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Topical Application of *Geniotrigona thoracica* Propolis Nanoparticle for Wound Healing in Streptozotocin-induced Type-1 Diabetic Rat Model

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ABSTRACT

Diabetic ulcers are common complications of diabetes. Herbal products such as propolis may accelerate wound healing because of their biological activity. The development of propolis using nanotechnology is expected to enhance its effectiveness and accelerate its absorption. This study aimed to explore the therapeutic potential of propolis nanoparticles in the treatment of diabetic ulcers in a streptozotocin-induced type I diabetic rat model. Male Wistar rats with diabetic ulcers were treated topically with propolis nanoparticles for 14 days, and outcomes were assessed based on wound closure rate, histological characteristics, and oxidative stress markers. Wound closure was measured on days 7 and 14 post injury. Histological analysis focused on epidermal thickness and subcutaneous tissue formation, whereas oxidative stress was assessed by serum malondialdehyde (MDA) levels at baseline, day 1, and day 7. The results showed that propolis nanoparticles accelerated wound closure by days 7 ($P < 0.05$) and 14 ($P > 0.05$). Histologically, the treated wounds exhibited improved structural restoration, with hair follicles and sebaceous glands. The treated wounds also had a thinner epidermis, indicating controlled proliferation and a reduced risk of abnormal scar formation. MDA levels were lower in the propolis-nanoparticle-treated group than in the untreated group ($P > 0.05$). Propolis nanoparticles demonstrated potential in promoting early-stage wound healing in diabetic rats, suggesting they could be a valuable addition to the therapeutic options for diabetic ulcer management.

Keywords: Propolis, Nanoparticle, Stingless bee, *G. thoracica*, Diabetic wound.

Introduction

Diabetes mellitus is a metabolic disease characterized by decreased insulin synthesis or action, which results in elevated blood sugar levels commonly called hyperglycemia.[1] Based on data from the World Health Organization, the disease was ranked among the top 10 causes of death worldwide, with approximately 537 million adults aged 20-79 years in 2021.[2,3] According to the American Diabetes Association, a diabetic condition is reached at random blood glucose levels above 200 mg/dL. [4] Hyperglycemic conditions in diabetic patients often lead to comorbidities that affect multiple organs and significantly prolong wound healing.[1] Among which diabetic ulcers are the most common, particularly in patients who are not properly treated. These ulcers have an incidence risk of 19%–34% among patients with diabetes. Additionally, untreated chronic ulcers can lead to the risk of amputation.[5–7]

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Diabetic ulcers have a longer inflammatory phase of wound healing than non-diabetic ulcers.[8] When inflammation persists without adequate treatment, healing is delayed, often leading to chronic ulcerative conditions.[9] Applying ulcer care to reduce inflammation is vital to aid recovery.[10] For diabetic ulcers, it is crucial to minimize the healing duration to lower the risk of infection and reduce overall treatment costs. By speeding up recovery, the chances of complications such as infections decrease, along with the need for extended medical interventions. This approach lessens the financial burden on both patients and healthcare systems.[11] Herbal products have the potential to accelerate the wound healing process owing to their biological properties.[11] Natural or herbal medicines have gained popularity because of their advantages over conventional medical therapies. These benefits include accessibility, cost-effectiveness, and minimal side effects.[12,13] For example, propolis, a natural substance produced by bees, is composed of various plant components mixed with pollen, wax, and enzymes.[14] Stingless bee generally lives in tropical forests with several species of above 500 worldwide discovered in some subtropical areas.[15] Among the widely cultured species is *Geniotrigona thoracica,* known to be the largest among others and is distributed across Cambodia, Thailand, Myanmar, Brunei, Malaysia, Singapore, and Indonesia.[16–19] Stingless bees may produce up to 6 times the amount of propolis as honey bees.[20] The two primary constituents of its propolis are phenolics and flavonoids, which possess anti-inflammatory and antioxidant properties with significant implications for wound healing. These compounds are frequently present in higher concentrations than in honeybee propolis.[21,22] Despite the acknowledged anti-inflammatory and antioxidant properties of propolis, the use of *G. thoracica* propolis

nanoparticles (GTPN) in enhancing diabetic wound healing remains underexplored. This study aims to bridge this gap by providing empirical evidence on its efficacy and mechanisms of action. GTPN was produced using a top-down method that combined melt-emulsification with ultrasonication. However, this study conducted an *in vivo* experiment to investigate the effectiveness of GTPN in topical diabetic wound treatment. The assessment was performed based on wound healing time, histological appearance, and oxidative stress level.

