Advances in Pharmacology and Therapeutics Journal
Shahid Sadoughi University of Medical Sciences, Yazd, Iran

Original Article

Healing effects of *Solanum nigrum* fruit extract on second-degree burn wounds and its antibacterial activity against common pathogens of burn infection

Running Title: *Solanum nigrum* fruit extract for burn wounds

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ARTICLEINFO

Received: 6/04/2021

Accepted: 8/19/2021

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Abstract

Aims: *Solanum nigrum* (*S. nigrum*) is a species of flowering plant from *the Solanaceae* family and one of the indigenous plants of Eurasia. Given the biological activities of this plant, like antimicrobial, antioxidant, and anti-inflammatory ones, this study assessed its effects on the healing process of second-degree burn wounds in rats. We also evaluated its antibacterial activity against common pathogens of burn wound infection (i.e., *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Acinetobacter baumanni*).

Methods: *S. nigrum* fruit extract was prepared by percolation and reflux methods. The extract was applied for the treatment of animal models with second-degree burn wounds. Parameters of wound healing and maturation, including collagen deposition, epithelialization, reduction of neutrophil migration, and angiogenesis, were evaluated. The antimicrobial activity of *S. nigrum* fruit extract against common pathogens of burn wound infection was assessed by the agar well diffusion method via measurement of zones of microbial growth inhibition.

Results: Histological analysis showed a significant reduction in neutrophil migration by the 20% hydroalcoholic extract vs. control group (normal saline). In addition, we found that the 20% hydroalcoholic extract was more efficient than silver sulfadiazine in augmenting collagen deposition. *S. nigrum* hydro alcoholic extract also showed an inhibitory effect on *S. aureus*.

Conclusion: *S. nigrum* 20% hydroalcoholic extract improved some of the wound healing parameters such as collagen deposition and inflammation. It also shows an inhibitory effect on *S. aureus*. So, it may have therapeutic effects on burns.

Keywords: *Acinetobacter baumanni*; Burn; *Pseudomonas aeruginosa*; *Solanum nigrum*; *Staphylococcus aureus*; Wound healing

Citation: Rashidi Ashjerdi P, Zabihi M, Ranjbar AM, Hekmatimoghaddam S, Fatahi Bafghi M. Healing effects of *Solanum nigrum* fruit extract on second-degree burn wounds and its antibacterial activity against common pathogens of burn infection. Adv Pharmacol Ther J. 2021;1(1): 1-12. https://doi.org/10.18502/aptj.v1i1.7915.

Introduction

Skin, the largest organ of the body, acts as a biological barrier against foreign hazards, retains electrolytes and water, and plays crucial roles in sensation, vitamin D biosynthesis, etc. Thermal injury can be threatening damage leading to impaired skin functions. Epidemiological studies show that burns affect many people of all ages in the world (1). According to World Health Organization (WHO) estimation, 11 million burn injuries occur annually worldwide, of which 180000 cases are at risk of death (2).

Burn is a multicausal injury given that multiple agents such as electricity, chemicals, radiation, and heat can cause burn (3). Burn severity is a crucial factor in thermal burns that determines the patients' chance of survival and is divided into four degrees. Second-degree (partial-thickness) burns may be either superficial or deep burns. In the former, the epidermis and superficial dermis are damaged. These kinds of burns appear as pink, wet and painful blisters but usually do not cause scarring. Deep partial-thickness burns involve the epidermis and entire dermis (4).

Burn wound healing is a multistage and complicated process during which blood clotting, edema, immune cells migration, the release of biochemical mediators, inflammation, neovascularization, collagen synthesis, and regeneration of epidermal layers occur. According to burn pathophysiology, treatment is efficient when it influences different parts of the wound healing process and ameliorates various tissue regeneration factors such as epithelialization and collagen synthesis. In addition, such treatment should allow completion of the stages of wound healing and maturation, the symptoms of which are a decrease in inflammation and immune cell migration in addition to the disappearance of emerging and undifferentiated new vessels. Medicinal herbs are reported to have antimicrobial, anti-inflammatory, and antioxidant properties. Given the allergic reactions and adverse effects of chemical medications, herbal products may be a safe and suitable treatment for burn wounds (4).

