia lentiscus* Fruit Oil

In the present work, the fatty acid (FA) composition (%) of *Pistacia lentiscus* fruit oil was studied within the official limits established for olive oil referred to in the International Olive Oil Council: Trade standard applied to olive oils and olive pomace oils [44]. The acid composition of *P. lentiscus* fruit oil is summarized in Table 1. Monounsaturated FA (MUFA) represented the major class of FA in the oil, accounting for 48.68%, and was followed by saturated FA (SFA) and polyunsaturated FA (PUFA), which accounted for 26.56 and 24.74 of the whole FA, respectively (Table 3).

The limit acids present in *Pistacia lentiscus* fruit oil were: Myristic acid (C14:0) 0.05%, Palmitic acid (C16:0) 24.93%, Palmitoleic acid (C16:1) 2.76%, Margaric acid (C17:0) 0.04%, Margaroleic acid (C17:1) 0.11%, Stearic acid (C18:0) 1.37%, Oleic acid (C18:1) 45.66%, Linoleic acid (C18:2) 24.21%, Arachidic acid (C20:0) 0.17%, and Gondoic acid (C20:1) 0.15%.

3.6. Sterols Composition

The results presented in Table 3 revealed that β-Sitosterol was the major sterol (78.1%) identified in the *P lentiscus* fruit oil, followed by Δ-5-avenasterol (8.02%) and Campesterol (5.26%).

Table 3. *Sterol composition of P. lentiscus fruit oil.*

Sterol (%)	Pistacia fruit oil
Cholesterol	0.21
Brassicasterol	0.00
24-methylene-cholesterol	0.03
Campesterol	5.26
Campestanol	0.18
Stigmasterol	2.34
Δ -7- Campesterol	1.57
Δ-5-23- Stigmastadienol	0.00
Clerosterol	1.13
β -sitosterol	78.33
Sitostanol	1.23
Δ-5-Avenasterol	8.02
Δ-5-24- Stigmastadienol	1.02
Δ -7- Stigmastenol	1.30
Δ -7- Avenasterol	0.59
Total Sterols (ppm)	1374
β –sitosterol (ppm)	1071

3.7. General Characteristics of Animals

Table 4 illustrates the mean (± SEM) weights of the rats. The results revealed no significant differences in this parameter between the studied groups. No significant ($p<0.05$) differences were also observed between the weights of all rat groups before and after treatment (Table 4).

Table 4. *Effect of «CICAFLORA®» and P. lentiscus fruit oil on the body weight of rats before and after cutaneous wound excision.*

Day	Group I	Group II	Group III
Before treatment	182.50 ± 3.271	184.50 ± 3.271	184.33 ± 3.204
After treatment	193.00 ± 3.265	194.33 ± 3.559	193.00 ± 3.847

3.8. Wound Healing Evaluation

3.8.1. Chromatic Evaluation

Wounds photos of representative rat of the same group were illustrated in Figure 1. We choose the days 1/3/7/10/12/14 and 21 which correspond respectively to the day of wound induction, inflammatory phase, granulation tissue formation and re-epithelialisation.

The chromatic study of the wounds of the 3 groups showed a similar coloration: we observed a bright red coloration on the day of wound induction, a dark red coloration on the second and third day which gives evidence of the formation of blood clot with cellular debris.

From the fourth day, among the control group we noted a more important thick inflammatory pad with whitish punctuation. The coloration is homogeneous brown at parts treated.

On the 6th and the 8th days, the control group presents a white to grayish color, whereas for the treated groups (reference and experimental) the coloration was brown.

Toward the 9th day, the cloth of granulation began to fall to let appear a red coloration for the control group and a pink coloration for the wounds treated by CICAFLORA® and *P. lentiscus oil*. For the remainder of days of up to 13th day the coloration was pink blade.

