

ORIGINAL ARTICLE

Grape seed extract promotes *Staphylococcus aureus* infected skin wound Healing in diabetic rat model

¹Hanaa A. Mansour, ¹Wedad A. Hassan, ²Mai Hammam*

¹Pharmacology Department, National Organization for Drug Control and Research (NODCAR); Egyptian Drug Authority (EDA), Giza, Egypt

²Zoology Department (Immunology), Faculty of Science, Cairo University

ABSTRACT

Key words:

Diabetes mellitus, streptozotocin, grape seed, vaseline

*Corresponding Author:

Mai Hammam
Zoology Department
(Immunology) Faculty of
Science, Cairo University
Tel.: 01009957972
maimahmoud18@gmail.com

Background: Open wounds leave the wound bed at risk for colonization by opportunistic pathogens. Therefore, the understanding and control of the microbial infections are of great importance for the enhanced healing and management of wounds. Reducing the wound healing time is important as it lowers the risk of infection and decreases possible complications. Grape seed extract is rich in powerful antioxidant compounds. Accordingly, grape seed extract (GSE) alone or in combination with chemical agents might be of beneficial value in wound healing. **Objective:** The present study aimed to evaluate the topical application of grape seed extract on wound healing of induced *Staphylococcus aureus* infected skin wound in diabetic rats. **Methodology:** Ninety male Sprague-Dawley rats weighing 150–200 grams were divided into five groups. All groups were anesthetized, shaved, and exposed to round full-thickness punch biopsy on the back: group I (control); group II (wounded); group III (wounded *Staphylococcus aureus* infected); group IV (wounded STZ diabetic) and group V (wounded STZ diabetic infected with *Staphylococcus aureus*). These last four groups were subdivided into Vaseline base treated or GSE- Vaseline treated groups. Macroscopic examination were observed at 0, 1, 3, 6, 8, 12 and 16 days after skin biopsy. Wound contraction data were expressed as a percentage of the initial wound area. Skin homogenates were prepared for determining the immunological parameters TNF- α , IL-10 TGF- β 1. **Results:** Grape seed extract topical application improved the skin homogenate contents of TGF- β 1, IL-10 and TNF- α denoting the immunological effect of GSE on wound healing in different experimental groups. Skin histopathological examination was in agreement with the immunological results. **Conclusion:** Grape seed extract topical application promoted skin wound contraction and closure. Furthermore, it possesses antioxidant and antibacterial properties. Grape seed extract has also strong immunoregulating properties of the skin immune system in *Staphylococcus aureus* infected diabetic wounded rats.

INTRODUCTION

Incision wounds leave the wound bed at risk for colonization by opportunistic bacteria ¹ that leads to delayed wound repair ^{2,3}. The wound bed provides a surface for growth; poor blood flow and hypoxia impairing native defenses ⁴, creating an ideal environment for bacterial growth. Several potential factors contribute to impaired antimicrobial efficacy in a rat MRSA infected wound model. These factors include the components within wound exudates, bacterial re-colonization, a high bacterial load, systemic diseases such as diabetes mellitus and immunosuppression ^{5,6}. Methicillin-resistant *Staphylococcus aureus* by injured patients not only increases the risk of sepsis but also delays wound healing ^{7,8}. Granulation tissue formation

in a wound may be accelerated if high bacterial load is treated properly allowing enhanced wound healing.

Grape seed extract has bactericidal effect on methicillin-resistant *Staphylococcus aureus* (MRSA) ⁹. Substantially decreased bacterial growth in a partial-thickness skin wound model decreases the level of wound infection that was associated with an attenuation of the local dermal inflammatory response ¹⁰.

The present study aimed to evaluate the efficacy of grape seed extract in treatment of *Staphylococcus aureus* infected wounds of diabetic Sprague Dawley rats. This was proficient through measurements of wound closure percentage in all animal groups. The anti-inflammatory effects of grape seed extract was assessed through the measurements of immunological, inflammatory markers and histopathological evaluation.