Solanum nigrum (*S. nigrum*) is a species of the Solanaceae family and one of the indigenous medicinal plants of Eurasia brought to America, Australia, and Africa. This weedy plant has been indicated for various illnesses in traditional medicine since ancient times till nowadays. It grows in different lands and weathers, such as tropical and mid-tropical climates (5). Ethanolic and aqueous extracts of S. nigrum fruit contain steroids, flavonoids, saponins, terpenoids, alkaloids, and polyphenolic compounds (6). Anti-inflammatory, antimicrobial, and antioxidant properties are reported for this plant. Methanolic extract of *S. nigrum* fruit has bactericidal activity against a wide spectrum of gram-negative and gram-positive bacterial strains, such as *Pasteurella multocida*, *Salmonella typhi*, *Micrococcus luteus*, *etc*. Furthermore, it has a fungicidal effect on some types of Aspergillus species (7). Therefore, the present study aimed to assess the effectiveness of topical *S. nigrum* fruit aqueous and hydroalcoholic extracts in the burn wound healing process and its antibacterial activity against common pathogens of burn wound infection.

 DOI: https://doi.org/10.18502/aptj.v1i1.7915

Materials and methods

Plant collection

S. nigrum fruit was purchased on February 14, 2019, from the medicinal herbal market in Yazd, Iran. The quality and authenticity of the plant product were certified, and a voucher number (SSU0032) was registered in the herbarium of Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Preparation of hydroalcoholic extract through percolation method

Eight hundred grams of dry powder of *S. nigrum* fruit was used to prepare the hydroalcoholic extract using the percolation method, while 80% ethanol was consumed as solvent. The extraction process lasted for one month. Finally, the extract was filtered and concentrated. The extraction efficiency was also calculated.

Preparation of aqueous extract using reflux method

Thirty grams of dry powder of *S. nigrum* fruit with 100 mL water, as a solvent, were prepared in a reflux system at 70 ºC temperature for 90 minutes. Eight reflux systems were used to prepare an aqueous extract of *S. nigrum* fruit. The obtained extract was then filtered and concentrated. Finally, the extraction efficiency was calculated (8).

Standardization of the extract

Determination of total phenolic content: Total phenolic content of *S. nigrum* fruit extract was measured using Folin-Ciocalteu reagent. Folin-Ciocalteu reagent (1.5 mL) was added to 200 µL of the test sample (0.5 mg/mL). After 5 minutes, 1.5 mL of saturated sodium bicarbonate (6% w/v in water) was added, and the mixture was kept at room temperature for 90 minutes. Sample absorbance was measured by using a spectrophotometer at 725 nm wavelength against the blank. Different concentrations of gallic acid solution (25-150 µg/mL) were used to produce the standard curve ($y = 0.0049x - 0.0016$, $R^2 = 0.9979$), and the equation calculated the total phenolic content of the extract. Then, the extraction efficiency was expressed as gallic acid equivalents GAE/g of dry plant product (9).

Pilot experiment

Burn wound induction was performed on several male Wistar rats using a hot metal plate at different temperatures and durations of heat exposure. Histopathological analysis was done to determine the best temperature and exposure time for second-degree burn wound induction.

In-vivo animal tests

In this study, 48 male Wistar rats weighing 200-250 g were evaluated, and the ethics committee approved the whole study in Shahid Sadoughi University of Medical Sciences, Yazd, Iran (ethics code:NREC: IR.SSU.MEDICINE.REC.1398.190). The animals were randomly assigned into 8 groups of 6. Throughout the experiment, all standard situations for keeping the laboratory animals were observed entirely (i.e., 25 ºC temperature, 60% dampness, 12-hour alternating periods of light and darkness, and animal feeding with standard food and water), and animals were kept apart in separate cages (10).

Induction of anesthesia was performed by intraperitoneal injection of 50 mg/kg ketamine and 10 mg/kg xylazine (produced by Alfasan Company, Netherlands). Then, 2-cm diameter burn wounds were made by applying a heated (100 ºC) round metal plate on shaved dorsal areas of rats for 8 seconds.