Materials and Methods

G. thoracica Propolis Nanoparticle Preparation

GTPN was produced at the Faculty of Pharmacy, Padjadjaran University, using a top-down method that combined melt-emulsification with ultrasonication. In this study, the propolis nanoparticles used were sourced from stingless bees, such as *G. thoracica*, harvested from bee cultivation sites in Kebun Efi, North Sumatra, Indonesia (3° 0' 29.908" N, 98° 28' 5.797" E) in July 2022 (batch number 0722). The propolis was identified by Felix Zulhendri and deposited at Kebun Efi, where it can be made available upon request.

Propolis extraction from raw materials was performed using the maceration method with 95% ethanol for 48 hours. The use of 95% ethanol aimed to maximize extraction yield. From an initial sample weight of 5.001 g, a total extract of 2.415 g was obtained, resulting in an extraction yield of 48.24%. Based on phytochemical screening, the extract contained phenolics, tannins, flavonoids, saponins, and triterpenoids.

GTPN was prepared using a nanostructured lipid carrier (NLC) drug delivery system. In this study, the water phases adopted were 2.06 g polysorbate 80 (Tween 80) and phosphate buffer (pH 7.4) added to 20 mL, whereas the lipid phases were 0.9 g propolis extract and 0.24 g lecithin, resulting in a 4.5% w/v propolis concentration. Based on the Particle Size Analysis characterization test, GTPN had an average particle size of 72.7 nm with a polydispersity index of 0.211 and a zeta potential of -5.1 mV.[23]

Animals and Diabetes Induction

A total of 30 sexually matured male Wistar rats, aged ranging 12-15 weeks and weighing approximately 200-300 grams, were obtained from the Biofarma Animal Breeding Facility (Bandung, Indonesia). The animals were acclimated to cages (40 cm × 20 cm × 20 cm) with wood shavings as bedding and maintained under standard laboratory conditions (21-25^{o}C, 70% relative humidity, and 12 h light/dark cycle). Feed and drink were provided *ad libitum*, while procedures were performed according to the 5F (5 of Freedom) and 3R principles. This study was approved by the Padjadjaran University Research Ethics Committee (approval number 216/UN6).KEP/EC/2024.

The samples were divided into six treatment groups: a control group (A), normal rats administered GTPN (B), untreated diabetic rats (C), diabetic rats administered PVP-I (D), diabetic rats administered normal propolis at a concentration of 10% w/v (E), and diabetic rats administered GTPN (F). All treatments were topically administered at 0.05 a concentration of.

To create a model of type I diabetes mellitus in rats, this study utilized an induction method including repeated low doses of streptozotocin (STZ) from Cayman Chemical, administered at 50 mg/kg body weight.[24] STZ was diluted in a mixture of dimethyl sulfoxide and sterile water before being injected intraperitoneally. Random blood glucose levels of the rats were periodically monitored using an Autocheck Multi-Monitoring System. Blood samples were collected from the tail vein every two days following STZ injection and administered three times. Diabetes was defined as a random blood glucose level of 200 mg/dL.

Wounding and Macroscopic Observation

Wound preparation was conducted under anaesthesia using a combination of xylazine and ketamine at 75 and 125 mg/kg body weight. Subsequently, the hair was cut into an area of 2 × 2 cm, and the skin was cleaned with 70% alcohol before being wounded using a 6 mm biopsy punch at a full-thickness depth. The wound was cleaned with blood, photographed, treated accordingly, and covered with a flexible dressing made of non-woven polyester (Fixomull Stretch).

The treatment was administered daily for 14 days, with careful attention given to each step to ensure optimal healing conditions during the study. Each day, the process included careful removal of the existing dressing, thorough cleaning of the wound area, and reapplication of treatment. The procedures comprised cleaning the surrounding skin with normal saline, photographing the wound, administering 0.05 mL of the respective topical treatment using a 1 mL syringe, and finally closing with a fixomull stretch.

The wound closure rate was determined by measuring the reduction in diameter over time. The diameter was measured using the ImageJ software based on the photographed area. The percentage of closure was calculated by subtracting the final wound diameter from the initial wound diameter and dividing the result by the initial diameter.

Histological Examination

A histological examination of the ulcer tissue was conducted. Histological slides were prepared using the paraffin embedding method, followed by Hematoxylin and Eosin (HE) staining. The healed wound samples were observed under a microscope at magnifications ranging from 100x to 400x.