METHODOLOGY

Preparation of grape seed extract (GSE):

Seeds of Muscat of Alexandria grapes was isolated and dried at room temperature in the shade before being crushed into a fine powder. 100 g of grape seeds powder was blended with 40 mL H₂O and 360 mL ethyl alcohol. The alcohol was evaporated below 40°C under reduced pressure after the GSE was filtered with filter paper. Finally, GSE was evaporated to dryness. The final yield was then homogeneously combined with pure vaseline to produce a vaseline-based ointment (2 %).

Animals:

Ninety healthy male Sprague Dawley rats, age of 10 weeks and weighing 170-200 g, were used in the experiment. They were obtained from the breeding colony from the animal house of the National Organization for Drug Control and Research (NODCAR, Cairo, Egypt). All rats were housed under a constant temperature (25±/-2) °C and 40-60% humidity with an artificial 12-h light/dark cycle and allowed free access to food and water ad libitum. All the surgical and experimental procedures were approved by Cairo University Institutional Animal Care and Use Committee (CU-IACUC) under the number (CU-I-S-34-16).

Experimental induction of diabetes:

Rats were made diabetic by a single I.P injection of 60 mg/kg body weight of streptozotocin (STZ; Sigma-Aldrich) dissolved in citrate buffer (0.01 mol/l, pH 4.5). Rats serving as controls were given the same volume of sodium citrate. Diabetes was confirmed by determination of fasting blood glucose (FBG) level every 3 days. Animals were considered diabetic if they had fasting blood glucose glycaemia values greater than or equal to 250 mg/dl ¹¹.

Excision wound model:

Under anesthesia with ketamine (30 mg/kg, IP) and xylazine (10 mg/kg, IP), the dorsal skin of the animals was shaved and cleaned with 70% ethanol. A full-thickness skin wound was made according to ¹² that was approximately 2cm in diameter. Wound was created after marking the area with a wooden ink stamp. The

entire wound was left undressed. GSE/vasline or vaseline only was topically administrated to the wounds twice daily.

Staphylococcus aureus inoculation:

Staphylococcus aureus was obtained from Microbiological Resources Center (Cairo MIRCEN); Designations: ATCC 6538. Bacteria were grown on nutrient agar (Oxoid) for an incubation period of 24 hours at 37° C in aerobic conditions. After verifying their viability and purity, the colonies were used for turbidity in nutrient broth (Oxoid) and incubated for 8 hours. Turbidity was then adjusted in the 0.5 Mac Farland range (1x10⁸ UFC/mL). Ten microliters of the bacterial suspension were applied on the wound and spread with the aid of a sterile swab to wounds of infected animal groups ¹³.

Rate of wound contraction

The rate of wound contraction was measured as percentage reduction of wound size until wound closure in 16th day of the study. Progressive decrease in the wound size was monitored periodically (every 3 days) using transparency graph paper and a marker. Wound closure was indicated by the formation of new epithelial tissue to cover wound. The wound healing rate was calculated according to ¹⁴ with a formula as following:

$$\text{Wound contraction \%} = \frac{\text{initial wound size} - \text{specific day wound size}}{\text{initial wound size}} \times 100$$

Experimental design:

Ninety healthy male Sprague Dawley rats were assigned into five main groups as shown in Table 1. Each of the main five groups were subdivided into 8th day groups and 16th day groups. At the end of the study, the rats were anesthetized and from the healed wounds, specimen samples of tissue were collected from each rat, leaving a 5mm margin of normal skin around the edges of the healed wound. Blood was collected after exsanguinations for serum preparation. Specimen tissues were stored in 10% formalin solution and preserved for histological examination and the other were homogenized in Tris buffer saline to obtain a 10 % homogenate for assessment of the chosen biochemical parameter used for biochemical studies.