One day after the wound induction, the case groups were treated with different concentrations of *S. nigrum* aqueous and hydroalcoholic extracts (0.2%, 2%, and 20%) twice a day for 21 days. The control group was treated with normal saline, and the standard group was treated with silver sulfadiazine (SSD) 1% cream.

Slide preparation and histopathological analysis

At the end of the experiment, the animals were sacrificed, and burnt areas of skin were cut out and fixed in 10% formalin. Then, tissue sections at 5-µm thickness were made. Staining of sections was performed by Ehrlich's hematoxylin and eosin (H&E) and Masson's trichrome (11). On microscopic examination, central areas of each section were scored for the reduction in angiogenesis (a criterion of complete wound healing and maturation), neutrophil accumulation (an indicator of inflammation severity), epithelialization, and collagen filament deposition. Microscopic images were taken under various magnification powers.

Based on our previous experiments, specific numerical scoring criteria were considered to describe the quantity or quality of the tissue repair indicators. For collagen deposition status, 0 indicated the absence of collagen filaments, 1 = few collagen filaments, 2 = moderate collagen filaments, and 3 = a high amount of collagen synthesis. For evaluation of neutrophil migration rate, the number of neutrophils in the affected tissues was analyzed in 10 high power fields and averaged. Then, numerical scores were allocated to different numbers of neutrophil as follows: 0 = 10 or more cells/HPF, 1=4-9 cells/HPF, 2=1-3 cells/HPF, and 3=no cell. The intensity of angiogenesis was scored from 0 to 2 (0=moderate or severe, 1=mild, and 2=lack of neovascularization). Epithelium status was checked and scored based on the thickness of newly formed epithelial layers (0=no epidermis, 1=a partial repair of the epidermis, and 2=complete renovation of epidermal layers). A pathologist blindly determined the above-mentioned histopathological parameters.

Determination of the antibacterial activity of the extract

To assess the antibacterial effect of different concentrations (25, 40, 50, 75, 100 mg/mL) of the extract by agar well diffusion method, three common pathogens of burn wound infection, *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853) and *A. baumanni* (ATCC 19606) were selected and cultured on nutrient agar (made in Q-lab Company, Canada) by streaking technique. After incubation at 37 ºC temperature for 24 hours, microbial suspensions were prepared by adjusting to 0.5 McFarland standard (Darvash Company, Iran) to obtain a 1.5 × 108 CFU/mL microbial concentration. A 100 µL inoculum of each bacterial suspension was cultured on Mueller Hinton agar (Q-lab Company, Canada). In any inoculated culture media, 7 wells of 5 mm

 DOI: https://doi.org/10.18502/aptj.v1i1.7915

diameter were created using a 10 mL Pasteur pipette and their bottom sealed by 15 µL melted Mueller Hinton agar. The wells were filled with 50µL of either normal saline (N/S) as a negative control, ciprofloxacin (20 µg/mL) as a positive control, or different concentrations (25, 40, 50, 75, and 100 mg/mL) of aqueous or hydroalcoholic extract. The culture media were incubated at 37 ºC for 24 hours. After incubation, the diameters of bacterial growth inhibition zones were measured. The procedure was repeated three times for each variable factor (kind of extract and pathogen). Finally, the means of diameters were evaluated (12).

Statistical analysis

At the end of the experiment, mean scores of wound healing parameters were calculated for each group (N=6 rats), and means of triplicate microbial tests were reported. Statistical analysis of histological data using the Kolmogorov-Smirnov test showed that data were not normally distributed, so multiple comparisons of materials in the animal test were performed by Kruskal-Wallis test within a 95% confidence interval.

