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In vitro and in vivo evaluation of the diabetic wound healing properties of Saffron (*Crocus Sativus* L.) petals

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Wound healing is a complex process orchestrated by interactions between a variety of cell types, including keratinocytes, fibroblasts, endothelial cells, inflammatory cells, and bioactive factors such as extracellular matrix (ECM) components, growth factors, and cytokines. Chronic wounds exhibit delayed proliferative phase initiation, reduced angiogenesis, impaired ECM synthesis, and persistent inflammatory response. Chronic wounds are one of the main challenges to the healthcare system worldwide, with a high cost for medical services. Hence, investigation of new approaches to accelerate wound healing is essential. Phytomedicines are considered as potential agents for improving the wound healing by accelerating epithelization, collagen synthesis, and angiogenesis. These natural compounds have various advantages including availability, ease of application, and high effectiveness in wound managment. This study aimed to investigate the biological effects of saffron or *Crocus sativus* L. (*C. sativus*) petal extract on cell survival, migration, and angiogenesis using MTT, scratch and in vitro tube formation assays. Moreover, the expression of collagen type I alpha 1 (COL1A1) and vascular endothelial growth factor (VEGF) were evaluated in human dermal fibroblasts (HDF)s and human umbilical vein endothelial cells (HUVEC)s, respectively. The effect of the *C. sativus* extract on the skin of diabetic mice was also monitored. The results showed that *C. sativus* petal extract promoted the viability and migration of HDFs and HUVECs. Moreover, *C. sativus* petal extract enhanced the formation of tube-like structures by HUVECs cultured on the Matrigel basement membrane matrix, indicating its potential to stimulate angiogenesis. Gene expression studies have shown the the *C. sativus* extract increases wound healing by upregulation of COL1A1 and VEGF, which are crucial factors involved in collagen deposition, epithelialization, and angiogenesis. Histological analysis revealed that *C. sativus* petal extract enhanced vascularity and increased the number of fibroblasts and collagen synthesis, ultimately accelerating wound closure compared to wounds treated with eucerin and commercial ointment in diabetic mice. Therefore, *C. sativus* petal extract has potential as a herbal treatment to improve the healing of diabetic wounds.

Keywords Phytomedicine, *Crocus sativus* L., Wound healing, Viability, Migration, Angiogenesis, Collagen synthesis

Wound healing is a dynamic physiological process involving four intimately linked stages: hemostasis, inflammation, proliferation, and remodeling, which are aimed at re-establishing the cellular integrity and function of damaged tissue[1]. Matrix deposition and the formation of microvascular network are important events in wound healing. Among the various cell types involved, fibroblasts play a crucial role throughout all phases of wound healing by interacting with other cells and mediators at the site of injury[2]. As the main cell type in the dermis, fibroblasts proliferate and secrete various extracellular matrix (ECM) components such as type I and III collagen

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which can replace fibrin clots by generating a new ECM[3]. Angiogenesis provides a supply of nutrients, oxygen, and growth factors during wound healing[4]. The Proliferation and migration of microvascular endothelial cells are required for angiogenesis process.

In the patients with diabetes, the process of wound healing is impeded due to excessive inflammation, infection, and impaired angiogenesis. The presence of wounds carries significant implications owing to their impaired immune response, protracted recovery period, and heightened vulnerability to infections and related complications. The confluence of impaired circulation, neuropathy, and dysregulated immune function frequently precipitates delayed wound healing and an augments susceptibility to the development of chronic wounds, such as diabetic foot ulcers. These particular wounds entail severe ramifications, including infectious complications, tissue necrosis, and the potential necessity for amputation. Consequently, a comprehensive understanding of the precise impact of wounds on individuals with diabetes is a paramount importance, facilitating the implementation of tailored interventions and optimizing outcomes for wound healing[5].

Phytomedicines derived from different parts of plants have been used for the treatment of various diseases such as chronic wounds[6,7]. Medicinal plant extracts contain bioactive constituents with proliferative, migratory, antioxidative, antibacterial, pro-angiogenic, and immunomodulatory properties that promote healing process[8–12]. The safety pharmacology, high efficacy, and affordability of herbal remedies have attracted attention in pharmaceutical research[13]. Recent studies have revealed the potential of extracts of *Crocus sativus L.*, commonly known as saffron, and its compounds such as crocin, crocetin, and safranal in skin wound healing[14,15]. Although the stigma of saffron plants has been predominantly used in the food industry, cosmetics, and disease treatment, attention has recently been directed toward its petals as a low-cost waste component containing effective pharmacological compounds[16]. In this study, we investigated the biological effects of saffron or *Crocus sativus L. (C. sativus)* petal extract on cell survival and scratch wound closure. The angiogenic potential of *C. sativus* was assessed by tube formation assay. The expression of collagen type I alpha 1 (COL1A1) and vascular endothelial growth factor (VEGF) expression were investigated in human dermal fibroblasts (HDFs) and human umbilical vein endothelial cells (HUVECs), respectively. The purpose of this study was to determine in vitro effects of *C. sativus* extract through evaluating fibroblast migration and endothelial cell angiogenesis. This study was also intended to investigate the wound healing efficacy of this extract in mouse excisional wound models.

