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Original Article

The Effects of Olive Leaf Extract Ointment on Third-Degree Burn in Rat

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ABSTRACT

Due to the prevalence of burns and the necessity of effective treatment with low and optimal complications, in the present study, the efficacy of olive leaf extract ointment was evaluated as a therapeutic substance for burn wounds. Moreover, the healing effects of olive leaf extract ointment were compared to Silver Sulfadiazine. 36 rats were used in the present study. To create a burn wound, a rectangular piece of copper (2 × 1 cm) was put in 94° C water for 20 minutes and then placed on the rats' skin for 30 seconds. Then, the rats were randomly divided into three groups; Sham group: Rats did not receive any treatment and just the wound was washed with distilled water. Control group: In this group rats were treated with silver sulfadiazine. OLE group: 10% olive leave extract ointment was employed to treat the wounds. Wounds were macroscopically examined during days 4, 7, 14, and 21. Histopathological assessments were performed on days 4, 7, 14, and 21 in various studied groups. Results revealed that wound contraction was higher in the OLE group compared to the sham and control groups and histopathological examinations indicated that olive leave extract improved wound healing in comparison to the sham group.

Introduction

Skin is the broadest and one of the most important organs in the body which plays an important role in different aspects including avoiding evaporating electrolytes and macromolecules, regulating the body temperature, protecting the body from ultraviolet (UV), releasing some materials like fat, sweat, or milk, etc.[1-3] It is made of three layers including epidermis, derma, and hypoderm. Once it is damaged, by burning or laceration, this protecting layer is disrupted and the body is predisposed to be infected by various microorganisms.[4]

Burn wounds are still considered as one of the most hazardous conditions in medicine in all countries, resulting in physical and psychological scars and may cause chronic problems.[5] Multiple factors affect morbidity and mortality of burnings such as the depth and size of the burn and also type of it.[6] There are three different kinds of burning: 1) burning by hot metals/objects, 2) burning by electricity, and 3) burning by chemical agents.[7] However, burns triggered

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by hot metals/objects are accompanied by the most mortality. Based on the research conducted by Hasan *et al*., the reason for death in 50% of cases is secondary infections.[8] Therefore, the high probability for secondary infections should be considered and appropriate antibiotic therapy should be done.

Plenty of substances, both chemical and herbal, are used to help to heal burns. They are administered systematically or topically. One of the most prevalent topical drugs is Silver Sulfadiazine ointment. It possesses several beneficial effects including, a broad antibacterial effect, calming effect, and low cost.[9] In addition to available commercial ointments, researchers are investigating high-value substances and they have worked on many plants or chemical stuff like *Plantago major,*[10] curcumin,[11] *Madeira vine,*[12] *Aloe vera*[13], etc.

Olive leaf extract (OLE) is one of the prominent sources of therapeutic active compounds. It is demonstrated that crude OLE is composed of oleuropeosides, flavones, flavanols, flavan-3-ols, and substituted phenols.[14] This polyphenolic structure is one of the most promising compounds which has great activity against free radicals. Oleuropein, being the most abundant phenolic compound in the extract also has a protective effect against pathogens like *E. coli* and *P. aeruginosa* which is one of the most important pathogens in nosocomial infections.[15,16] Besides the antibacterial and antioxidant effects of the olive leaf extract, it has anti-inflammatory agents that may be beneficial in the first phase of wound healing.[17]

This study aimed to evaluate the protective effects of olive leaf extract on third-degree burn wounds in Wistar rats.

Materials and Methods

Ointment Preparation

The fresh leaves of *Olea europaea* (variety: Sevillano) were collected from the Faculty of Agriculture, Lorestan University, Iran. They were washed, shade dried at room temperature, and milled. The leaves powder was extracted with 70% ethanol. The collective ethanol extract was filtered and placed under reduced pressure in a rotary evaporator to produce dried residues. The high-performance liquid chromatography (HPLC) technique was used to quantification of phenolic compounds in the olive leaf extract. In this study, to make a 10% ointment using Eucerin, 10 g of pure olive leaf extract was dissolved in 100 g of Eucerin, which was formulated as a 10% by weight ointment. Silver sulfadiazine ointment was utilized to compare the healing effects of alcoholic extract of olive leaf with a commercial sample. The main phenolic compositions of the olive leaf extract were oleuropein (356 mg/g), tyrosol (3.73), hydroxytyrosol (4.89), and caffeic acid (49.41) in dry extract. The extract was kept at −20° C before use.[18,19]