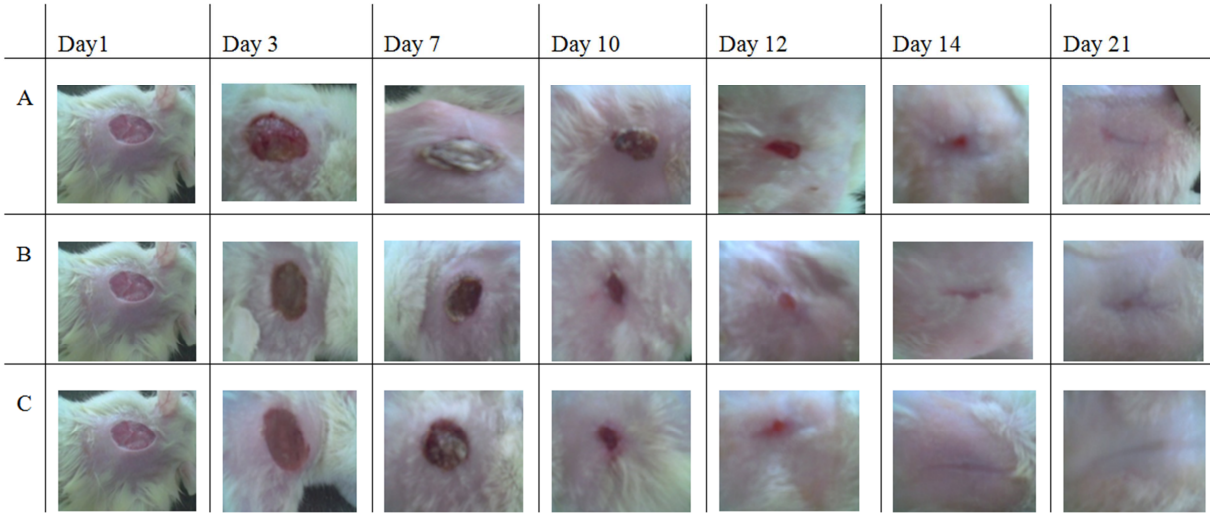

Figure 1. *Visual observation of wound healing experiment on days 1, 3, 7, 10, 14 and 21. (A) Untreated group (control), (B) group treated with «CICAFLORA», and (C) group treated with P. lentiscus.*

3.8.2. Wound Surface Evaluation

The wound surface areas observed for the different groups of rats are presented in Figure 2. The results showed that the wound contracting abilities displayed by the CICAFLORA® and *P. lentiscus* oil treated groups were more significant than that of the control group. The findings also indicated that a 50% wound contraction was achieved on the 5th, 6th and 8th days for the reference, *P. lentiscus* oil, and untreated groups, respectively.