Table 1: Experimental animal groups

Group	Experimental design	
Group I	Normal control group	
Group II	Wounded rats received treatment with either:	Vaseline GSE/ Vaseline
Group III	Wounded rats infected with <i>Staphylococcus aureus</i> followed by treatment with either:	Vaseline GSE/ Vaseline
Group IV	Wounded diabetic rats followed by treatment with either:	Vaseline GSE/ Vaseline
Group V	Wounded diabetic rats infected with <i>Staphylococcus aureus</i> followed by treatment with either:	Vaseline GSE/ Vaseline

Immunological parameters measurements:

Rat TGF- β 1 ELISA Kit (CSB-E04727r), rat IL-10 ELISA Kit (CSB-E04595r) and rat TNF- α ELISA Kit (CSB-E11987r) were used for the measurement of TGF- β 1, IL-10 and TNF- α , respectively, to evaluate the immunological effect of GSE on wound healing in different experimental groups.

Statistical analysis:

Statistical analyses were carried out using SPSS v.15 software. All data were expressed as mean \pm standard deviation (mean \pm SD). The independent variables of individual comparisons were illustrated by Duncan's multiple range test using post hoc test of one-way ANOVA to determine the similarities and differences of mean values between different groups significantly. P values less than 0.05 were considered statistically significant.

RESULTS**Characterization of grape seed**

The chemical composition of grape seeds was represented in table 2. The antioxidant activity of grape seeds was 88.3% with IC50 of 35.7 μ g/ml (Table 2).

Table 2: characterization of GSE

Item	Content
Moisture (%)	9.5 \pm 1.2
Ash (%)	2.3 \pm 0.9
Carbohydrates (%)	29.1 \pm 2.7
Fibers (%)	34.7 \pm 1.9
Protein (%)	12.5 \pm 0.9
Total lipids (%)	11.7 \pm 3.4
Total phenolic compounds (mg gallic acid/ g dry seed)	10.5 \pm 1.6
Flavonoids (mg quercetin/g dry seed)	12.7 \pm 6.1

Macroscopic analysis

Figure (1) presents the macroscopic aspects of the wounds over the time course of the experiment. Control Vaseline / Vaseline grape seed extract treated group (II) showed no sign of abscess formation in the early phase or hypertrophic scars in the final phase (16 days) was observed. The digital images of the wounds permitted to evaluate wound area progression in the groups of the experimental times of days 0, 1, 3, 6, 9, 12 and 16. *Staphylococcus aureus* infected wounds (groups III & V) showed pus that persisted throughout the days of the study in Vaseline treated group that showed big deformed scar in 16th day. Those treated with Vaseline grape seed extract revealed markedly improved wound healing and disappeared pus formation on 6th day that left with clean wound closure on 16th day. Diabetic wounds (groups IV & V) showed delayed wound healing and wound closure in Vaseline treated groups whereas those treated with Vaseline grape seed extract presented enhanced wound healing and wound closure.