Animal Experiment

36 mature male Wistar rats weighing 200-250 g were used in this study. This study was carried out after obtaining approval of the Animal Ethics Committee form Lorestan University (Ethical number: LU. ECRA.2018.6). Before experiments, the rats were accustomed to the environment for one week in a standard condition with a temperature of 23 ± 2° C, humidity 60%, and 12 light/dark cycles.

Afterward, they were divided into 3 groups randomly (n=12) including: Sham group (S): In this group, the burn was induced and the wound region was washed using distilled water; Positive control group (C): Same as the sham group, the burn was induced and rats received silver sulfadiazine; and Treatment group (OLE): Rats in this group received 10% olive leaf extract ointment.

Experimental Protocol

The rats were anesthetized using ketamine 10% (Alfasan Inc., Utrecht, Holland) and xylazine 2% (Alfasan Inc., Utrecht, Holland) with a dose of 80 mg/kg and 10 mg/kg IP, respectively. After shaving the hair of the back of rats, the skin was disinfected with 10% povidone-iodine. Subsequently, a hot flat metal object with 1 × 2 cm size which was in a 94° C for 20 min, was placed on the bare skin for 30 seconds.[20] For Confirmation of the third-degree burn wound, one sample was sent to the laboratory for pathological tests.

Afterward, all wounds were rinsed with sterile normal saline every 12 h for 21 days. In the treatment group, after washing the wounds, it was completely covered with olive leaf extract ointment and in the control group, silver sulfadiazine cream was used instead, but for the sham group, nothing was added.

Macroscopic Evaluation

Macroscopic assessment of the burn region was performed on days 4, 7, 14, and 21 post-operations, and photographs were taken using a digital camera with a ruler on one side as a scale. All photos were analyzed by

AutoCAD software and their area was measured, then, according to the following formula, the wound healing contraction percentage was calculated: Wound healing percentage = difference between the area on day x to surgery day / the area on surgery day.

Microscopic Evaluation

For histopathological evaluation, three rats from each group were selected randomly on days 4, 7, 14, and 21 post-surgeries, and the rats were euthanized using an overdose of sodium pentobarbital (250 mg/kg). Then, the hairs were shaved, and using a scalpel blade N #11 a piece of skin containing both healed and injured regions were removed and they were transferred to 10% formalin containers. Subsequently, they were processed routinely for Hematoxylin and Eosin (H&E) staining and they were assessed by an optical microscope (Olympus, Germany). Ultimately, for histopathological parameters in each slide five fields (1 mm^2) were randomly selected and numbers of neutrophils, macrophages, fibroblasts, and endothelial cells were counted for each rat and the final values were expressed as mean ± SD.

Statistical Analysis

All data were tested for normality followed by Levene's static test for homogeneity of variances. Then they were analyzed using one-way ANOVA and univariate test, followed by post hoc pairwise comparison using the Tukey test. All analyses were done by SPSS version 25 for Windows (SPSS, Inc, IBM Company, Chicago, USA). $p < 0.05$ was considered statistically significant. Results are expressed as the mean ± deviation (SD).

Results

Macroscopic Evaluation

According to Table 1, the area of healed wound (mm^2) in experimental groups is shown. Briefly, the healing percentage was statistically significantly higher in C and OLE groups than sham group ($p < 0.05$). Moreover, the OLE group had a statistically higher significant healed percentage in comparison with the Sham group ($p < 0.05$) (Figure 1).

Histological Parameters

On the 4th day post-surgery, there was no significant difference between the groups in the number of neutrophil and macrophages ($p > 0.05$). On days 7, 14, and 21, the number of neutrophils in the OLE and control groups were significantly lower than that in the sham group ($p < 0.05$) (Figure 2A). On day 7, the number of macrophages was significantly higher in the control group compared to the sham group ($p < 0.05$). On day14, there was a significant difference between both treatment groups and the sham group ($p < 0.05$). On day 21, the number of macrophages in the OLE group was remarkably lower than that in the sham group ($p < 0.05$) (Figure 2B).