Malondialdehyde (MDA) Analysis

MDA analysis was performed to assess oxidative stress levels by measuring their concentrations in the blood serum of the rats. Blood samples were collected from the tail vein on days 0, 1, and 7 after injury to monitor changes in the levels of this organic compound over time. The analysis relies on the reaction between MDA and Thiobarbituric Acid (TBA), forming a colorimetric product that can be quantitatively measured using a spectrophotometer. MDA analysis was conducted using a Sigma-Aldrich kit (code MAK085-1KT).[25] The data obtained provide a detailed measure of lipid peroxidation and oxidative damage in the wound healing process. [26] MDA levels were used as a biomarker to assess the extent of oxidative damage. This allows for the evaluation of how the treatments affects oxidative stress, providing a basis for further understanding its role in potentially mitigating oxidative damage during the healing stages.

Statistical Analysis

The data obtained were analyzed using the One-Way Analysis of Variance (ANOVA) test, followed by Tukey and Dunnett tests to compare the averages of all pairs of treatment groups. Data analysis was performed using GraphPad Prism software, version 8.0.2.

Results and Discussion

Diabetic rats were successfully induced using streptozotocin at a dose of 50 mg/kg body weight, resulting in random blood glucose levels exceeding 200 mg/dL, as shown in Table 1. The traits observed were increased urine output, which led to bedding becoming wet and soiled more rapidly, a dirtier hair colour, and bedding with a distinct sweet or fruity scent.

Table 1: Average Random Blood Glucose After Streptozotocin Induction

Groups	Average Random Blood Glucose (mg/dL)
A	94.25
B	87.50
C	351.50
D	403.50
E	335.00
F	421.25

The experiment was conducted for 14 days, and the wound was documented on day 0, 7, and 14, as shown in Figure 1. In this study, PVP-I was used as the benchmark for comparison. This is because it

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possesses a broad antimicrobial spectrum, anti-inflammatory properties, and low cytotoxicity.[27]

The wounds of untreated diabetic rats showed signs of slight infection, characterized by a yellowish colour, indicative of exudate. Exudated tissue is classified as necrotic, containing dead cells and dirt that need to be cleaned.[28] Persistent infection, often associated with biofilms formed by *P. aeruginosa* and *Staphylococcus* biofilms can lead to chronic wounds that are difficult to cure.[29] Additionally, *G. thoracica* propolis showed antibacterial qualities against bacteria such as *B. subtilis*, *S. aureus, E. coli*, and *P. aeruginosa*, which frequently infected diabetic wounds.[15,30]

The wound treated with GTPN tended to be red-pink, indicating a proliferation or granulation stage. At this stage, the wound was stronger, less prone to bleeding, and painless. The pink hue was attributed to remodeling or epithelialization, which is the final stage of healing.[28] Propolis is believed to enhance tissue repair by promoting re-epithelialization and reducing inflammation through the restoration of the natural flow of neutrophils and macrophages into the injured region.[31,32]

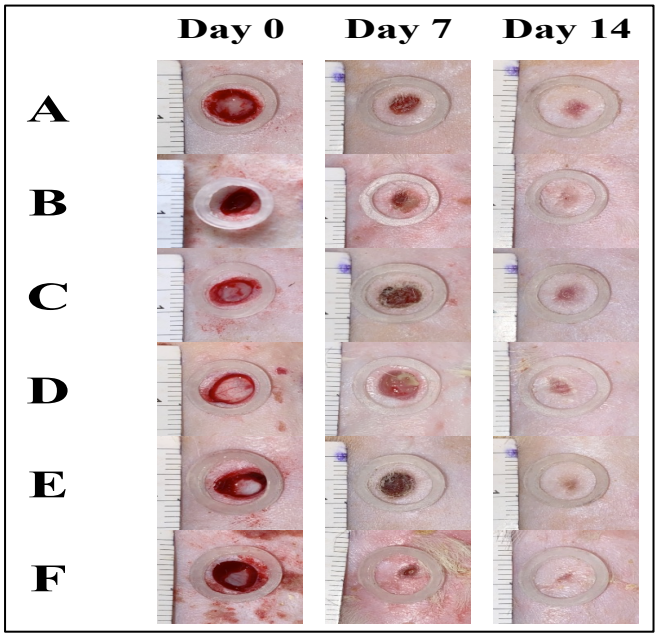

Figure 1: Wound closure. (A) Control group; (B) non-diabetic with GTPN group; (C) untreated diabetic group; (D) diabetic with PVP-I group; (E) diabetic with normal propolis group; (F) diabetic with GTPN group.