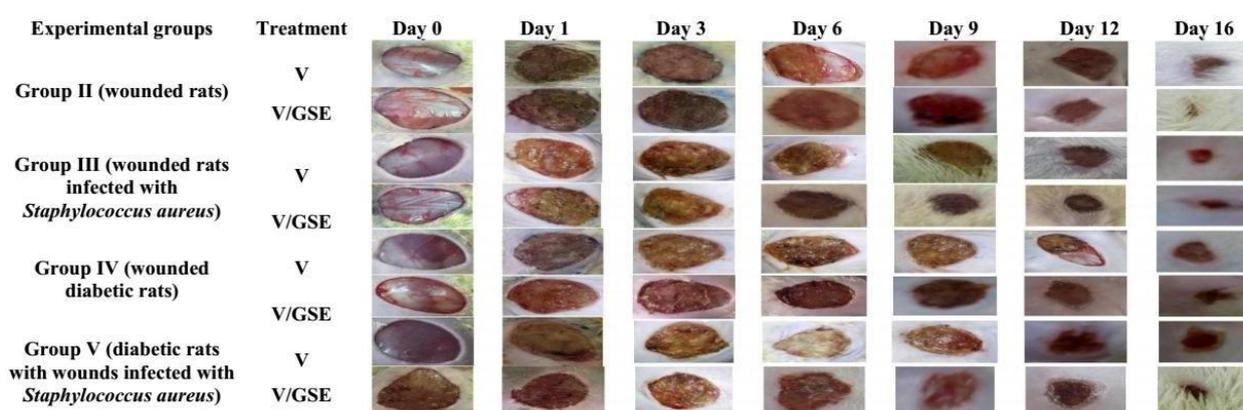


Fig. 1: The gross appearance of wound healing at 0, 1, 3, 6, 8, 12 and 16th day in groups I, II, III, IV and V post-treatment with V only or V/G in all experimental groups. Group I (healthy unwounded control rats), group II (wounded rats), group III (wounded rats infected with *Staphylococcus aureus*), group IV (wounded diabetic rats) and group V (diabetic rats with wounds infected with *Staphylococcus aureus*).

Measurement of cytokines in skin tissue homogenate

Excision wound induction resulted in an increase in skin homogenate content of TNF- α , on the first day in wounded rat groups as compared to control group. This increase reached a peak on 8th day after Vaseline /GSE treatment and subsequently dropped to almost half on the sixteenth day. When compared to uninfected groups, *Staphylococcus aureus* bacterial infection increased the content of TNF- α in wounded infected rats (III) and

also, in wounded infected diabetic rats (V). On the first day, a significant rise in TNF- α content was seen in diabetic *Staphylococcus aureus* infected animal groups (V) compared to control group. TNF- α content kept rising in Vaseline-treated diabetic groups throughout the study period. Treatment with Vaseline /GSE significantly decreased TNF- α content on the 16th day compared to 8th day in all studied groups (Figure 2).