The number of fibroblasts was significantly higher in the control and OLE groups compared to the sham group on days 4, 7, and 14 ($p < 0.05$). No significant difference was observed between the experimental groups in the number of fibroblasts on day 21 ($p > 0.05$) (Figure 2C).

The number of endothelial cells or neovascularization (the new vascular endothelial cells which present at the site of granulation tissue) was increased in the control and OLE groups compared to the sham group on all experimental days, but the difference was not significant ($p > 0.05$) (Figure 2D).

Microscopic Evaluation

Histopathological evaluation of the wound healing during the studied days is shown in Figure 3. According to histological assessments, on the 4th day, some blood

Table 1. Percentage of burn area improvement (mean ± SD) between experimental groups on different days.

Groups	Day 4	Day 7	Day 14	Day 21
Sham	8.2 ± 1.4*#	12.2 ± 1.6*#	25.2 ± 1.7*#	55.6 ± 3.95*#
Control	12.6 ± 1.8	18.08 ± 3.38	38.64 ± 2.70*	71.18 ± 3.60*
OLE	12.6 ± 1.8	20.18 ± 5.8	45.08 ± 2.08	80.58 ± 4.08

*Shows significant difference with the OLE group and # shows significant difference with control group ($p < 0.05$).

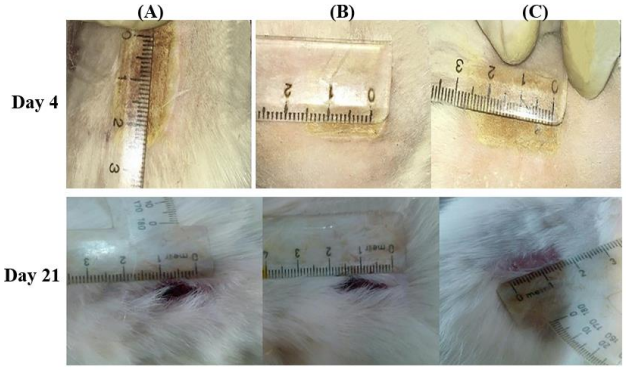

Figure 1. Wound size on days 4 and 21. (A): OLE group. (B): Control group. (C): Sham group.