Materials and methods

In vitro study

Preparation of C. sativus (Saffron) petal extracts

The *C. sativus* (Saffron) petals collection follows all the applicable international standards, guidelines, and laws. Saffron petals were obtained from the medicinal plant company. Subsequently, the saffron aqueous extract was prepared using the maceration method at room temperature with ultrasonication (5 min) and incubated on shaker for 72 h. This step was repeated three times and the extract was then filtered through a 0.45 µm polytetrafluoroethylene (PTFE) filter membrane and concentrated using a rotary evaporator (Heidolph, Germany) at low temperature and under vacuum conditions. The extract contain several compounds, including crocins, crocetin, picrocrocin, and safranal[17]. The concentrated extract was dried using freeze dryer and stored at 4 °C until further use.

Cell culture

The Human umbilical vein endothelial (HUVEC) and human dermal fibroblast (HDF) cells were obtained from the National Cell Bank of Iran (Pasteur Institute, Iran) and cultured in DMEM medium supplemented with 10% (v/v) FBS (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco). Cells were then incubated at 37 °C in a humidified atmosphere of 5% CO_2 and sub-cultured every 2 days. For the experiments, cells in passages 3–5 were used.

Cell viability assay

To evaluate the proliferative effect of *C. sativus* petal extract, cells were seeded in a 96-well plate at a density of 10^4 cells/well and incubated overnight. After 24 h, the cells were treated with a medium containing different concentrations of *C. sativus* petal extract (0–320 µg/mL) for 24, 48, and 72 h. After the indicated time points, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed to evaluate the percentage of viable cells. MTT is a tetrazolium salt can be reduced by the mitochondrial metabolic function of viable cells, forming a purple formazan product. For the MTT assay, the culture media were removed and 100 µL of DMEM containing 10 µL MTT solution (5 µg/mL) was added to each well, and the plates were incubated at 37 °C for 4 h. After removing the MTT solution, 100 µL of DMSO was added to dissolve the formazan crystals. Absorbance was measured at 570 nm using microplate reader and the percentage of viable cells was calculated relative to the control cells. The results were obtained from three independent experiments, each performed in triplicates.

Scratch wound healing assay

To evaluate the effect of *C. sativus* petal extract on wound healing, an in vitro scratch assay was performed as previously described[18]. Briefly, HUVEC and HDF cells were cultured in 6-well plates and incubated until reaching 80–90% confluence. A small linear scratch was then gently made using a sterile 100 µL pipette tip, and the cells were washed with phosphate-buffered saline (PBS) to remove cellular debris. Subsequently, cells were treated with different concentrations of *C. sativus* petal extract and incubated for an additional 24 h. The migration and wound closure ability of cells were quantified by microscopy. The migration and wound closure ability of cells were quantified by microscopy. Images were captured using a digital camera connected to an inverted microscope. The area between the scratch edges was measured by ImageJ analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA). Data are presented as the average of three independent experiments.

Tube formation assay
The 96-well plates were coated with 10 mg/mL Matrigel (Corning, USA) treated and non-treated HUVECs (10^4/well) were seeded in the coated plates with 10 mg/mL Matrigel (Corning, USA) and incubated for 24 h. The formation of tube-like structures was observed and images were captured under three random microscopic fields per well. The images were quantified using the WimTube Image Analysis software (ibidi GmbH, Germany).

RNA extraction, and cDNA synthesis, quantitative real-time PCR (qRT-PCR)
For the analysis of mRNA expression, cells were harvested after treatment with 10 µg/mL of *C. sativus* petal extract for 24 h. Total RNA was extracted from cells with RiboEx LS reagent (Geneall, South Korea). RNA concentration of was determined by NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA integrity was assessed using gel electrophoresis. cDNA synthesis was performed using Easy cDNA Synthesis Kit (Parstous Biotechnology, Iran). The expression of candidate genes was measured using a SYBR green-based assay kit (Parstous Biotechnology, Iran) according to the manufacturer's instructions. The RT-PCR cycling conditions were initial denaturation at 95 °C for 5 min, followed by 35 cycles of amplification at 62 °C for 30 s and extension at 72 °C for 20 s. Relative gene expression was calculated based on using the $2^{-\Delta\Delta CT}$ quantification method with B-actin as the reference gene. The primer sequences of genes were: VEGF forward (5′-ATCTTCAAGCCATCCTGTGTGC-3′); reverse (5′-TATGTGCTGGCCTTGGTGAGG-3′), COL1A1 forward (5′TAGACATGTTCAGCTTTGTGGACC-3′); reverse (5′-TCTGTACGCAGGTGATTGGTGG-3′); β-actin forward (5′-AGACGCAGGATGGCATGGG-3′), and reverse (5′-GAGACCTTCAACACCCCAGCC-3′).

Western blot
To evaluate protein expression, cells were treated with 10 µg/mL of *C. sativus* petal extract for 24 h, and cell lysate was prepared in RIPA lysis buffer containing protease inhibitor cocktail and the total protein concentration was determined by the Bradford protein Assay. 20 µg of proteins were separated on sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membranes. The membrane was blocked with 5% non-fat dried milk (NFDM) in Tris Buffered Saline-Tween (TBS) containing Tween-20, and then incubated overnight at 4 °C with primary antibodies including VEGF, COL1A1, and β-actin. The membrane was then washed and incubated goat anti-mouse HRP-conjugated secondary IgG for 1 h at room temperature. After washing with TBS proteins were detected by chemiluminescence using ECL western blotting detection reagents.