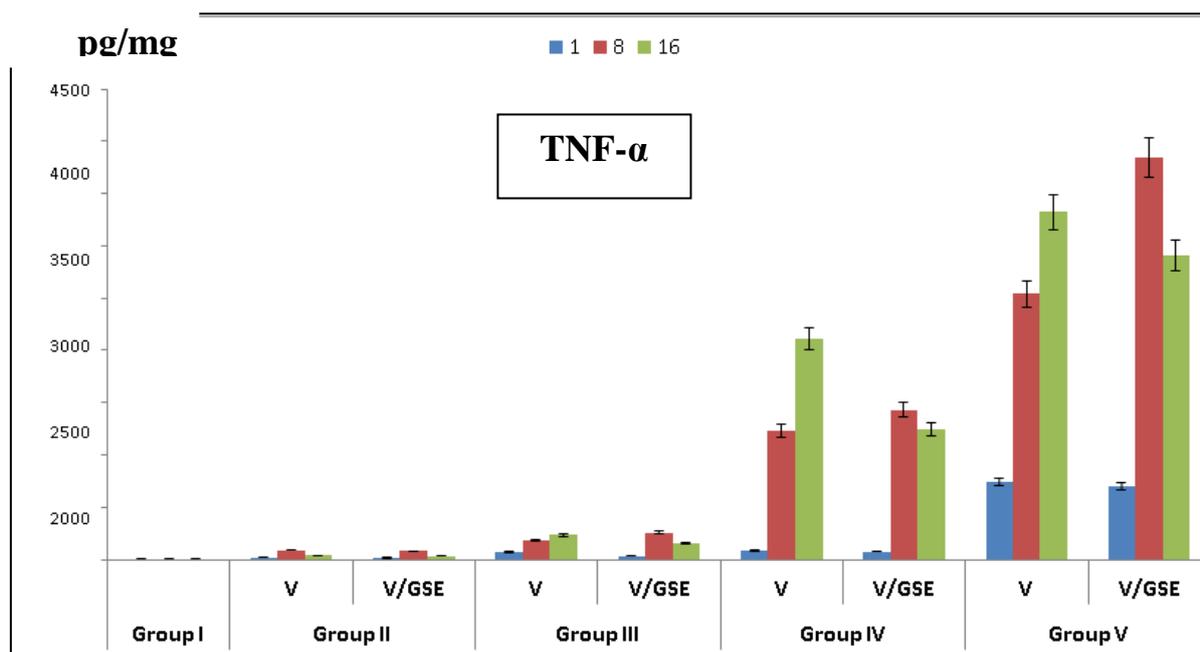


Fig. 2: The levels of TNF- α (pg/mg protein) in the skin homogenate of rat groups on the 1st, 8th and 16th days post treatment with Vaseline (V) or Vaseline / grape seed extract (GSE). Group I (healthy unwounded control rats), group II (wounded rats), group III (wounded rats infected with *Staphylococcus aureus*), group IV (wounded diabetic rats) and group V (diabetic rats with wounds infected with *Staphylococcus aureus*). [Two way Anova]

The skin homogenate content of IL-10 in the healthy control group showed no significant changes during the course of the experiment. Wounded diabetic groups (IV & V) had considerably higher IL-10 levels on the first day than wounded group (II) and wounded *Staphylococcus aureus* infected group (III). On the eighth day, the levels of IL-10 in all wounded groups was considerably greater than those on the first day. When compared to the first day, the IL10 levels of diabetic groups had significantly increased by the

sixteenth day. Only on the 8th day following treatment with Vaseline, the greatest amount of IL-10 was observed in diabetic infected group. Although the greatest statistically peak increase of IL-10 level was seen in all rat groups on 8th day after treatment, it remained higher than the equivalent level on the first day after treatment and totally greater than that of the control group (I). This means that wound induction and infection with *Staphylococcus aureus* has a direct effect on IL-10 level (Figure 3).

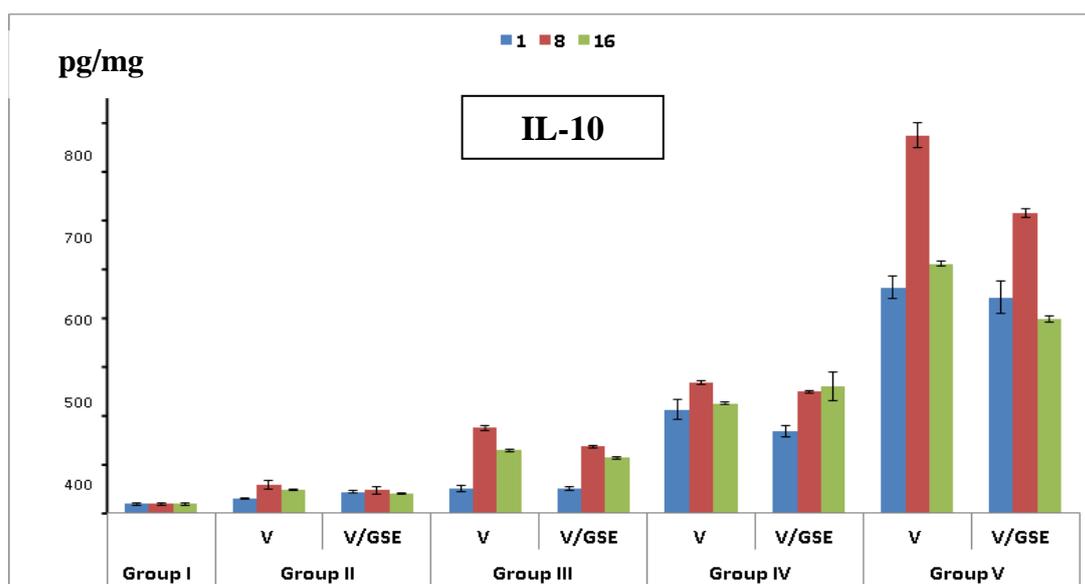


Fig. 3: The levels of IL-10 (pg/mg protein) in the skin homogenate of rat groups on the 1st, 8th and 16th days post treatment with Vaseline (V) or Vaseline / grape seed extract (GSE). Group I (healthy unwounded control rats), group II (wounded rats), group III (wounded rats infected with *Staphylococcus aureus*), group IV (wounded diabetic rats) and group V (diabetic rats with wounds infected with *Staphylococcus aureus*). [Two way Anova]