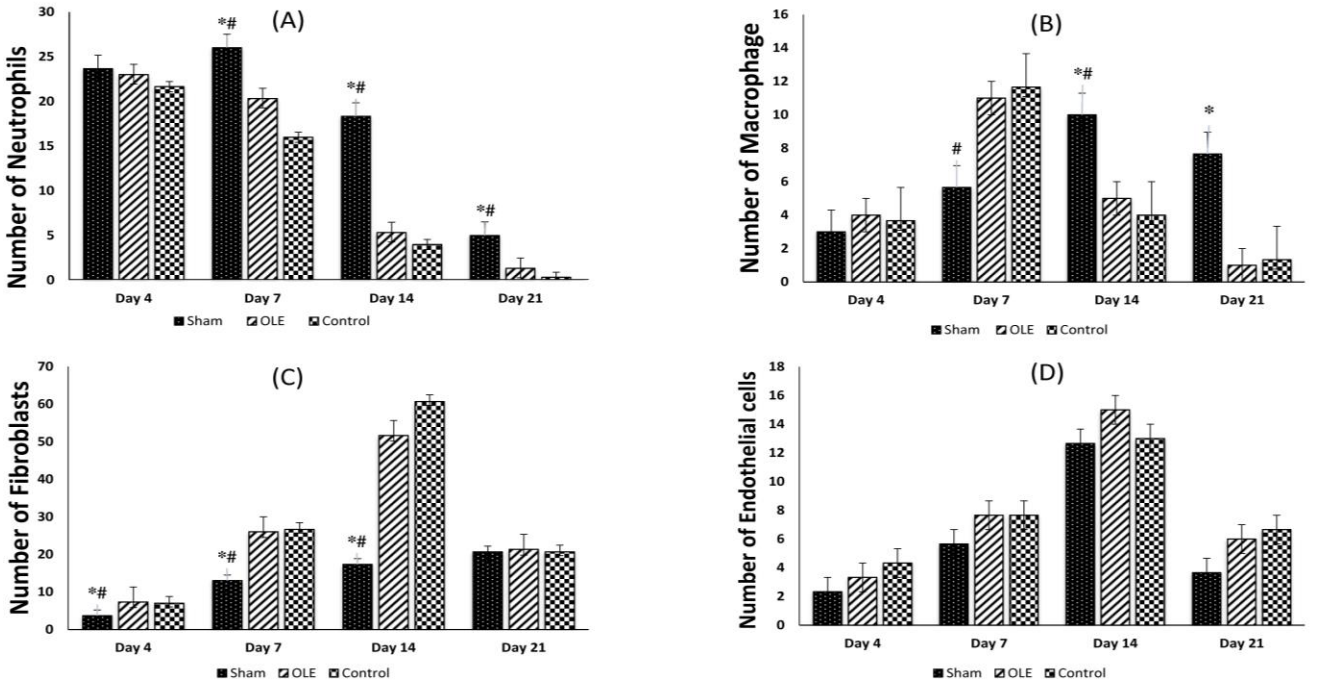

Figure 2. (A): Number of neutrophils as a healing factor. (B): Number of macrophages as a healing factor. (C): Number of fibroblasts as a healing factor. (D): Number of endothelial cells as a healing factor. * Indicates significant difference with OLE group, and # shows the significant difference with Control group.

clots were observed in the wound region, which was higher in the sham group. Besides, there was the infiltration of inflammatory cells into the dermis layer. On the 7th day, some fibrin and inflammatory cells were observed in the wound region. On the 14th day, the re-epithelialization was observed, and still, the inflammatory cell was observed in the wound site. Eventually, the epidermis formation was completed on the 21st day (Figure 3).

Discussion

The skin is the largest system of the human and animal body, which protects the body against many external factors, including bacterial invasion, chemical agents and mechanical damage, water loss, and electrolytes.[21] Burns are the fourth most common trauma worldwide. Burn wounds become infected shortly after injury. The most important complication of burns, which is responsible for 50 to 75% of hospital deaths, is infection.[22] In general, wound healing is divided into three dynamic stages: 1) inflammatory phase or lag phase 2) proliferation phase 3) remodeling phase. Any substance that can shorten the time of these phases will accelerate the healing process.[23] The purpose of research in the field of wound healing is to gain access to the ideal treatment in which injuries heal in the shortest possible time with the formation of healthy tissue. Hence, reducing the length of the healing period, increasing the speed of healing, the low complication of the drugs used, and also decreasing treatment costs are considered. It should also be noted that administration of many synthetic and chemical drugs leads to several problems such as allergies and drug resistance.[24] In the present study the healing effects of the Olive Leaf extract on third degree in rat was investigated.

Silver sulfadiazine is one of the topical creams that is commonly used to treat burns in animals due to its antibacterial properties. However, this drug triggers neutropenia and is also contraindicated in cases of burns where the skin graft technique is used. Silver sulfadiazine stains the skin and may cause photosensitivity. It may also cause pain, irritation, skin rash, and itching.[25] In the present study, the rate of wound contraction in the experimental groups was faster and better than in the sham group. The wound contraction rate is due to the contractile properties of fibroblasts and myofibroblasts in the wound.[26] In tissue factors, the number of fibroblasts in the olive extract and silver sulfadiazine ointment groups on days 4, 7, and 14 were higher than the sham group, which indicates that the olive leaf extract ointment has a