The highest percentage of wound closure on day 14 was achieved in the normal GTPN group (79.65%). On the same day, the lowest value was observed in the untreated diabetic group at an average of 63.48%, as shown in Figure 2. ANOVA showed a significant difference among the averages (ρ= 0.007) on day 7. Further analysis was conducted using Dunnett's multiple comparisons test between the treated (B, D, E, and F) and untreated groups (A and C). The diabetic group treated with PVP-I and GTPN (D and F) showed a significant difference compared with the untreated diabetic group (C). The group treated with GTPN showed better wound closure on day 14, with no significant difference. A study by Mujica et al.[33] showed good results in diabetic wound healing of foot ulcers after the daily administration of 3% propolis in propylene glycol. Nano-sized GTPN particles also helped accelerate absorption, ensuring rapid healing. It is important to acknowledge that the particle size has a significant influence on the solubility, absorption, and distribution of drugs. A smaller drug particle size results in a higher surface area to volume ratio, which increases the rate of dissolution and accelerated absorption.[34]

Microscopic observations were then conducted on histological sections of healed wound tissue on day 14. These observations included the measurement of epidermal thickness and the formation of subcutaneous tissue in the wound area.

The epidermis of the skin on the healed wound had different thicknesses. Groups B, E, D, and F had a thinner epidermis of 72 µm compared to A and C at 42–43 µm, as shown in Figure 3. ANOVA revealed significant differences in epidermal thickness ($P < 0.0001$). Groups B, E, D, and F showed significant differences from the untreated groups (A and C), without any significant difference from each other.

Epidermis thickening on healed skin was indicative of excessive proliferation and the potential for abnormal scars such as keloids or hypertrophic wounds.[35] The high flavonoid content in propolis was known to reduce the formation of hypertrophic wounds.[36]

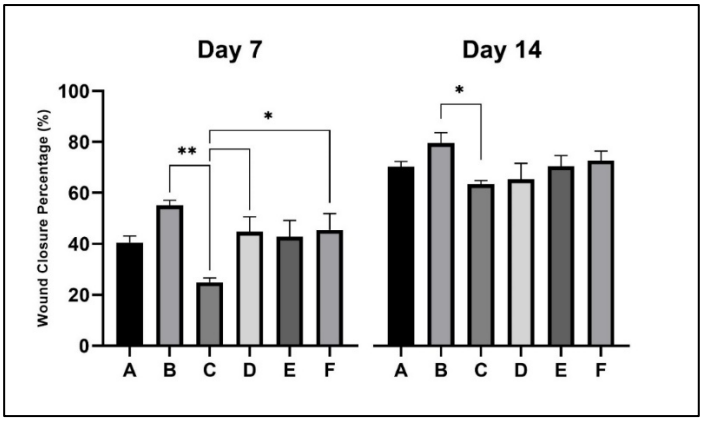

Figure 2: Wound closure percentage. (A) Control group; (B) non-diabetic with GTPN group; (C) untreated diabetic group; (D) diabetic with PVP-I group; (E) diabetic with normal propolis group; (F) diabetic with GTPN group

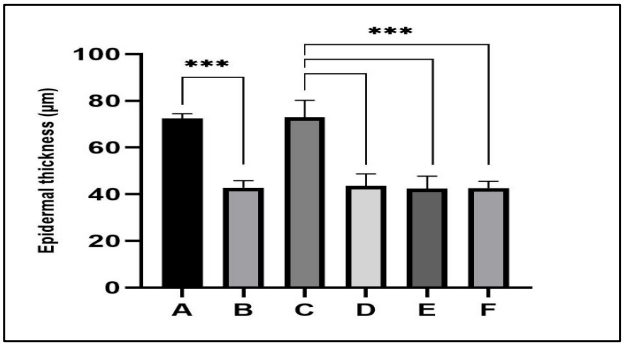

Figure 3: Epidermal wound thickness (A) Control group; (B) non-diabetic with GTPN group; (C) untreated diabetic group; (D) diabetic with PVP-I group; (E) diabetic with normal propolis group; and (F) diabetic with GTPN group.