Results

According to the phytochemical analysis of *S. nigrum* fruit extract and using the standard gallic acid curve (**Figure 1**), the amount of total phenolic content in *S. nigrum* fruit extract as gallic acid equivalents was determined to be 41.96 mg/g of dry hydroalcoholic extract and 29.38 mg/g of dry aqueous extract. Extraction efficiencies for percolation and reflux methods were 16.6% and 20.83%, respectively, so the total amount of phenolic compounds was determined to be 6.11-6.96 mg/g of dry plant material. As is shown in **Tables 1** and **2**, statistical analysis of tissue repair data by Kruskal-Wallis test showed that the rate of neutrophil migration to the wound tissues treated by 20% hydroalcoholic extract was significantly lower than in wounds treated by normal saline ($P=0.018$). In other words, the 20% hydroalcoholic extract could markedly reduce the inflammation in the affected tissues. Furthermore, the rate of neutrophil migration in the wounds treated by 20% hydroalcoholic extract of *S. nigrum* fruit was significantly lower than the other treatments ($P<0.05$). Despite 21 days of treatment by *S. nigrum* aqueous extract, 0.2% and 2% hydroalcoholic extracts, no advantage was detected in reducing the rate of neutrophil infiltration compared to controls ($P>0.05$). According to the histopathological evaluation and statistical analysis done by the Kruskal-Wallis test, the 20% hydroalcoholic extract of *S. nigrum* fruit enhanced the collagen deposition more than silver sulfadiazine 1% cream ($P<0.05$). It was also revealed that 0.2% and 20% aqueous extracts and 0.2% hydroalcoholic extract increased the amount of collagen deposition in the affected tissues, but these effects were not statistically significant in comparison to the control group ($P>0.05$). The 2% aqueous and hydroalcoholic extracts showed no efficacy for collagen synthesis. In the wounds treated by 0.2% aqueous and 20% hydroalcoholic extracts, partial repair of epithelial layers happened, but it proved that these effects were not significantly different from those of the control ($P>0.05$). Other treatments showed no evidence of efficacy for new epidermal layer generation.

Statistical evaluation on the reduction of angiogenesis severity, as a criterion of wound maturation, showed that 2% and 20% aqueous and hydroalcoholic extracts slightly reduced angiogenesis, but these effects were not significantly greater than those of controls (P>0.05). Angiogenesis was not diminished in tissues treated with 0.2% aqueous and hydroalcoholic extracts (**Figure 2**).

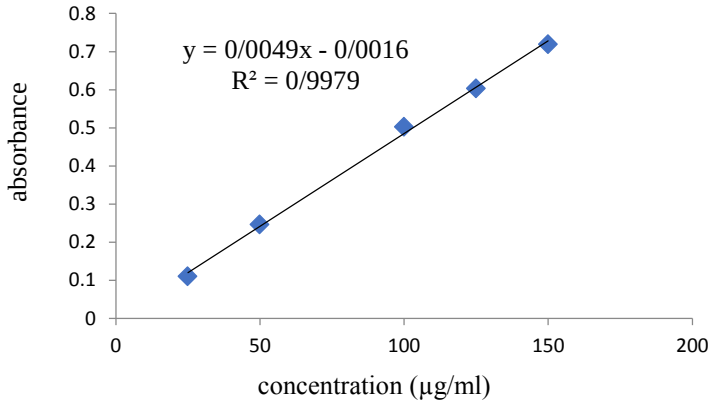

Figure 1. Standard gallic acid curve

Table 1. Effects of different treatments on wound healing parameters

	Normal saline	**Silver Sulfadiazine**	**0.2% HA Ext**	**2% HA Ext**	**20% HA Ext**	**0.2% Aq Ext**	**2% Aq Ext**	**20% Aq Ext**
neutrophil migration	0.2 ± 0.2	0.5 ± 0.2	0 ± 0	0 ± 0	2.2 ± 0.2*	0 ± 0	0 ± 0	0.33 ± 0.2
Collagen deposition	0.17 ± 0.2	0 ± 0	0.4 ± 0.2	0 ± 0	1.2 ± 0.2**	0.4 ± 0.2	0 ± 0	0.67 ± 0.2
Epithelialization	0.17 ± 0.2	0 ± 0	0 ± 0	0 ± 0	0.67 ± 0.4	0.2 ± 0.2	0 ± 0	0 ± 0
Angiogenesis	0 ± 0	0.83 ± 0.2	0 ± 0	0.2 ± 0.2	0.33 ± 0.2	0 ± 0	0.17 ± 0.2	0.33 ± 0.2

HA Ext, hydroalcoholic extract; Aq Ext, aqueous extract. Data are displayed as mean ± SEM (N=6). *P<0.05, in comparison to controls (normal saline); **p<0.05, in comparison to silver sulfadiazine 1% cream. Statistical analysis of data was performed by Kruskal-Wallis test.