In vivo study

Animals and experimental design
A total of 36 male NMRI mice, weighing approximately 20–30 g, were obtained from the Pasteur Institute, Tehran, Iran. All animal studies were carried out in accordance with relevant guidelines and regulations and ARRIVE guidelines. The methods and protocols utilized in the animal study received ethical approval by the Ethics Committee of Hamadan University of Medical Sciences, Hamadan, Iran (IR.UMSHA.REC.1400.654). The mice were housed in an animal facility under optimal conditions, maintaining a temperature of 23 ± 2 °C, relative humidity of 50%, and a 12 h light–dark cycle. During the study, mice had unrestricted access to water and food[19]. After one week of acclimation to the environmental conditions, to ensure adaptation, the mice were randomly divided into four groups:

Group 1: NDW (Non-Diabetic Wounds) or Healthy individuals without any treatment.
Group 2: DW-E (Diabetic Wounds treated with Eucerin).
Group 3: DW-P (Diabetic Wounds treated with commercial ointment (Purilon® Gel).
Group 4: DW-S (Diabetic Wounds treated with experimental (saffron) ointment.

The animals were subjected to topical treatment with both the experimental ointment and the commercial ointment through gentle massage within a time frame of 24 to 48 h prior to the completion of the experiment.

Induction of diabetic animal models
To induce diabetes, the experimental procedure was conducted as follows: Initially, a nicotine amide solution was administered via intraperitoneal injection at a dose of 100 mg/kg of body weight. After a 20 min interval, a solution of streptozotocin (STZ) (Sigma-Aldrich, USA) prepared in 5 mM sodium citrate buffer (pH 4.0) was administered via injection at a dose of 55 mg/kg body weight. Five days after injection, blood samples were obtained from the tail of the animals to measure the fasting blood glucose concentration using a glucometer GlucoDr. Plus, AGM-3000, Korea). The criterion for confirming the establishment of diabetes was a fasting blood glucose concentration exceeding 250 mg per deciliter (mg/dl)[20,21].

Wound induction
The animals were anesthetized using a combination of 100 mg/kg Ketamine HCl administered intramuscularly (i.m.) and 10 mg/kg Xylazine HCl administered intraperitoneally (i.p.)[22,23]. Prior to the procedure, the fur on the dorsal area of the animals was shaved and the back area was disinfected with povidone-iodine solution. Following aseptic precautions and adhering to surgical principles, a one-centimeter-deep wound was created in the dermis and hypodermis using a 5 mm punch tool (Biopsy Punch, Kai Industries Co., Ltd, Gifu, Japan). The subcutaneous muscles were not excised during the procedure.. The day of surgery was designated as day zero for subsequent assessment and analysis.

Wound recovery assessment

To evaluate wound size, digital photographs of the lesions were taken on days 0, 3, 7, and 14. The wound area was quantified using ImageJ software. The percentage of wound recovery was determined by employing the following formula[24]:

$$\text{Percentage of wound recovery} = (\text{Wound area}\ (\text{day 0}) - \text{Wound area}\ (\text{day x})/\text{Wound area}\ (\text{day 0}) * 100$$

Histological staining

Full-thickness skin samples were collected on days 3, 7, and 14 of the treatment periods in each experimental group for histological analysis. To obtain samples, animals were anesthetized using a carbon dioxide (CO_2) chamber, and the desired tissue was excised. After washing with physiological saline, all samples were fixed in a 10% formalin solution. The dehydration process was carried out using a series of ascending ethanol concentrations, starting from 60% and gradually reaching absolute ethanol. Subsequently, the tissues were cleared using xylene and embedded in paraffin. Thin sections containing the wound sites were prepared from paraffin-embedded tissues, mounted on glass slides, and stained with Hematoxylin and Eosin (H&E) and Masson's trichrome. Finally, the stained sections were examined and compared under a light microscope to assess parameters, such as collagen content and angiogenesis.

Statistical analysis

The results were presented as the mean values ± standard deviations. Statistical analysis was performed by one-way and two-way ANOVA using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

Results

C. sativus petal extract moderately improved viability of HDFs and HUVECs

The results of the MTT assay indicated that the viability of HDFs and HUVECs did not show a significant difference after treatment with various concentrations of the extract, except for the concentration of 10 µg/ml, which demonstrated an increase in cell viability compared to the control group. Therefore, it seems that the extract not only lacks a toxic effect on the cells, but may also enhance survival at lower concentrations (Fig. 1).

C. sativus petal extract increased migration ability of HDFs and HUVECs

The cell migration assay demonstrated a significant enhancement in the migratory capacity of HDFs and HUVECs following treatment with an extract concentration of 10 µg/ml. Specifically, the closure percentage in treated HDFs exhibited a threefold increase ($P < 0.05$) compared to the control group, while treated HUVECs showed a 1.6-fold increase ($P < 0.05$) compared to their respective controls (Fig. 2).

C. sativus petal extract induced angiogenesis

The in vitro angiogenesis assay demonstrated a significant increase in the number of loops ($P < 0.05$) and tube length ($P < 0.01$) in HUVECs treated with an extract concentration of 10 µg/ml after 24 h, as compared to the control cells (Fig. 3).