Propolis has been observed to promote epithelialization and tissue remodeling by modulating the expression of growth factors and antioxidant molecules.[37] A study showed a thinner epidermal layer in a wound treated with topical application of Brazilian propolis.[35] The nanoparticles were discovered to enhance healing by inducing blood vessel and collagen formation, leading to faster closure and improved tissue regeneration.[38] Nano-sized particles may provide some advantages such as enhanced penetration to the wound, improved bioavailability, and increased surface area, which contributes to better therapeutic effects and wound healing outcomes.[37]

The groups treated with GTPN (B and F) and normal propolis (E) developed hair follicles and sebaceous glands, slightly more new blood vessels, and fewer inflammatory cells than the other groups (Figure 4). Studies have observed that regions with hair follicles typically recover more quickly.[39] Sebaceous glands are present on nearly the entire body's surface, and are responsible for producing and secreting sebum that protects the skin. However, these glands do not directly signify better wound healing. However, maintaining the skin barrier and contributing

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to overall skin health is crucial for facilitating an optimal environment for healing.[40] The groups treated with propolis (B, E, F) also showed fewer inflammatory cells and developed several blood vessels. A study by Tamara et al.[41] stated that flavonoid extracts from *G. thoracica* propolis reduced neutrophils on days 3 and 5 of periodontitis induction. Several types of saponins were known to accelerate wound healing, improve the proliferation of epithelial cells, decrease inflammation, and promote vascularization.[22] Triterpenoids contained in the propolis also contribute to the process of wound healing. Based on several studies, triterpenoids are known to speed up wound closure, regulate the production of reactive oxygen species (ROS), and promote cell proliferation.[42] These compounds can also enhance healing and improve the appearance of wounds.[43]

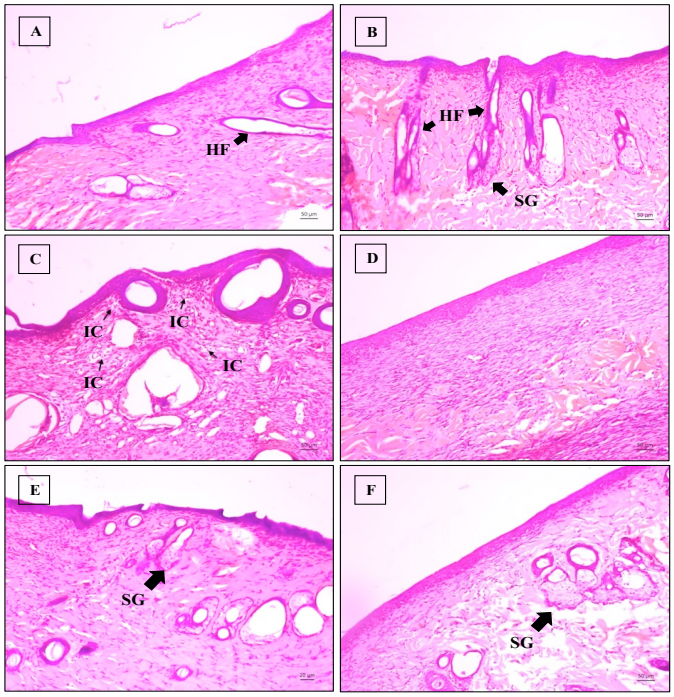

Figure 4: Representative histological examination at 200x magnification. (A) Control group; (B) non-diabetic with GTPN group; (C) untreated diabetic group; (D) diabetic with PVP-I group; (E) diabetic with normal propolis group; (F) diabetic with GTPN group. SG, sebaceous gland; HF, hair follicle; IC, inflammatory cells.

Oxidative stress levels in the blood were assessed using MDA as a biomarker. Both the diabetic condition and the presence of wounds are known to induce oxidative stress.[44,45] ROS are crucial for cell survival, differentiation, signaling, as well as cell death, and are closely associated with inflammation.[46–48] These species are formed from cellular processes such as mitochondrial electron transport and aerobic respiration, as well as inflammatory processes that can cause oxidative stress.[46,49,50] MDA is a byproduct of lipid peroxidation resulting from the reaction of ROS with fatty compounds discovered in cell membranes and is often used as an oxidative stress marker.[51]

In this study, oxidative stress was measured using the MDA parameter obtained from the blood serum. MDA levels in the control and untreated diabetic groups (A and C) continued to increase from day 1 to day 7 after injury, whereas a decrease was observed in the treated groups on day 7 (B, D, E, and F). The treatment groups (B, D, E, and F) also showed a smaller increase compared to the untreated group (A and C) on day 1 after injury, as shown in Figure 5. There was no significant difference between the treatments (p = 0,123). However, high antioxidant activity in propolis reduced the levels of oxidative stress, as observed from the lower levels of MDA compared to the untreated group. The normal group treated with GTPN had the best outcome in lowering MDA levels. Based on the results obtained, GTPN was comparable to PVP-I and normal propolis at higher concentrations (10% for normal propolis vs. 4.5% for GTPN).