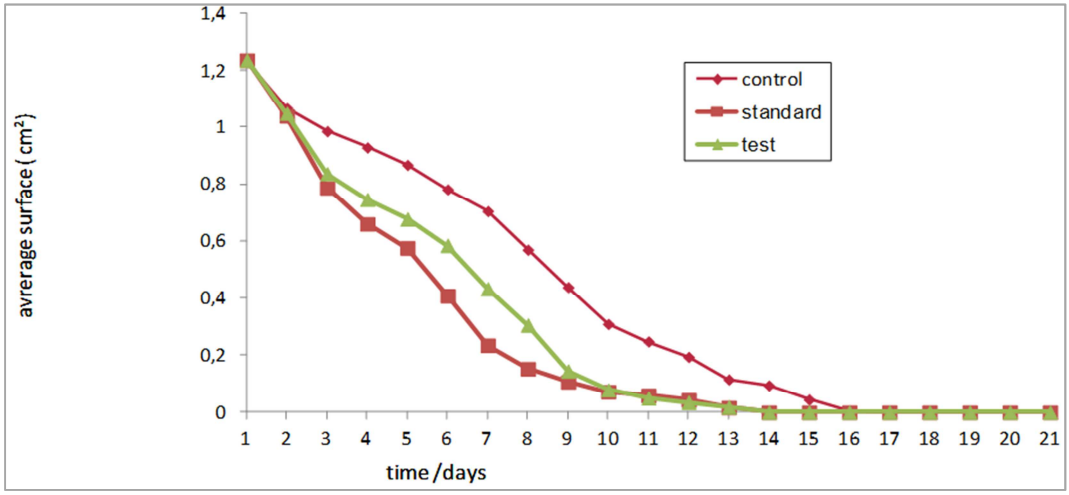

Figure 2. *Effects of CICAFLORA® and P. lentiscus oil on wound's evolution.*

3.9. Histological Evaluation

The results from histological analysis are shown in Figure 3. The results revealed an enhancement in the wound healing process the 3rd, 7th, 12th, 14th and 21st days in the treated and untreated groups. Day 3 represented the coagulation and inflammatory phase. This involved the migration of neutrophils at the borders of the incision towards the fibrin clot. The inflammatory cell recruitment (granulocytes, macrophages, lymphocytes) on the site of the lesion was noted to begin very early (Figure 3A). An incomplete full-thickness epithelialization of epidermis with debridement crust overlying the area of the wound was observed on day 7 in all groups. However, the regeneration of dermal architecture (revealing large amounts of deposited extra-cellular matrix elements and narrow capillary-sized blood vessels) was more significant in the test than in the reference and control groups of rats (Figure 3B). On days 14, a progress was observed in the maturation of granulation tissue in the dermis and on-going epithelialization of the treated and untreated wounds (Figure 3C).

The progressive changes observed in the epidermal and dermal architecture included keratinization and full-thickness epidermal regeneration. They also involved a significant improvement in the maturation and organization of epidermal layers, with no debridement crust covering the epidermal surface. The changes also included an increase in the deposition and organization of extra cellular matrix elements and the presence of highly vascularized areas in the

granulation tissue that were associated with several empty vacuoles, particularly at the wound site of the rats treated with CICAFLORA® or *P. lentiscus* fruit oil.

The microscopic examination of the wounds treated by the *P. lentiscus* fruit oil and colored by the Eosin Hematoxylin revealed the presence of a relatively normal and organized epithelium. In this section, the results showed the proliferation of the epithelial tissue covering the wound area. Further results revealed that the fibrous connective tissues in the dermis started to multiply (Figure 3C). In fact, the treatment with *P. lentiscus* fruit oil tended to yield into more contracted scars than the reference drug, which showed thin and insufficiently structured epithelial patterns.

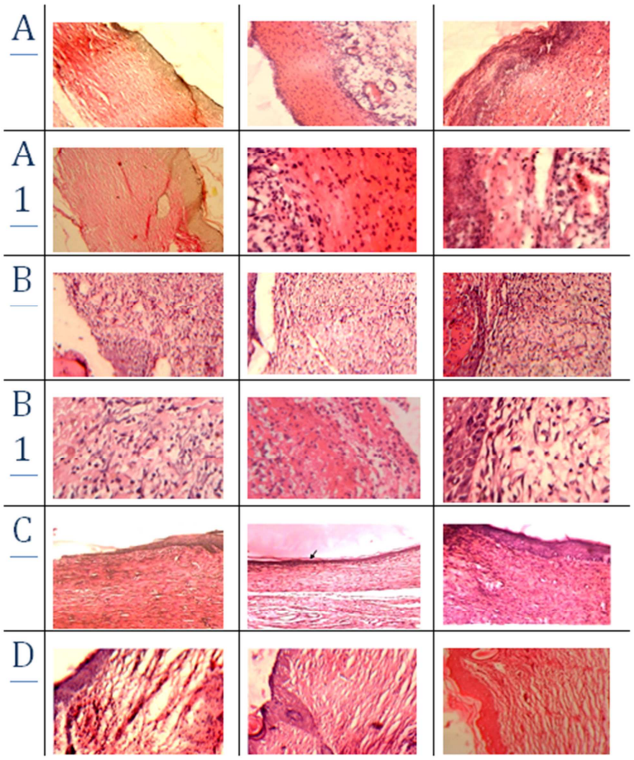

Figure 3. *Hematoxylin and eosin staining histological sections of cutaneous wounds site obtained from the controls, CICAFLORA® and Pistacia lentiscus treated, revealing epidermal and dermal architecture of wounds on days 3, 7, 12 and 21. The original magnification was ×100 for figures in A, B, C and D and the original magnification was ×250 for both A1 and B1. (A and A1) 3-day -old wound tissue of controls, treated with CICAFLORA® and treated with Pistacia lentiscus. (B and B1) 7-day-old wound tissue of controls, treated with CICAFLORA® and treated with Pistacia lentiscus. (C) 12-day-old wound tissue of controls, treated with «CICAFLORA®»and treated with Pistacia lentiscus. (D) 21-day-old wound tissue controls, treated with CICAFLORA® and treated with Pistacia lentiscus.*