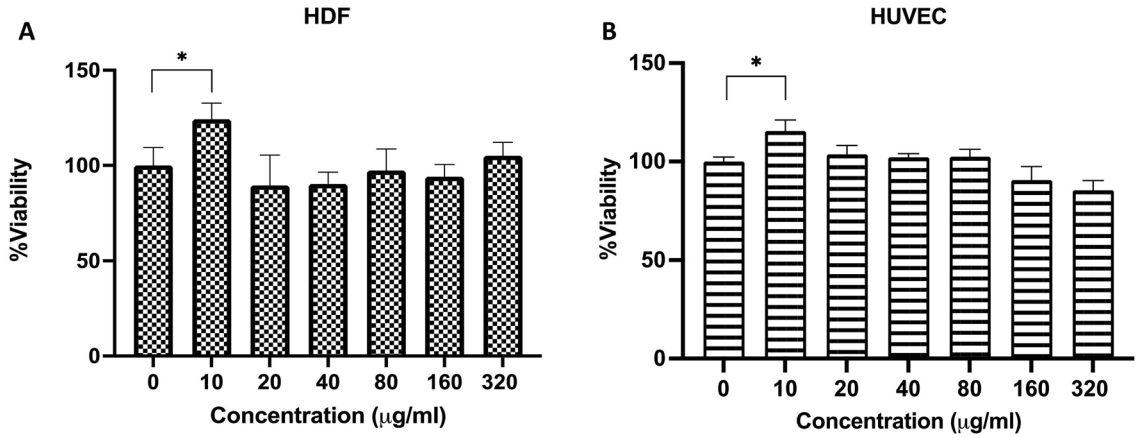

Figure 1. Effects of *C. sativus* petal extract on the viability of HDF (**A**) and HUVEC (**B**). Cells were seeded in 96-wells plate and allowed to attach for 24 h, then treated with different concentrations of *C. sativus* extract (0–320 µg/mL) for 24 h. Absorbance was measured at 570 nm using a ELISA plate reader at 570 nm. Results are presented as the means ± SEM from three independent experiments performed in triplicates (*$p < 0.05$).

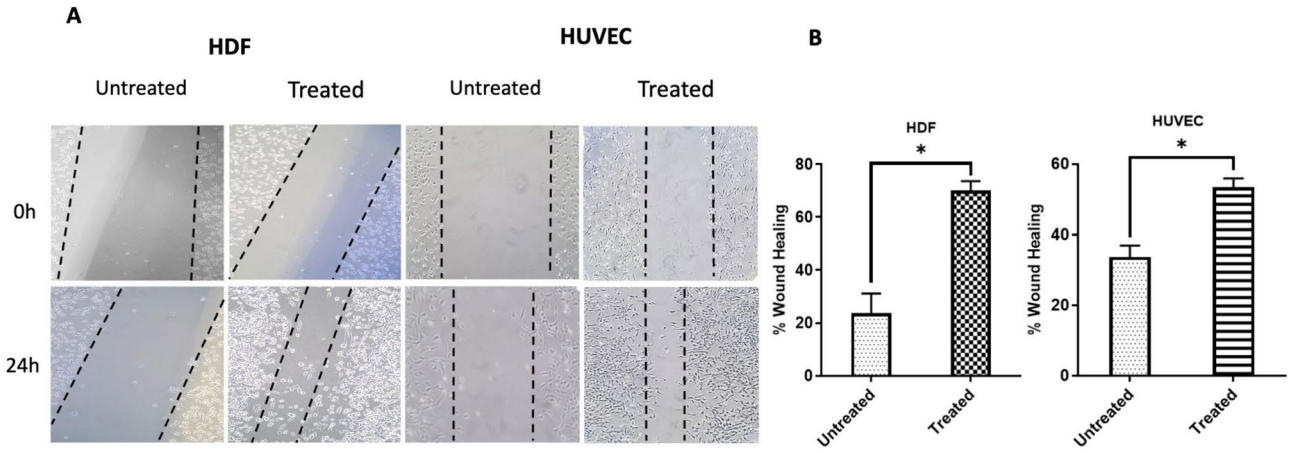

Figure 2. Effects of *C. sativus* petal extract on migration were measured using scratch assay. (**A**) Representative images of the in vitro scratch migration assay in HDF and HUVEC cells immediately and 24 h after wound induction. (**B**) The quantitative evaluation and statistical analysis of wound closure percentage in wound scratch assay measured using ImageJ software. Data are expressed as means ± SEM from three independent experiments (*$p < 0.05$, **$p < 0.01$).