Previous analyses showed the presence of phenolics, tannins, flavonoids, saponins, and triterpenoids in the *G. thoracica* propolis extract.[23] These compounds contained in GTPN may aid wound healing. It was important to acknowledge the dominance of phenolics and flavonoids in stingless bee propolis.[21] They may serve as anti-inflammatory and antioxidant which strongly influence the process of wound healing.[22] Tannin has been shown to play a significant role in promoting wound healing, including antioxidant, antimicrobial, and anti-inflammatory activity.[52]

Propolis was reported to lower blood serum MDA levels and inflammation in a rat model of sepsis and diabetes.[53,54] This is because of the anti-inflammatory, antioxidant, and immunomodulatory properties that prevent the production of inflammatory cytokines and secondary mediators.[54,55] Another study confirmed that the administration of propolis to a rat model of skin graft significantly reduced MDA levels. The antioxidant content of propolis such as terpenoids, flavonoids, and cinnamic acid, contribute to reducing ROS levels in the body.[56]

The application of GTPN demonstrated significant benefits in the early stages of wound healing. The enhanced wound closure rate observed on day 7 aligns with the histological improvements seen in treated wounds, particularly the presence of hair follicles and sebaceous glands. These findings suggest that propolis not only accelerates the initial wound healing process but also promotes more complete tissue regeneration, which is critical for restoring normal skin function. The presence of these structures in propolis-treated wounds indicates a more advanced level of healing compared to the untreated group, where such features were less developed.

Although the reduction in MDA levels in GTPN-treated wounds was not statistically significant, this trend suggests a potential for oxidative stress mitigation. This observation warrants further investigation, particularly with larger sample sizes or different concentrations of GTPN, to fully elucidate the antioxidant capacity of GTPN in the context of diabetic wound healing.

The lack of a significant difference between normal propolis and GTPN suggests that the nanoparticle formulation may not provide additional benefits under these experimental conditions. This could be due to the effective bioavailability and penetration of normal propolis in this model, which might already deliver sufficient bioactive compounds to support the healing process. Further optimization of the dosage for GTPN could help to reveal any subtle differences in efficacy that were not detected in this study. Our findings suggest nanoparticle formulations may allow for lower dosages while maintaining or improving therapeutic outcomes due to their enhanced delivery efficiency.

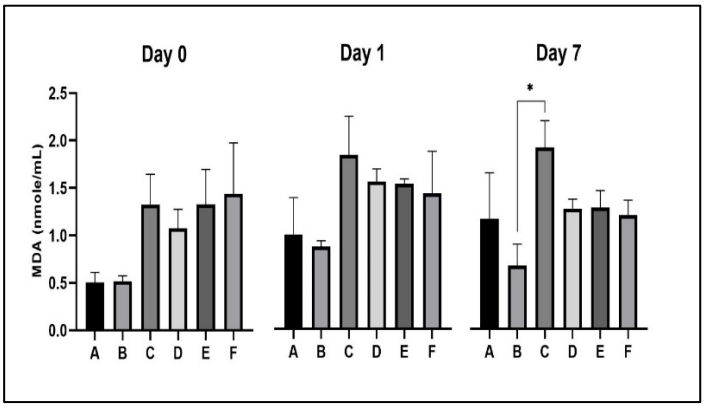

Figure 5: Malondialdehyde levels before (day 0) and after wound (day 1 and day 7). (A) Control group; (B) non-diabetic with GTPN group; (C) untreated diabetic group; (D) diabetic with PVP-I group; (E) diabetic with normal propolis group; (F) diabetic with GTPN group.

Conclusion

In conclusion, GTPN significantly improved wound healing in diabetic ulcers, particularly in the early stages, as evidenced by faster wound closure and better tissue regeneration. The presence of hair follicles and sebaceous glands in treated wounds suggests that propolis supports better healing, with a reduced risk of abnormal scar formation. The decrease in oxidative stress markers was not significant but indicated a potential antioxidant benefit of GTPN. The results of the treatment with GTPN were comparable to those of PVP-I and normal propolis at higher concentrations. These results confirm the potential of propolis nanoparticles as a promising therapeutic option for diabetic ulcers. Further studies are needed to optimize dosage and application frequency for enhanced efficacy.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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