TGF-β1 levels in group II were similar to those in control group I and wounded diabetic group IV on the first day, but substantially lower than those in group III (rats with infected wound) and group V (diabetic rats with infected wound). TGF-β1 levels in groups II, IV, and V were similar and substantially higher than those in control group I by 8th day, but significantly lower than those in group III. TGF-β1 levels were the highest with Vaseline /GSE therapy. In group V, there were

strong positive associations between time and TGF-β1 levels. On 16th day, the TGF-β1 levels were in the following order: group I= II < IV < III < V. The greatest TGF-β1 level was noticed in *Staphylococcus aureus* infected group III either after treatment with Vaseline (108.50 pg/mg protein) or Vaseline /GSE (129.00 pg/mg protein) on 8th day; however, TGF-β1 level during Vaseline /GSE treatment was the highest (129.00 pg/mg protein) (Figure 4).

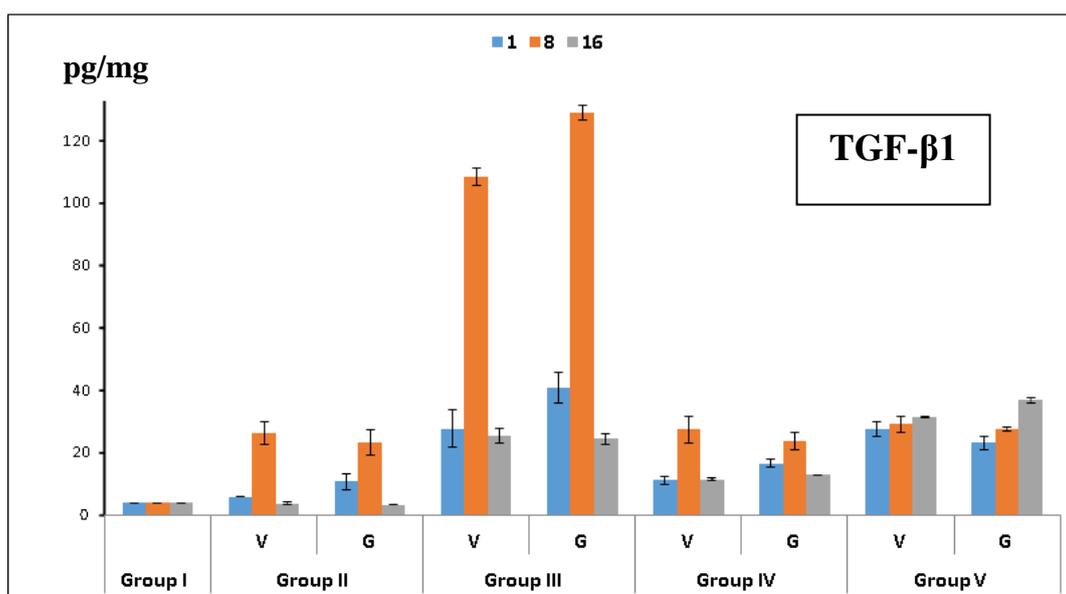


Fig. 4: The levels of TGF-β1 (pg/mg protein) in the skin homogenate of rat groups on 1st, 8th and 16th days post treatment with Vaseline (V) or Vaseline / grape seed extract (GSE). Group I (healthy unwounded control rats), group II (wounded rats), group III (wounded rats infected with *Staphylococcus aureus*), group IV (wounded diabetic rats) and group V (diabetic rats with wounds infected with *Staphylococcus aureus*). [Two way Anova]

DISCUSSION

The present study aimed to evaluate the effect of topical application of grape seed extract (GSE) on *Staphylococcus aureus* infected wound healing of streptozotocin (STZ) induced diabetic rats. Ninety healthy male Sprague Dawley rats were used in the study. Diabetes was induced by injecting animals of diabetic groups with 60 mg/kg streptozotocin (STZ) for the induction of type 1 diabetes¹⁵. Following the induction of diabetes, a full-thickness skin wound was created under anaesthesia. Infected wounds were induced by inoculation with 10 µl of bacterial suspension of *Staphylococcus aureus*.