The colorization by the Van Gieson technique showed horizontal and well-organized collagen fibers, with a tinctorial difference in the treated groups. The biopsies of the control group disclosed a sample tissue consisting of a granulation cloth that included several vessels and polymorphous inflammatory cells, testifying an obstinate chronicle inflammation (Figure 4). The scars of the *P. lentiscus* oil treated group of rats showed, on the other hand, very advanced patterns, with no hypertrophy, fewer inflammatory cells, and stronger collagen density than those of the reference group. Van Gieson colorization also revealed that the biopsies of the *P. lentiscus* oil treated wounds displayed marked angiogenesis, with more open vascular structures than the ones of reference group. On the 21st day, the histological studies of the tissues obtained from the test group showed a significant increase in collagen deposition and more fibroblastic cells. The results from the histological analysis of the granulation tissue of the control group of animals (Figure 4A) also showed angiogenesis, but with fewer collagen fibers and cellular inflammatory infiltration.

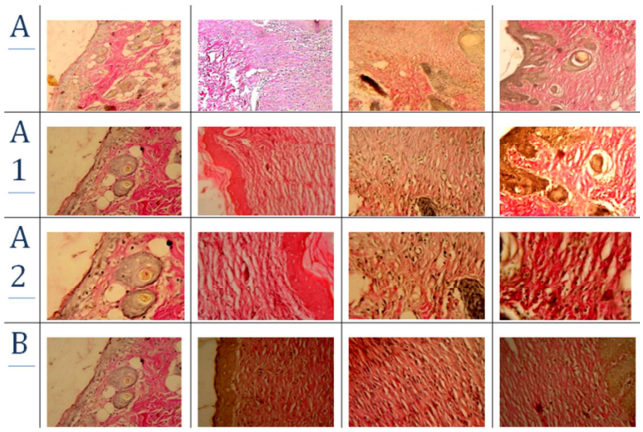

Figure 4. *Van Gieson staining histological sections of cutaneous wounds site obtained (from left to right) from the normal skin, controls, CICAFLORA® and Pistacia lentiscus treated, revealing epidermal and dermal architecture of wounds on 12 and 21 days. The original magnification was ×100 for figures in A and B. The original magnification was ×250 for figures in A1 and the original magnification was 400×for figures in A2. (A1, A2 and A3) 12-day -old wound tissue of normal skin, controls, treated with CICAFLORA® and treated with Pistacia lentiscus. (B) 21-day-old wound tissue of normal skin, controls, treated with «CICAFLORA®» and treated with Pistacia lentiscus.*

4. Discussion

Wound care is continuously evolving with the advances in medicine. Various plants and their extracts have been used for wound treatment and management. The literature presents several phytoconstituents, polyherbal formulations, and nutraceuticals from plant origins for application in wound care. Some of the medicinal plants traditionally used in folk medicine, including Pistacia lentiscus L., need to be investigated to understand their modes and mechanisms of action. The present study was undertaken to explore the potential relationships between the chemical composition and wound healing effects of *Pistacia lentiscus* L.

Pistacia lentiscus fruit oil was extracted from fresh and healthy fruits by traditional processes and without solvents, heat treatment or preliminary refining. The stability of the chemical quality of this oil was evaluated by monitoring some quality markers, including acid, peroxide, and ultraviolet absorption (K232 and K270) values. The results indicated that the title oil had a good quality and stability.

Oil acidity is the result of the degree of triacylglycerol distribution due to a chemical reaction, called hydrolysis or

lipolyse, in which free fatty acids are formed. The indexes of quality give a general idea about the chemical state of the major compounds and, subsequently, the degree of their biological actions. The acidity measured in the oil sample under investigation was in the order of 3.12%. This value is close to the one previously reported by BOUKELOUA Ahmed (2009) for Algerian *Pistacia lentiscus* fruit oil, which was in the order of 2,955±0.03% [45]. This acidic PH promotes the inhibition of bacterial growth, which accelerates the wound healing process especially during the inflammatory phase. In fact, the capacity of microorganisms to grow at a low pH depends on their ability to prevent protons from entering the cytoplasm. Most bacterial species have optimum moisture content close to neutrality (pH 6.5 to 7.5). In general, most bacteria require a pH ranging between 5.5 and 8.0 for normal growth. Beyond this range, there is a slowdown in their development, reaching a complete stop of growth at a pH below 4.5 or above 9.0. Acidic pH contributes to the ideal environment for fibroblastic activity, migration, proliferation, and organization of collagen, which results in the stimulation of wound healing [46].