Table 2. P-values of the differences between the groups regarding neutrophil accumulation and collagen deposition

Group 1 – Group 2	**Neutrophil**	**Collagen**

 DOI: https://doi.org/10.18502/aptj.v1i1.7915

	migration	deposition
0.2% HA Ext – normal saline	1.000	1.000
2% HA Ext – normal saline	1.000	1.000
20% HA Ext – normal saline	0.018##	0.066
0.2% Aq Ext – normal saline	1.000	1.000
2% Aq Ext – normal saline	1.000	1.000
20% Aq Ext – normal saline	1.000	1.000
0.2% HA Ext – silver sulfadiazine	1.000	1.000
2% HA Ext – silver sulfadiazine	1.000	1.000
20% HA Ext – silver sulfadiazine	0.643	0.017##
0.2% Aq Ext – silver sulfadiazine	1.000	1.000
2% Aq Ext – silver sulfadiazine	1.000	1.000
20% Aq Ext – silver sulfadiazine	1.000	0.643
0.2% HA Ext – 2% HA Ext	1.000	1.000
0.2% HA Ext – 20% HA Ext	0.004##	0.742
2% HA Ext – 20% HA Ext	0.004##	0.017##
0.2% Aq Ext – 2% Aq Ext	1.000	1.000
0.2% Aq Ext – 20% Aq Ext	1.000	1.000
2% Aq Ext – 20% Aq Ext	1.000	0.643
0.2% HA Ext – 0.2% Aq Ext	1.000	1.000
0.2% HA Ext –2% Aq Ext	1.000	1.000
0.2% HA Ext –20% Aq Ext	1.000	1.000
2% HA Ext – 0.2% Aq Ext	1.000	1.000
2% HA Ext – 2% Aq Ext	1.000	1.000
2% HA Ext –20% Aq Ext	1.000	0.643
20% HA Ext – 0.2% Aq Ext	0.002##	0.742
20% HA Ext –2% Aq Ext	0.002##	0.017##
20% HA Ext –20% Aq Ext	0.048##	1.000

HA Ext, hydroalcoholic extract; Aq Ext, aqueous extract; Asymptomatic significances are displayed; the significance level is 0.05; ##There is a significant difference between 2 groups; statistical analysis was performed by Kruskal-Wallis test.

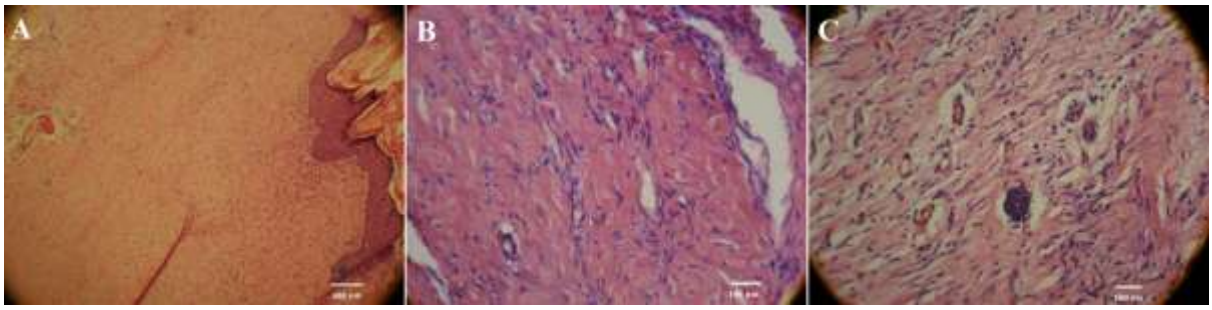

Figure 2. (A) Regeneration of epidermal layers by 20% hydroalcoholic extract; (B) Marked collagen formation without neutrophil migration by 20% hydroalcoholic extract; (C) Mild angiogenesis by silver sulfadiazine. H&E staining, light microscopy.