Throughout the study, the wound area % was monitored together with the blood glucose level. According to our findings, diabetic groups had significantly higher glucose levels than non-diabetic groups during the whole experimental periods. This was in agreement with¹⁶. Furthermore, diabetic rats with infected wounds showed higher blood glucose level than diabetic wounded rats. Diabetes induced relative disorganization of skin wounds. The experimental time and type of treatment had a substantial impact on the wound area percent in different groups of the study. Treatment with V/GSE decreased wound area % more than treatment with Vaseline alone. The significant decrease in wound area from day 8 forward in V/GSE treated groups indicated an early healing process. Early healing was explained by¹⁷ who mentioned that in incision wound, the observed increase in tensile strength of treated wounds may be due to the increase in collagen concentration and stabilization of the fibers.

The present study showed that *Staphylococcus aureus* infection of a wound seriously delayed the healing process by causing the formation of poor-quality granulation tissue, reducing the tensile strength of the connective tissue as well as the loss of epithelization and the appearance of pus and odor. These observations were in accordance with^{18,19}. Contamination with pathogenic microbial flora can lead to infection and sepsis, which disrupts wound healing continuum^{20,21}. Wound microbial infection can induce excessive inflammation²².

The current study revealed significantly delayed wound healing in diabetic animals that was more prominent in diabetic *Staphylococcus aureus* infected animal groups. Diabetic mice exhibited delayed wound closure that was explained by a marked elevation in free radical levels and a prolonged elevation in the levels of inflammatory cytokines, interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) and tumor necrosis factor-alpha (TNF-α).

The Vaseline based grape seed extract used in this study showed a significant increase in the percentage of

wound closure. According to Ejaz et al.,²³ wound contraction accounts around 88% of the healing process. The total phenols, flavonoids, and tannins found in the grape seed have been shown to be important for wound healing and antimicrobial activities²⁴. Also, topical use of grapes seed hydroethanolic extract cause decreases in healing period²⁵. Similarly, Izadpanah et al.,²⁶ results suggest that 5% grape seed extract may have beneficial therapeutic effects in promoting skin CS wound healing. Curing skin lesions with grape seed extract caused proliferation areas with protected boundaries in epithelium, increased cell density and increased deposition of connective tissue at the wound site which in general improves cellular structure in wound^{27,28}.

The existing study gross examination revealed that *Staphylococcus aureus* infected excision wounds were characterized by redness, swelling, purulent exudate, temporally associated with contact bleeding and tissue breakdown. Similar observations were recorded by Healy & Freedman²⁹

In this context, grape seed extract has been shown to promote diabetic wound healing^{30,31}. Vázquez-Armenta et al.,³² applied grape stem extract as disinfectant due to its antibacterial and antioxidant properties. Similarly, Kim et al.,³³ proved the enhanced wound healing by an epigallocatechin gallate-incorporated collagen sponge in diabetic mice; that represent some of the components of grape stem analysis approved by this study. Grape seed extract is said to have potent anti-oxidant properties that protect DNA from oxidative damage³⁴, improved circulation through strengthening arteries and veins, and reduced blood pressure have all been mentioned in several researches^{34,35,36}.