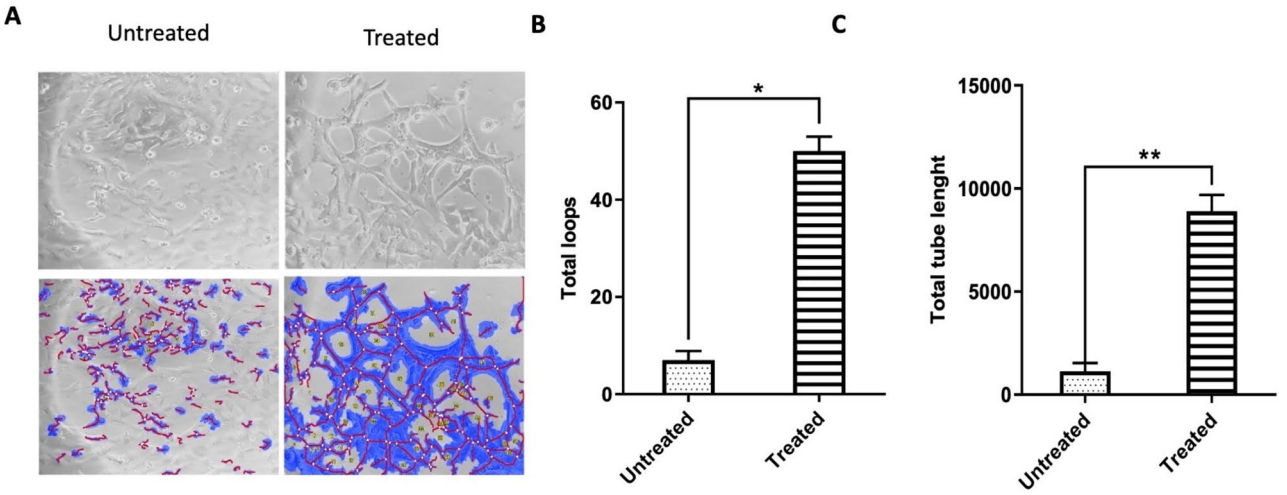

Figure 3. Effects of *C. sativus* petal extract on tube formation in HUVEC cells. (**A**) The images of tube formation on Matrigel in HUVECs treated with *C. sativus* petal extract. (**B**) The total tube length and loops in HUVEC cells. Scale bar = 100 µm. (*$p < 0.05$).

C. sativus petal extract increased COL1A1 and VEGF expression in HDF and HUVEC, respectively

The qRT-PCR analysis demonstrated a 3.5-fold increase in the expression level of COL1A1 in treated HDFs and a 46-fold increase of VEGF in treated HUVECs compared to the respective control groups ($P < 0.05$, $P < 0.01$) (Fig. 4A). Moreover, the protein expression levels of COL1A1 and VEGF in the treated HDFs and HUVECs showed a significant elevation when compared to the corresponding control groups ($P < 0.05$) (Fig. 4B).

Wound closure

Application of *C. sativus* petal extract-significantly improved wound closure in diabetic mice (Fig. 5A). Based on the obtained results, the group of mice with normal wounds (NDW) demonstrated a more pronounced wound healing trend compared to the other groups, likely because of the absence of diabetes in the mice within this group. In excision diabetic wound models, a significant difference was observed in the percentage of wound closure in group 4 (DW-S) compared with the eucerin-treated (DW-E) group on days 3, 7, and 14 ($P < 0.01$, $P < 0.001$, and $P < 0.0001$, respectively) (Fig. 5B). The wound healing rate was also enhanced in models treated with commercial ointment (DW-P); however, the wound contraction was faster in G4 group. At day 14, no significant differences were observed among the group 3 and group 4.

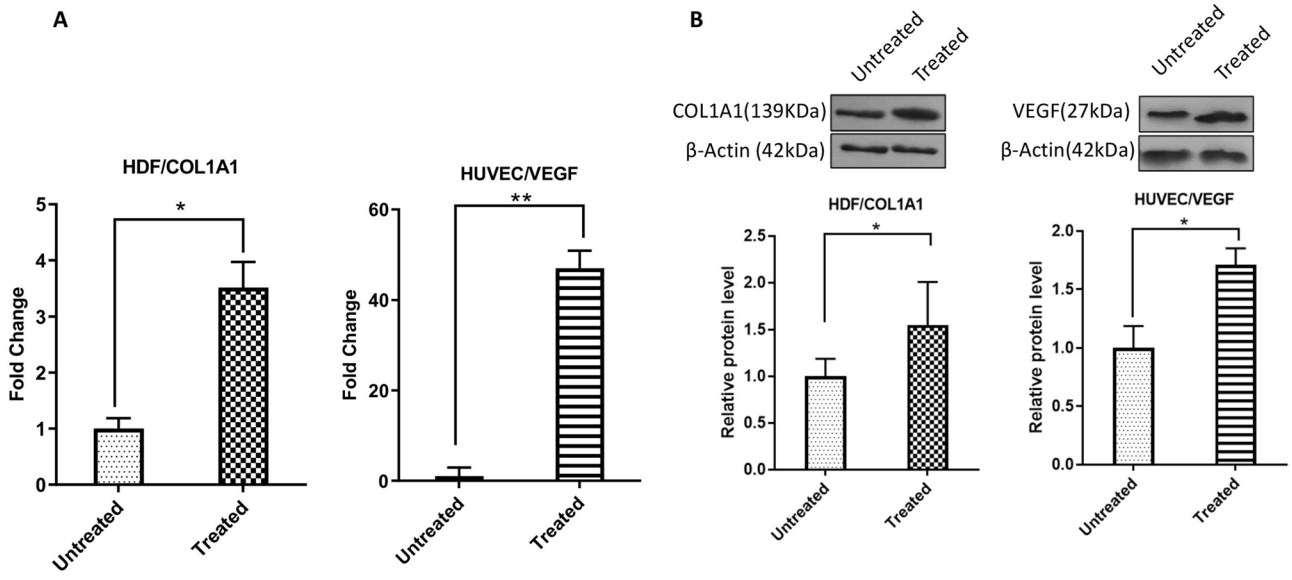

Figure 4. Quantification of collagen1A1 and VEGF expression at (**A**) mRNA and (**B**) protein level in HDF and HUVEC cells treated with *C. sativus* petal extract. The values are presented as the means ± SEM from two independent experiments. Full-length images are included in a Supplementary Information file (*$p < 0.05$, **$p < 0.01$).