Regarding antimicrobial evaluation, the inhibitory effect of *S. nigrum* hydroalcoholic extract on *S. aureus* at the concentrations of 50, 75, and 100 mg/mL was observed (**Figure 3**). *P. aeruginosa* and *A. baumanni* were completely resistant to *S. nigrum* aqueous and hydroalcoholic extracts, and no zone of inhibition was observed (**Table 3**).

Table 3. Growth inhibition zones (as the average of triplicates, in mm ± SEM) of common pathogens of burn infection exposed to different concentrations of aqueous and hydroalcoholic extracts of *S. nigrum* fruit

		Pathogen (mm)		
Material	**Concentration**	***S. aureus***	***P. aeruginosa***	***A. baumanni***
Normal saline	0.9%	0.0	0.0	0.0
Ciprofloxacin	20 µg/ml	34.7 ± 0.3	25 ± 0.0	22.3 ± 1.4
Hydroalcoholic extract	25 mg/ml	0.0	0.0	0.0
	40 mg/ml	0.0	0.0	0.0
	50 mg/ml	14.0 ± 0.6	0.0	0.0
	75 mg/ml	16.3 ± 1.2	0.0	0.0
	100 mg/ml	19.7 ± 0.3	0.0	0.0
Aqueous extract	25 mg/ml	0.0	0.0	0.0
	40 mg/ml	0.0	0.0	0.0
	50 mg/ml	0.0	0.0	0.0
	75 mg/ml	0.0	0.0	0.0
	100 mg/ml	0.0	0.0	0.0

Figure 3. Antimicrobial, the dose-dependent activity of different concentrations (25, 40, 50, 75, and 100 mg/mL) of *S. nigrum* hydroalcoholic extract on *S. aureus* compared with negative (C-, normal saline) and positive (C+, ciprofloxacin)

 DOI: https://doi.org/10.18502/aptj.v1i1.7915

Discussion

The wound healing process is a complex event and includes 4 phases. The hemostasis phase is the first event to stop bleeding and coincides with vasoconstriction, coagulation pathway activation, and platelet clumping. The second phase is the inflammatory phase taking place within 24 hours after the injury and is associated with a generalized increase in capillary permeability that leads to plasma leakage, the release of systemic inflammatory mediators and cytokines, and the migration of immune cells. The release of pro-inflammatory factors such as interleukins, prostaglandin E2, and tumor necrosis factor-alpha triggers inflammation. Several types of growth factors, such as angiogenesis factors, stimulate new capillary generation. In addition, transforming growth factor-β, platelet-derived growth factor, and epidermal growth factor promote the recruitment of fibroblasts with consequent collagen production. In the proliferation phase, angiogenic growth factor secretion induced by some microenvironmental changes (decrease in O_2 pressure and pH and other biochemical changes) leads to neovascularization or angiogenesis. Epidermal growth factor and transforming growth factor-β secreted by macrophages, keratinocytes, and platelets stimulate epithelization. Keratinocytes proliferate and then migrate to cover the whole wound surface. The remodeling phase is the last and includes collagen rearrangement and the elimination of dead tissues. It also contains all incidents leading to a return to a normal physiological state (13, 14).

Burn wound treatment protocols are based on pharmacological and non-pharmacological remedies. One of the most convenient and non-pharmacological treatments for first and second-degree burn wounds is to cool the affected areas immediately by 10-25 ºC water. This method reduces pain and damage severity in mild thermal burns. In chemical burns, washing the affected areas with soap and water is a critical action that removes the corrosive materials from the skin. Unfortunately, there is no specific plan for the treatment of blisters. Systemic analgesic and anxiolytic agents and topical treatments are the main parts of pharmacological treatment. Analgesics such as opioids and benzodiazepines can be used to reduce pain and anxiety (15). Topical treatments such as honey, *Aloe vera*, and antibiotics can be applied for mild burns (16).