Wound healing is assisted by proinflammatory cytokines such as IL-1, IL-6, and TNF-α. Their production increases keratinocyte and fibroblast proliferation, extracellular matrix protein synthesis and degradation, fibroblast chemotaxis, and immune response control^{37,38}. TNF-α is a cytokine produced by skin cells (keratinocytes, fibroblasts, and endothelial cells) as well as inflammatory cells engaged in wound repair. Skin-resident T cells recognize and respond to infected, stressed, or damaged cells by secreting cytokines IL-1, IL-6, IL-10 and TNF-α and growth factors such as TGF-β that stimulate cellular proliferation, induce cytolysis, and/or activate other cells to infiltrate the affected region, later skin-infiltrating T cells arrive to fight infection and secrete cytokines including IFN-γ³⁹.

In this investigation, it was evident that wound induction resulted in an increase in skin homogenate content of TNF-α, on the first day in wounded rats as compared to control group. This increase peaked on 8th day after treatment and subsequently dropped to almost half on the sixteenth day. When compared to uninfected

groups, bacterial infection increased the level of TNF- α in wounded infected rats and also, in wounded infected diabetic rats. On the first day, a significant rise in TNF- α content was seen in diabetic *Staphylococcus aureus* infected animal groups compared to control group. TNF- α content kept rising in non-treated diabetic groups throughout the study period. Treatment with V/GSE significantly decreased TNF- α content on the 16th day compared to 8th day in all studied groups. Pierce⁴⁰ referred the delayed repair of diabetic wound to the persistently elevated TNF- α . He mentioned that leukocytes in non-healing diabetic wounds trigger inflammatory cell activation, leading to synthesis of pro-inflammatory cytokines such as interleukin-1 β and tumor necrosis factor (TNF)- α .

The skin homogenate content of IL-10 revealed a significant increase in all wounded groups as compared to control group. Peak level was achieved on 8th day of the study and that was considerably greater in diabetic and *Staphylococcus aureus* infected groups. V/GSE application significantly decreased IL-10 content on 16th day. This study demonstrated that wound induction and *Staphylococcus aureus* infection have a direct effect on IL-10 content. These findings were consistent with those of Maniam et al.,⁴¹ who discovered that bacterial infection caused increased levels of cytokines (TNF-, IL-12, and IL-10) in diabetes and nondiabetic rats. IL-10 is also a powerful anti-inflammatory cytokine that affects both the innate and adaptive immune systems.

The present study recorded significantly elevated TGF- β in all skin wounded groups, reaching a peak mostly on 8th day post-wounding, then significantly decreased by 16th day. Similarly, Stadnicki et al.,⁴² found substantially greater TGF- β 1 in active inflammation. A substantial rise in TGF- β 1 level resulted in a considerable decrease in wound area⁴³. Ishida et al.,⁴⁴ investigated the role of TGF- β in wound healing and found that cross-talk between IFN- γ and TGF- β 1 is critical in the skin wound healing process. TGF- β has a dual role in wound healing, acting as a pro-inflammatory factor in the early phases and then helping to the resolution of inflammation later on⁴⁵. The current study showed significantly increased skin content TGF- β in grape seed extract treated diabetic group on 8th day that declined on 16th day.

In conclusion, the current study revealed that Vaseline based grape seed extract successfully treated *Staphylococcus aureus* infected skin wound in diabetic and non-diabetic rats. The Vaseline based grape seed extract topical application to full thickness excision skin wound, can significantly accelerate the wound healing process. Also, Vaseline based grape seed extract topical application depressed tissue TNF- α along with sustaining both the IL-10 concentration and TGF β -1. The formulation possessed anti-microbial properties against *Staphylococcus aureus* infected skin wound groups besides anti-inflammatory effects. The Vaseline

based grape seed extract topical treatment is a promising pharmacological agent for diabetic *Staphylococcus aureus* infected wound.

This manuscript has not been previously published and is not under consideration in the same or substantially similar form in any other reviewed media. I have contributed sufficiently to the project to be included as author. To the best of my knowledge, no conflict of interest, financial or others exist. All authors have participated in the concept and design, analysis, and interpretation of data, drafting and revising of the manuscript, and that they have approved the manuscript as submitted.

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