Peroxydation is the first stage of fat autoxydation, which is slow but inevitable. Precautions taken during oil manufacturing and storage allows for the deferral of autoxydation and the reduction of its effects. According to common regulations, the peroxide index of extra virgin olive oil must be lower than or equal to 20 meq O_2 / kg. The results revealed that the *P. lentisus* fruit oil had a peroxide value of 7.21 meq O_2/Kg. This low peroxide values indicated that the oil was quickly extracted after harvest and maintained good quality after storage in good conditions, thus suggesting that it would not oxidize prematurely and preserve good quality over time.

The specific UV absorbance in the wavelength 232 nm indicated the primary oxidation of the oil. According to common standards, the UV absorbance in 232 nm of extra virgin olive oil must be lower than 2.5 [47]. The K232 value recorded for *P. lentiscus* fruit oil was 6.856. This value can be attributed to insufficiently precautious steps followed either during the harvest or storage procedures.

The constant K270 value represents the percentage of oxidation resistance reduction as tested by the chromatography of the gas phase in some oils with a light wavelength of 270 nm. The results revealed that the *P. lentiscus* fruit oil had a constant K270 value of 0.458. In fact, the value of this constant depends on the freshness of the extracted oil. Old oils or mixtures with used (worn out) oil often present high K270 values. The value of this constant immediately after bottling is generally low, increasing with the increase of oil age. Exposure to sun at high temperatures accelerates the ageing process.

According to standard regulations, *Pistacia lentiscus* fruit oil exhibited a good quality, but because of its fragility, it needed protection from oxidation and hydrolytic changes during storage.

The present study is the first to report on the biochemical composition of *P. lentiscus* fruit oil using GC-MS analysis. The results revealed that the oil contained a complex mixture of several components, with the predominance of hydrocarbons, monoterpens and sesquiterpens. The major components identified were α-Pinene (13.35%), β-Phellendrene (10.45%), α–Phellendrene (10.12%) Sabinene (7.01%), Germacrene-D (6.68%), 2-β-Pinene (5.57%), β-Caryophyllene (4.58%), and Myrcene (4.33%). These results are in agreement with previous reports on essential oils in the literature [48, 49], with slight quantitative and qualitative variations.

The compositional analysis of P. lentiscus essential oil revealed that hydrocarbonated monoterpenes amounted for 63.9% of the total oil and that the sesquiterpenic fraction amounted to 31.3%. The major compounds were monoterpene α-Pinene (20.6%), limonene (15.3%) and β-Pinene (9.6%), oxygenated monoterpene terpinen-4-ol (8.2%), and sesquiterpene germacrene D (8.4%) [50]. The findings revealed that the P. lentiscus essential oil exhibited significant antimicrobial activity, which is in accordance with the results reported in the work of Douissa et al. [45] on P. lentiscus essential oils grown in Tunisia.

Overall, the results showed a correlation between the antifungal activity and percentage of some major components, including α-Pinene, Limonene and α-Terpinene, which are known by their antimicrobial activity. In fact, the antimicrobial activity of monoterpenes suggested that they diffused into pathogens and damage cell membrane structures [51]. Moreover, α-Pinene, which was recorded in significant amounts in the oil under investigation, was previously reported to be a key contributor to the antimicrobial activity of oil from Pistacia lentiscus. The antimicrobial activity of essential oil was previously attributed to phytochemical components, including monoterpens [52].

In addition, The GC-MS composition of the studied oil could explain the mechanisms of the skin wound healing process. So, their considerable amount monoterpennes and sequiterpenes are important in the inflammatory cascade. These substances act as inflammatory mediators and accelerate the inflammatory process. For example humulene, caryophyllene, β-pinene and α-pinene can exhibit marked anti inflammatory effects in the cellular phase. These monoterpenes act as cyclooxygenase [53].