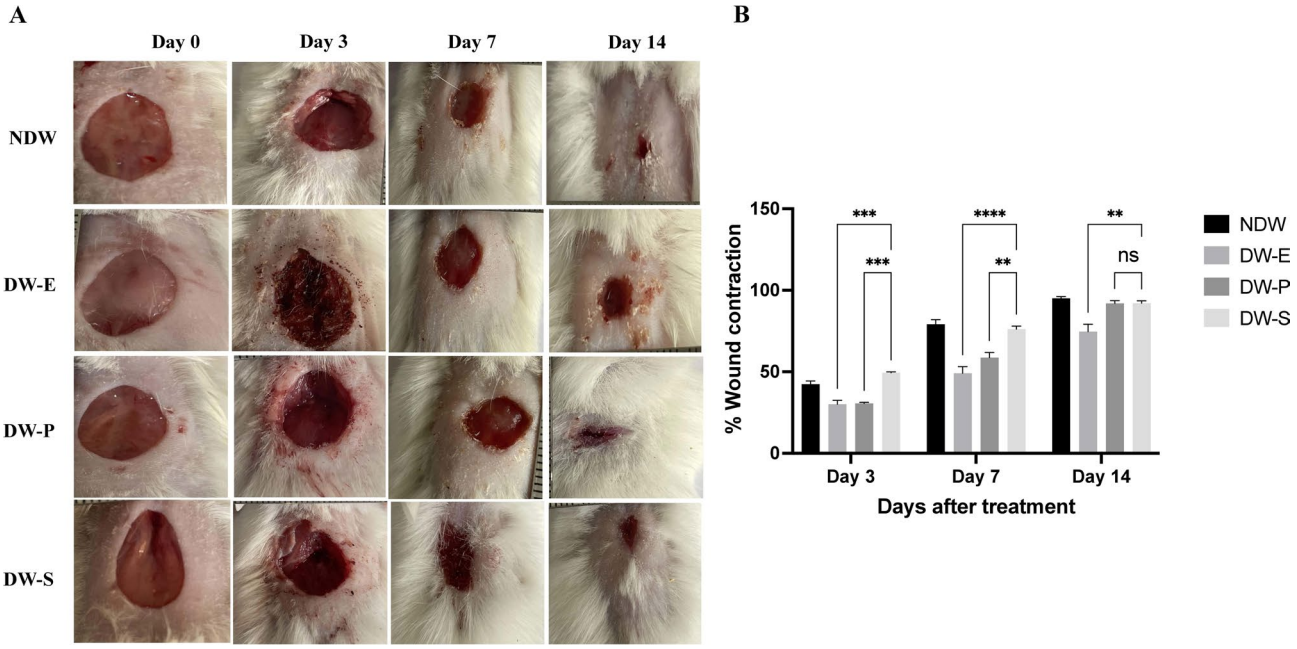

Figure 5. Effect of *C.* sativus petal extract on the diabetic wound healing. (**A**) The representative images of the wound on days of 0, 3, 7, and 14 in four different groups: normal wound (NDW), diabetic wound treated with blank ointment (DW-E), diabetic wound treated with commercial ointment (Purilon® Gel) (DW-P) and diabetic wound treated with *C. sativus* petal extract (DW-S). (**B**) The percentage of wound closure in four groups (**$p < 0.01$, ***$p < 0.001$).

Histological examination

Histological analysis revealed distinct patterns in the wound healing process across different treatment groups and time points, providing valuable insights. Macroscopically, the wound regeneration indicated by skin the black arrows in the images[25,26]. At day 3, the NDW and DW-E groups exhibited pronounced inflammation, limited angiogenesis, and tissue disruption. In contrast, the DW-P and DW-S groups displayed inflammation, lower levels of fibroblasts, tissue disruption, and early signs of tissue repair. By day 7, the NDW and DW-E groups showed variable levels of inflammation, reduced fibroblast presence, and limited tissue formation. In contrast,

the DW-P and DW-S groups demonstrated cell infiltration, fibroblast multiplication, and tissue formation. At day 14, NDW group exhibited ongoing repair processes, while DW-E group showed reduced fibroblast presence. The DW-P and DW-S groups displayed robust fibroblast proliferation, tissue formation, and involvement of hair follicles. Overall, DW-P and DW-S groups exhibited more favorable characteristics associated with tissue repair and regeneration compared to the NDW and DW-E groups (Fig. 6A).

In addition, the number of blood vessels was significantly higher in the DW-P and DW-S groups on day 3 ($P<0.01$, $P<0.05$) and day 14 ($P<0.05$) compared to the DW-E group. Evaluation of epithelization indicated that the extract-treated group had a higher ratio of epithelization compared to the DW-E and DW-P groups on day 14 after treatment ($P<0.01$, $P<0.05$) (Fig. 6B).To assess collagen density, trichrome Mason's staining was performed. The evaluation revealed the presence of collagen fiber bundles in certain areas of the dermis in NDW and DW-E groups. These fibers appeared irregular and clustered in their patterns (Fig. 6C). In contrast, DW-P and DW-S groups exhibited collagen fiber bundles throughout all sections of the dermis. These bundles appeared organized in some regions while intertwined in others. Collagen fibers were predominantly observed throughout most of the tissue. Notably, Fig. 6C demonstrated a considerable increase in collagen density in the extract-treated group compared to the DW-E and DW-P groups (both, $P<0.01$).