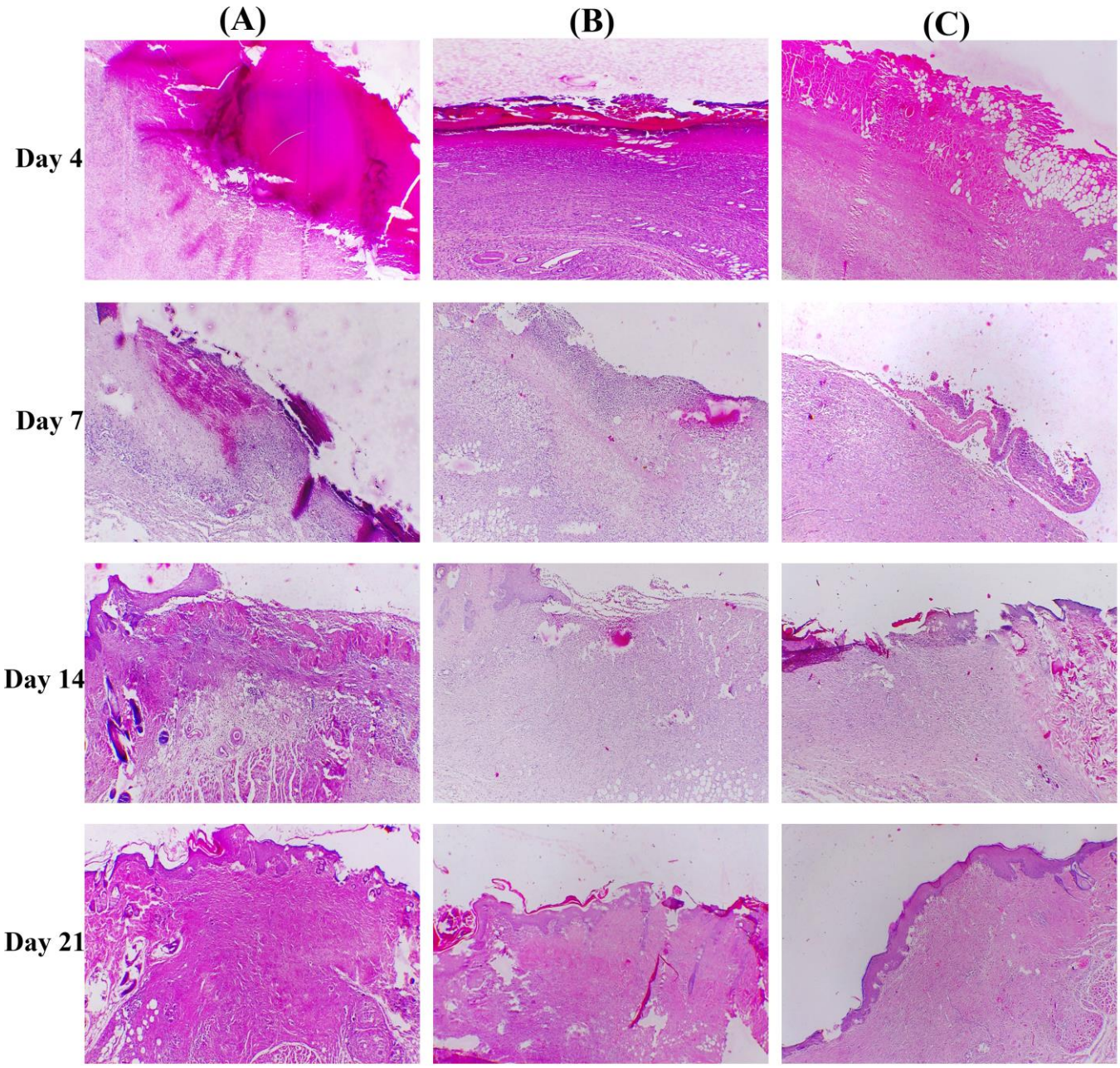

Figure 3. On day 4, Reduction of wound space and blood clots in the OLE and control groups (B and C) compared to sham group. On day 7, reduction of inflammatory cells in OLE and control groups (B and C) however granulation tissue was observed in sham group. On day 14, epithelial tissue formation (re-epithelialization) from wound edges and early formation of follicles in OLE and control groups. In contrast, the presence of granulation tissue is seen in the sham group. On day 21, the connective tissue filling the wound space showed more maturity in the OLE and control groups. The hair follicles are expanding, and the collagen fibers are more and orderly (H & E stain, ×40).