Knowing that sesquiterpenes have outstanding anti-inflammatory activities [54, 55], the anti-inflammatory activity of PLFO could be partly explained by the presence of some 408 sesquiterpenes especially, cadinene, amorphene, caryophyllene and muurolene.

Finally, the results reported in the work of Ben khedir et al. [56] concluded that *Pistacia lentiscus* fruit oil most likely decreased the paw edema by acting at both phases of the carrageenan-induced inflammation. The *Pistacia lentiscus* fruit oil exerts its anti-inflammatory effect by reducing the production of inflammatory mediators involved in the conduct of stages of the acute inflammatory response induced by the λ-carrageenan, and by inhibiting the leukocyte recruitment to the inflammatory site by exerting anti-

chimioattractants on these effects and blocking the synthesis of prostaglandins by inhibition of cyclooxygenase. [56].

The fatty acid composition of *P. lentiscus* fruit oil from Tunisia revealed that the three dominant FA were Palmitic (24.93%), oleic (45.66%), and Linoleic (24.21%) acids. The Tunisian oil contained a significant amount of unsaturated fatty acids 73.44%. Previous Algerian studies have previously reported on the presence of the three fatty acids at closely similar proportions, namely 16.3%, 55.3% and 17.6%, respectively, together with 78.8% of unsaturated fatty acids [57]. Earlier studies have demonstrated the efficiency of fatty acid agents in accelerating wound healing [58]. The results reported by Cardoso et al. [59] demonstrated the key role and potential therapeutic implications of fatty acids on skin wound healing. The pro-inflammatory action of oleic and linoleic acids have also been reported for their abilities to accelerate the wound healing process [60]. Containing a high concentration of oleic acid, *Pistacia lentiscus* fruit oil can, therefore, be considered a promising alternative therapeutic agent for wound healing. Fatty acids and triglycerides have been reported to have the ability to reduce trans-epidermal water loss and, hence, increase skin hydration [61].

Fatty acids have been reported to have the ability to reduce transepidermal water loss and increase skin hydration and supportive environment for accelerated skin wound healing [40].

Linoleic and Oleic acids are also known for their anti-inflammatory properties. Cell membrane repair and cellular respiration need the presence of Linoleic and α- Linoleic acids [62]. Furthermore, fatty acids has long been known to have attractive inhibitory effects on various micro- organisms [63]. Various studies have reported that Linoleic and Oleic acids, which are important constituents of *P. lentiscus* fruit oil, have promising antibacterial properties particularly due to their unsaturated long-chains [64].

Palmitic acid has been commonly employed in the preparation of some drug ingredients (Survanta, Multivitamin (UPSA), Renutryl, Fungisone, and Penticort) [65, 66] and to decrease the hydrophobicity of virginiamycin, a drug used Against Mycobacterium avium [67]. Linoleic acid is a vital unsaturated fatty acid (Omega 6) that is involved in lipid metabolism and maintaining integument integrity [68]. It has been widely used in combination with (vitamin E) against eczema and dermatitis [69, 70]. It has also been used as an excipient in some drugs, such as demangeaison, aphilan, and fongamil.

Further results revealed that the sterol components of the unsaponifiable fraction was dominated by β-Sitosterol (78.33%). The total sterol content (137.4 mg / 100 g oil) was compared to that of olive oil (119-268 mg / 100g). The results indicated that the total and individual sterol content present in the *P lentiscus* fruit oil were higher than that of olive oil, being characterized by the predominance of β-Sitosterol (58 -79.7%). The comparison between the total sterol content of the *P lentiscus* fruit oil with the standard reference and olive oil revealed that the *P lentiscus* fruit oil met all the quality criteria, which makes it a promising, efficient and cost-effective candidate for use in refining applications and processes.

The relationship between the high level of plant sterols and the healing properties and effects of plants has previously been demonstrated in the literature [71]. Several biological and pharmacological activities have been associated with these phytochemicals. Various studies have also attributes the oxidative, anti-inflammatory, and antimutagenic activities of plant extracts to their richness in phytosterol contents [72-74]. Other studies have shown the significant role of β-Sitosterol in the treatment of prostatic hyperplasia [75-76]. The latter was also reported to have attractive antioxidant properties, decreasing macrophage production of superoxide anions (O_2) and hydrogen peroxide (H_2O_2) [73].