Discussion

The pharmacological properties of *C. sativus L.* and its phytoconstituents have been -investigated in various in vitro and preclinical studies. *C. sativus* has shown neuroprotective, anti-hyperglycemic, anti-tumoral, anti-microbial, and anti-oxidant activities[27–31]. Although *C. sativus* petals are the main by-product of saffron, and they contain valuable bioactive compounds such as crocin, riboflavin, thiamine, kaempferol, proteins, flavonoids, and anthocyanin[32–34]. Mohaqiq et al. indicated that the extract of saffron stigma and petal increased serum level of adiponectin level, insulin sensitivity, as well as improved dyslipidemia and obesity-associated oxidative stress[35]. *C. sativus* petal extract has been found to decrease inflammatory factors such as TNFα, IL-1β, IL-6, and IL-18 in the serum of polycystic ovary syndrome (PCOS) mice induced by testosterone enanthate[36]. Prolonged inflammation contributes to impaired wound healing. It seems that saffron petals extract may exert an anti-inflammatory effect on wound healing. Another study showed that oral administration of saffron petals extract decreased fasting blood sugar (FBS), blood urea nitrogen (BUN), and urine in streptozotocin (STZ) induced diabetic rats[37]. Although the anti-angiogenic effect of this herb has been revealed in pathological conditions, mainly mediated by suppression of the VEGF pathway[38], this effect may be different compared to physiologic (normal) conditions. For example, it has been indicated that saffron extracts have less toxicity in non-cancerous cells compared to cancer cells due to a high IC50 in normal cells[39]. In this study, we investigated the angiogenic potential of saffron petal extract in endothelial cells. Endothelial dysfunction is associated with delayed wound healing. We showed that extract of saffron petal significantly promoted migration and proliferation of HUVECs compared to control cells (untreated cells). Goli et al. demonstrated crocin and safranal increased epithelium thickness, blood vessels density, and number of fibroblasts in diabetic wound healing[15]. However, to the best of our knowledge, there has been no study on the angiogenesis potential of saffron petal extract. The results of the present study showed an increased in the number of loops and the tube length in the treated HUVECs. Moreover, the expression of VEGF was elevated in treated HUVECs compared to untreated cells, which could be associated to angiogenesis augmentation in HUVECs. Hence, saffron petal extract may enhance endothelial cell function during the wound healing process via VEGF-dependent pathway. VEGF is involved in wound healing through angiogenic or extra-angiogenic mechanisms. Proliferation and migration of fibroblasts are critical contributors to the formation of granulation tissue during the wound healing process[40]. Fibroblasts produce ECM components such as collagen alpha-1(I) chain and collagen alpha-1(III) chain, which are encoded by COL1A1 and COL1A3 genes, respectively. Type 1 collagen is upregulated during wound healing and tightly controlled by the transforming growth factor beta (TGF-β1)/Smad signaling pathway[41]. This pathway could be impaired by the TNFα/nuclear factor kappa-B (NF-κB) axis[41]. We demonstrated that petal extract increased proliferation and migration of treated HDFs compared to control cells. Previous in vitro studies indicated saffron extract increased proliferation and migration of HDFs[17,42]. In addition, COL1A1 expression was significantly higher in the extract treated HDFs than in control cells. In accordance with our study, Xiong et al. showed increased collagen synthesis in primary dermal fibroblast normal human neonatal cells[17]. Since saffron extract has an inhibitory role on TNFα, it may increase the expression of COL1A1 in this manner. The expression of VEGF was elevated in treated HUVECs compared to untreated cells, which could be associated with angiogenesis augmentation in HUVECs. Hence, saffron petal extract may enhance angiogenesis in wound healing process through a VEGF-dependent pathway. VEGF is involved in wound healing through angiogenic or extra-angiogenic mechanisms[43]. VEGF could enhance epithelialization, migration of fibroblasts and collagen deposition[44,45].VEGF can mediate fibroblast induced angiogenesis and granulated tissue formation in wound healing[46]. Therefore, the increase in COL1A1migration and migration of cultured fibroblasts in this study may be partially related to the upregulation of VEGF.

The effects of *C. sativus* petal extract on excision wound models were measured by wound contraction and histopathological investigation. Our findings revealed that topical application of *C. sativus* petal extract in mouse excision wound models led to a significant acceleration of wound healing, as confirmed by faster wound closure after 3, 7, and 14 days. This may be due to an increase in the number of fibroblast cells, enhanced generation of new blood vessels, and accelerated epithelialization. Histopathologic data showed that the number of blood vessels increased in the early stage of wound healing process to provide oxygen and nutrients for tissue regeneration. However, vasculature decreased during the maturation and epithelialization phases. The histopathological data of the excision wound models on day 14 revealed full epithelization in the wounds treated with *C. sativus* petal extract, while the control group presented partial epithelization. The amount of epithelialization was similar