Silver sulfadiazine (SSD) cream is one of the usual treatments for burn wounds. SSD has beneficial effects on wound infection by inhibiting microbial division, but this treatment takes a long time and is associated with some problems, including pain and difficulties for patients (17).

Several studies were performed to evaluate the effect of some natural substances, such as honey, chitosan, and gelatin, on burn wounds. The results of these studies proved the wound healing activity of these compounds, such as their positive effects on epidermal cell renovation and wound contraction (18). Furthermore, it was shown that honey had antibacterial activity, ameliorating the inflammatory phase and reducing the pain in burnt cases (19).

Studies performed on different plants such as *Plantago major* (20), *Bauhinia purpurea* (21), *Arnebia euchroma* (22), and many other plants showed the beneficial effects of these herbal products on wound healing.

S. nigrum (black nightshade), a member of the *Solanaceae* family, is a medicinal herb that contains flavonoids, glycoalkaloids, and phenolic compounds. In terms of geographical distribution, it is native to Asia and Europe and has been brought to other world regions. The leaves of this plant are used for wart and wound treatment and applied to treat stomach ulcers in India. In some regions of Africa, people use the *S. nigrum* fruit as a traditional remedy for treating some ocular problems. Studies showed various therapeutic properties of different parts of this plant, such as antiproliferative, antioxidant, antiaging, and hepatoprotective properties (23).

A study on therapeutic effects of a polyherbal cream containing oily extract of *Rosa damascena* petals, aqueous extracts of *Malva sylvestris*, and *S. nigrum* leaves proved that topical application of this herbal composition for the treatment of second-degree burn wound has positive effects on the renovation of epidermis and reconstruction of blood vessels. In addition, it reduced the inflammation in the affected areas (24). In the present study, the efficacy of topical *S. nigrum* fruit extract in the treatment of second-degree burn wounds in rats was evaluated. Furthermore, its antibacterial activity was assessed by in vitro method. Based on our findings, topical application of *S. nigrum* fruit hydroalcoholic extract improved collagen synthesis and reduced inflammation in the burnt tissues. It also inhibited the growth of *S. aureus* as a common pathogen of burn infection. *S. nigrum* fruit contains many secondary metabolites, which may justify its demonstrated effects in this study.

Conclusion

According to our findings, topical application of *S. nigrum* fruit hydroalcoholic extract improves collagen synthesis and reduces inflammation in the burnt tissues. It also inhibits the growth of *S. aureus* as a common cause of burn infection. Although 20% hydroalcoholic extract of *S. nigrum* fruit could improve some of the wound healing parameters such as collagen deposition and tissue inflammation, using such a high concentration is not reasonable and economical. So, its usage in the form of a herbal formulation that enhances its absorption and delays its clearance from the skin can be a rational application to improve its therapeutic effects and complete wound healing. Therefore, more studies in the form of clinical trials should be made using a herbal product containing hydroalcoholic extract of *S. nigrum* fruit to provide more evidence for the effectiveness of this extract in burn wound healing.

Study limitations: Due to the apparent turbidity of the extract in the selected concentrations for the microbial test, determination of the minimum inhibitory concentration (MIC) by the microdilution method was not possible. On the other hand, turbidity removal by applying emulsifying agents was not a reasonable decision due to their destructive effects on the bacterial membrane, so the antibacterial activity of the extract was assessed by the agar well diffusion

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method instead of the micro-dilution method. Because of the lack of evidence-based information on the effective concentration of *S. nigrum* fruit extract for the treatment of burn wounds, a wide range of concentrations (0.2%-20%) was selected to determine the wound healing activity of *S. nigrum* fruit extract.

Conflict of interest: None to declare.

Funding: School of Pharmacy, Shahid Sadoughi University of Medical Sciences ,Yazd, Iran.

Acknowledgments: This work was supported by the School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

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 DOI: https://doi.org/10.18502/aptj.v1i1.7915