role in wound contraction. Regarding the neutrophil numbers, its level was high on the fourth day in all three groups, but on the 7th and 14th days in the groups of olive extract and silver sulfadiazine ointment, the number of neutrophils decreased more rapidly, which is due to the reduction in the inflammatory phase in these two groups and shows the efficacy of treatments in the healing process of burns. This decreasing process is due to the anti-inflammatory properties of olive leaf extract and the presence of compounds with anti-inflammatory and antioxidant properties in this extract, and this reduces the healing time by reducing the inflammatory phase.[27,28] The number of Fibroblasts was increased in both treatment groups from day 4 to 14 with the highest rate on day 14. This increment in the number of fibroblasts

occurred very slowly in the sham group.[28] In the healing process, fibroblasts play a vital role in the synthesis of collagen fibers and the reduction of wound size. The increase in the number of fibroblasts is due to the completion of the healing process and the formation of granulation tissue. The results showed that olive leaf extract with anti-inflammatory and antioxidant effects increased fibroblast cells at the burn site. Evaluation of the endothelial cells showed no significant difference between treatment groups and the sham group. However, the number of endothelial cells in the olive leaf extract treatment group was higher than that in the sham group on the studied days. This indicates the beneficial effects of the treatment group on wound healing. One of the wound healing problems is the chronic phase of inflammation which leads to prolonged wound healing. The inflammatory process is triggered by a set of cells (neutrophils and macrophages) and inflammatory factors (interleukins, TNF-α). Chronic inflammation is associated with increased inflammation of inflammatory cells and delayed angiogenesis. The study of cellular parameters and the process of angiogenesis indicate the success of the present study in the model simulation. Neutrophils and macrophages destroy the extracellular matrix, increase vascular permeability, and perpetuate the angiogenesis process during a positive feedback cycle upon entry into the wound site by releasing proteolytic enzymes, nitric oxide, and TNF-α and VEGF.[29,30]

The results of the present study revealed that the rate of wound healing with topical medications depends on the active ingredients of the drug. In the case of burn wounds, one of the main factors that slow down the healing process is oxidative stress, which causes the release of free radicals and leads to more tissue damage, and slows healing [31]. Therefore, the use of antioxidants to strengthen the immune system of cells is necessary to prevent damage.[32]

The most important ingredient in olive leaves is oleuropein, the main bitter ingredient in olives, discovered in 1908 by Vintilesco and Bourquelot. A 2006 study conducted by Andreadou *et al.* found that oleuropein had more antioxidant activity than other similar water-soluble tocopherols and was able to absorb hydroxyl radicals and superoxide anions.[33]

Numerous studies have been performed on the effects of olive oil and olive leaf extract on wound healing. In a 2018 study, the effects of olive leaf extract on diabetic wounds in rats were investigated and the results showed that this extract, in addition to its antioxidant properties, can increase the expression of hydroxyproline and transglutaminase, which are essential in the formation of collagen.[34] In another study in 2014, the effect of intradermal injection of oleuropein on skin wounds in mice was investigated and the results showed that this substance plays an important role in the production of type 1 collagen.[35] An investigation conducted by Erdogan *et al.* On the healing effects of olive leaf extract compounds on rat fibroblasts revealed that the rate of cell migration and wound closure improved with the use of oleuropein.[36] Moustafa and Atiba have assessed the effectiveness of a mixture of honey, beeswax, and olive oil in the treatment of canine deep second-degree burn and concluded that the healing effect of this compound was better than silver sulfadiazine cream and the probable reason was due to the antibacterial and antioxidant compounds of honey and olive oil.[37] Findings of the present study are in agreement with the previous studies and suggest that olive leave extract can be employed as an effective treatment in the curing the patients with burns.

Overall, the findings of this study indicate that the hydroalcoholic extract of olive leaf extract accelerates the healing process of deep burns in rats. The beneficial effects of the extract on wound healing are probably related to a significant reduction in inflammation time, increased angiogenesis, increased wound contraction, cell proliferation, and antioxidant and anti-inflammatory effects.

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Conflict of Interest

None.

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