Mezni et al. [34] reported that *P. lentiscus* fruit oils collected from different growing areas in Tunisia had a total tocopherol content ranging from 62.43 to 118.16 mg/kg oil. The α.-Tocopherol was the most abundant tocopherol fraction [34] compared to that of other oils, including sunflower [77, 78] and olive oils [79]. Its content varied from 44.76mg/kg oil to 96.77mg/kg oil. δ-Tocopherol was found in lower amounts, ranging between 14.56 and 22.12mg/kg oil [34].

Vitamin E is a major antioxidant in cell membranes and plasma lipoproteins. It inhibits the lipid peroxidation phenomenon by scavenging free radicals generated during oxidative stress [80]. The combination of sterols and vitamin E present in *P. lentiscus* fruit oil leads to the synergistic interaction of various components for antioxidant defense. This synergism significantly increases the antioxidative potential of the skin.

Mezni et al. investigated the total carotenoid content in *P. lentiscus* fruit oil harvested from eight different sites located in the North and centre of Tunisia and reported on values ranging between 5.8 and 10.57mg/kg oil [34]. The high carotenoid content of *P. lentiscus* fruit oil suggested that it is an important natural source of carotenoids compared to other suitable edible oils such as olive oil [81, 82]. β-Carotene and Lutein were also reported to represent the dominant carotenoids in the oil, ranging from 2.58 to 4.9 mg/kg and from 1.74 to 4.62 mg/kg oil, respectively [34]. Carotenoids are the most important source of vitamin A. The deficiency of vitamin A in human nutrients causes growth disturbances and reduces disease tolerance, in addition to disrupting the mucus membrane of gastrointestinal tract. The oily substances of carotenoids have been demonstrated to offer effective remedies against burns, frostbites, ulcers, skin cancers, and several other gynaecological illnesses [83, 85].

Overall, the results described above suggest that all the chemical compounds present in the *Pistacia lentiscus* oil are able to play promising individual and synergistic effects to accelerate the wound healing effect. The promoted wound contraction and shortened epithelialization period, in the *P. lentiscus* oil treated wounds, could presumably be attributed to the correlation between three mechanisms, namely antimicrobial, antioxidant and anti-inflammatory.

Wound evaluation involves several dimensions, including

the clinical, physical, physiological, biochemical, histological and genetic factors. The present study adopted histological parameters as well as morphological parameters, including wound contraction percentage and epithelialization period. The results revealed that, in the coagulation and inflammation phase, the inflammatory pad made of edema on the edges of the wound were more important in the control than in the treated groups of rats. This effect could presumably be attributed to the anti-inflammatory and antioxidant action of the CICAFLORA® healer [86] and *P. lentiscus* fruit oil [87]. The antimicrobial effect of those substances was previously reported in literature [88-91] and could presumably have contributed to the rapid contraction of scab as opposed to those in the rats of the control group which seemed to be oozing.

The wound healing evolution was marked by a reduction in the size of scabs. In fact, the important contraction of the wounds of the treated groups of rats could be due to advanced re -epithelialization. The epithelial cells of the wound borders proliferated and crossed towards the centre, leading to a complete closure of the wound towards the 14th day of treatment [92] (Figure 1).

The dimensions of the wounds of the rats treated with « CICAFLORA®» or *P. lentiscus* fruit oil were significantly smaller than those of the controls. The results of the present study revealed that the control group of rats presented an incomplete wounds contraction on the day of sacrifice, which is in accordance with several reports in the literature [93, 94]. Consequently, *P. lentiscus* oil seems to have a promising healing effect, which is rather, more important than that of «CICAFLORA®». However, and out of the 2 products used in the present study, «CICAFLORA®» seemed to have a faster dynamic healing activity between the 6th and 8th day. This dynamic healing effect could be attributed to a better retention of the active principle of «CICAFLORA®» due to its appropriate excipient, as opposed to a limited retention of the virgin oil of *P. lentiscus*.