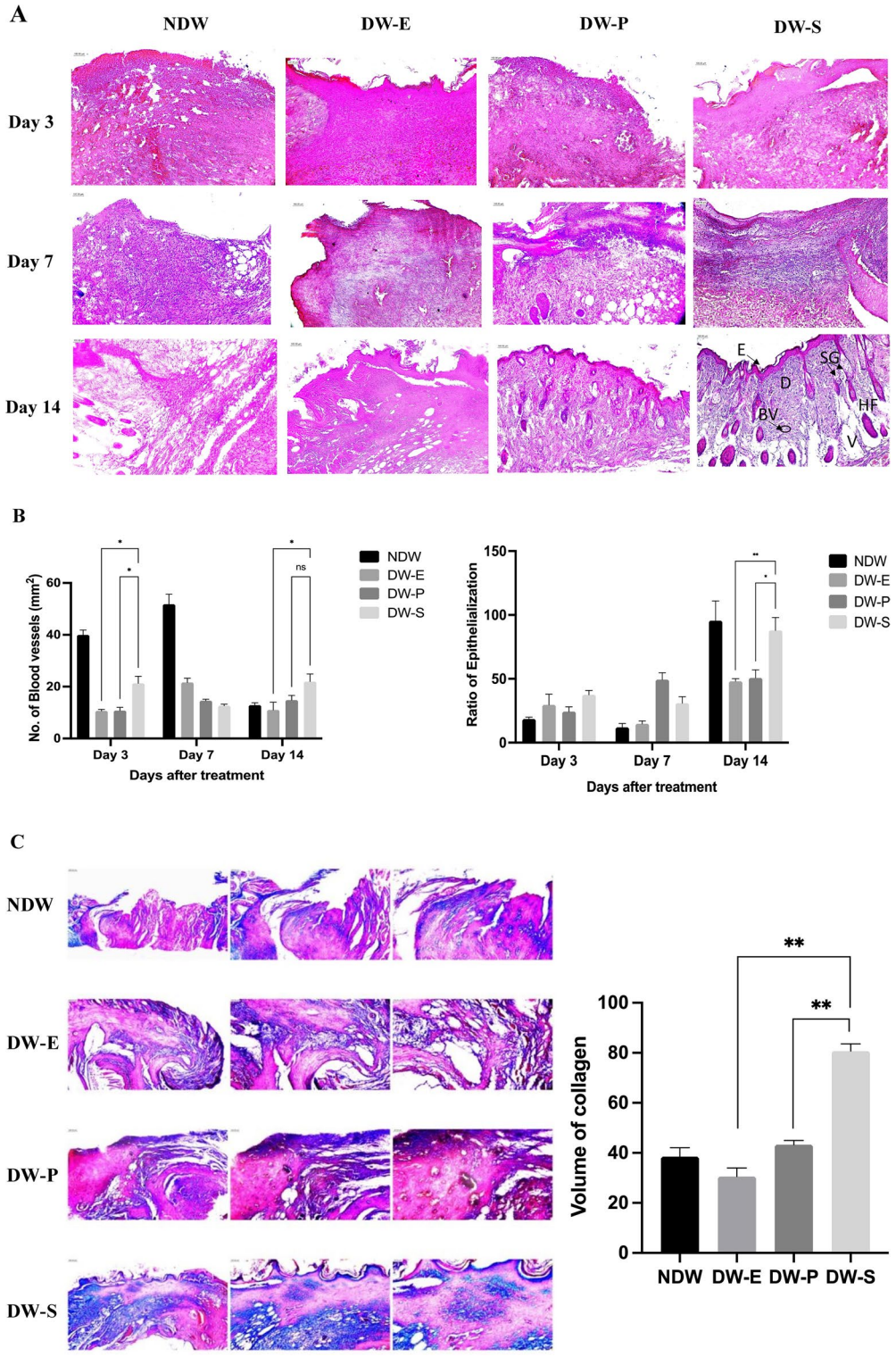

Figure 6. (**A**) Histological sections of wound tissues at 3, 7, and 14 days after treatment application in four different groups of normal wounds (NDW), diabetic wound treated with blank ointment (DW-E), diabetic wound treated with commercial ointment (Purilon® Gel) (DW-P), and diabetic wound treated with *C. sativus* petal extract (DW-S). Epi: Epidermis; D: Dermis; HF: hair follicles; SG: sebaceous glands; BV: blood vessels; V: Vacuole. (H&E staining, 200×). (**B**) quantification of histological parameters on day 3, 7, and 14-after treatment-. (**C**) Masson's trichrome staining of day 14 after wound treatment and quantitation of collagen deposition (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$).

to that is observed in non-diabetic wounds. Similar studies have confirmed that ointments containing herbal extracts can increase angiogenesis and accelerate epithelialization in the wound models[47–49].

Masson's trichrome staining revealed a remarkable increase in collagen deposition following the application of *C. sativus* petal extract on the mice excision wound models. Interestingly, the level of collagen deposition was more pronounced diabetic wounds than in non-diabetic wounds. Collagen is the main component of ECM and plays a critical role in wound closure. Therefore, collagen synthesis and remodeling are important steps in the wound healing process[50]. During wound healing, collagen is mainly produced by fibroblast cells. Therefore, the increased collagen observed in the excision wound models on day 14 after treatment was probably due to an increase in the number of fibroblast cells[51].

In conclusion, the findings of this study demonstrated that *C. sativus* petal extract has potential wound healing activity. This function may be attributed to the abilities of promoting the cell proliferation and migration and increasing the expression of COL1A1. This herbal extract upregulated the expression of VEGF and promoted angiogenesis in the endothelial cells. Topical application of *C. sativus* petal extract accelerates wound closure which is associated with increased new blood vessels, re-epithelization, and collagen deposition. These findings show that saffron extract has great potential as a herbal product, and can improve diabetic wound management.

Data availability

All data generated or analyzed during this study are included in this published article.

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Author contributions

M.H.S. carried out most of the experiments. M.H.S., D.D. and R.A. conceived the main idea and designed the experiments. MH.S. and N.M. participate in drafting and revising the article and analyzed the data. H.K.N., S.N. and S.M.T. contributed to data collection. M.H.S. and R.A. critically reviewed the study. All authors reviewed the results and approved the final version of the manuscript.

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Competing interests

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Additional information

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