Taken together, the results indicate that «CICAFLORA®» and *P. lentiscus* fruit oil exhibited comparable healing potential. In fact, the total closure of the treated wounds was achieved after 14 days. According to the literature, natural wound contraction normally takes place by the 21st day [95]. The results, therefore, highlight the promising abilities of *P. lentiscus* fruit oil and «CICAFLORA®» to accelerate the wound healing process, which could contribute to a faster recovery with a gain of 30% of the anticipated period (Figure 2). The literature also indicates that while the treatment with a plant extract from *Inula* viscose induced a total healing period of beyond 16 days [96], the treatment with the Madecassol healer, an extract from *Centella asiastica*, involved only 12 days [94]. This same healing period (12 d days) was also observed with two medicinal plant oils: *Cucurbita pepo. L* (Cucurbitaceae) and *Linum usitatissimum*

The results from histological analysis provided ample evidence for the suitability of *P. lentiscus* fruit oil in promoting wound healing as compared to the CICAFLORA® reference standard. The biopsies of the reference group showed fibro-conjunctive tissues with higher rates of collagen density than the *P. lentiscus* oil treated ones. This suggested that the CICAFLORA® healer stimulated fibroblastic cells in a more intensive way.

Furthermore, the invasion of the healing tissue of the reference biopsies by fibroblasts promoted the increase of collagen fibers, which limited the space reserved for neo vessels, thus preventing re-epithelization. *Mimosa Tenuiflora*, the principle active ingredients of « CICAFLORA®» seemed to stimulate the multiplication of fibroblasts and act in a minor way on keratinocyte [42]. The biopsies of the wounds treated with *P. lentiscus* fruit oil were, however, noted to display weaker levels of collagen density associated with better rates of blood irrigation and optimal epithelialization. It seems that the production of significant collagen fibers could disturb the tissue neovascularization and hinder the adequate reconstruction of epidermis epithelium. As a consequence, the incomplete epithelialisation noted in the control wounds of the present study and those reported in the work of Prasad and Dorle (2006) could be attributed to weak angiogenesis.

Although CICAFLORA® and *P. lentiscus* fruit oil displayed comparable healing abilities, in some cases the tissue healing process was accompanied by a deep density of collagen fiber, which can expose the individual to hypertrophying cheloides scars. Consequently, the neoformation tissue profile induced by *P. lentiscus* fruit oil seemed better than that of CICAFLORA® or any other fibroblast stimulating product. In order for these results to be confirmed, further studies are needed to envisage a histological quantitative evaluation and biochemical assays measuring the collagen in the normal skin and scarring zones of different groups. Taken together, however, the results provide strong support for the promising healing effects of *P. lentiscus* oil as compared to «CICAFLORA®» or other healers described in the literature.

5. Conclusion

The present study was undertaken to characterize an oil extract from *Pistacia L.* fruit collected from El Kef, North West of Tunisia and explore the correlations between its chemical composition and wound healing activities. The results revealed that *P lentiscus* fruit oil contained a high proportion of unsaturated fatty acids (Oleic, Palmitic and Linoleic acid), α-Tocopherol and β-Sitosterol. The title oil was noted to exhibit better wound healing activity than CICAFLORA®. This result could be attributed to the synergistic effect of all the constituent compounds present in *P. lentiscus* oil, particularly fatty acids, tocopherols, and sterols. These phytochemical agents exhibited attractive wound healing functions and positive activities at the different stages in the wound healing process via various mechanisms, including antimicrobial, anti-inflammatory, antioxidant, collagen synthesis stimulation, cell proliferative, and angiogenic effects. Overall, the results indicated that *P. lentiscus* fruit oil has a number of attractive wound healing

properties that make it a potential promising candidate for future application in the production of novel natural drugs for wound management and treatment. Accordingly, further studies, some of which are currently underway in our laboratory, are needed to explore the favorable conditions required for its production and for the achievement of optimal wound-healing effects.

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Competing Interests

"The authors declare that they have no competing interests".

Finding

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Authors' Contributions

Conceived and designed the experiments: SBk, SB, DM, ZS, and TR. Performed the experiments: SBK, SB, DM and ZG. Analyzed the data: SBK, SB, and ZS. Contributed reagents/materials/analysis tools: SBk, MM, SB, DM, ZS and TR. Wrote the paper: SBK and SB. All authors read and approved the final manuscript.

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