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The Effects of Propolis-Incorporated Nanofibers Produced by the Electrospinning Method on Skin Wounds in Rats

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Abstract

Electrospinning is a widely used process in various industries to create polymeric fibers with unique properties. In the context of wound healing, electrospun nanofibers can mimic the extracellular matrix structure, promote tissue regeneration, and enhance the wound healing process. Propolis, a natural substance with various biological properties, has shown potential in promoting healthy skin and wound healing. It has antioxidant, anti-inflammatory, antibacterial, antifungal, and antiviral effects. The study was conducted on male Wistar albino rats. The rats were divided into three group. The nanopropolis group received nanopropolis applied once daily, while the ethanol extracted propolis group received applied once daily. The control group did not receive any application after the wound was formed. The researchers evaluated the wound sizes throughout the study period. Macroscopically, a gradual healing was observed in all three groups. On the 11th day, the wounds in the nanopropolis and propolis groups healed completely, while the wounds in the control group healed on the 14th day. When the wound sizes were analyzed, the nanopropolis group showed a significant decrease in wound size compared to the control group. Histopathological analysis was performed on the wound samples collected at the end of the study. Microscopically, it was observed that the epidermis layer was more regular in the propolis and nanopropolis groups compared to the control group. In conclusion, the results of this study suggest that propolis-incorporated nanofibers produced by electrospinning (nanopropolis) have a positive effect on wound healing compared to propolis alone and the control group. The nanopropolis group showed a significant reduction in wound size and improved histopathological parameters. These findings highlight the potential of nanopropolis in promoting wound healing and tissue regeneration.

Keywords: Experimental, healing, nanopropolis, polyethylene oxide.

INTRODUCTION

Electrospinning is a commonly used electrohydrodynamic technique in a variety of industries to create polymeric fibers with diameters ranging from a few nanometers to a few microns (Luraghi et al., 2021). The technique involves the application of an electric field to a polymer solution or melt, which leads to the formation of ultrafine fibers through a process of electrostatic stretching and solidification. The process starts with a polymer either in the form of a solution or melt, is prepared. Polyvinyl alcohol, chitosan, polyurethane, poly (lactide-co-glycolicacid), silk fibroin, polyvinylpyrrolidone (PVP), cellulose acetate, and some other polymers have been used in production of nano/microfibers by electrospinning to formulate wound dressings and similar products (Tan et al., 2015). The polymer solution is essential for the formation of the fibers (Liu et al., 2021).

Electrospun nanofibers find applications in various fields, including biomedical, energy, environmental, and filtration. They have shown promise in tissue engineering scaffolds, drug delivery systems, supercapacitors, sensors, and air filtration, among others (Li and Xia, 2004). Electrospinning has shown great potential in the field of wound healing by fabricating nanofibrous scaffolds that mimic the extracellular matrix (ECM) structure of natural tissues. These scaffolds can support cell growth, tissue regeneration, and wound healing processes. Electrospun nanofibers can be designed to have a high surface area and porosity, which facilitates the absorption of wound exudates and creates a moist environment, known to be beneficial for wound healing. The nanofiber matrices can be functionalized with bioactive molecules like growth factors, antimicrobial agents, and extracellular matrix proteins to enhance the wound healing process (Sill and Von Recum, 2008). The porous structure of electrospun nanofiber scaffolds facilitates the diffusion of nutrients and oxygen, promoting angiogenesis and tissue regeneration at the wound site. This property is crucial for wound healing and tissue repair (Deeken and White, 2011)

Propolis is an important bee product created by honeybees by collecting resins and secretions of plants and mixing them with wax, pollen and their own enzymes. It contains more than 300 active compounds and these compounds give propolis many biological activities. Propolis is used as a building material in the hive and protects the hive against bacterial and fungal infections. In

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vivo studies on propolis have reported that propolis contains many components including flavanoids and hydroxycinnamic acids. Thanks to this content, propolis has been found to have antifungal and antibacterial properties. Propolis has been used in the treatment of wounds since ancient times (Bonvehi and Coll, 2000; Oryan et al., 2018).

Wound healing is a multi-stage process that depends on both internal and external factors. During the healing process, bacterial infections accompany the skin damage and delay the healing time. To eliminate these negative effects, antibiotics are often included in the treatment. However, recently, antibiotic-resistant strains have been developing as a result of excessive and incorrect use of antibiotics. Propolis, on the other hand, is effective on wound healing because it is a natural substance produced from bees and has antibacterial properties. In addition, propolis accelerates the process of reepithelialisation in histology (Medellin-Luna, 2019; Rojczyk, 2020).

Propolis has been used in skincare for its potential benefits in promoting healthy skin and addressing various skin concerns. According to studies by Schnitzler et al. (2010); Seven et al., (2011); Funakoshi-Tago et al., (2015); Gul Baykalir et al., (2016) and Shokri et al., (2017) propolis has a number of biological properties, including antioxidant, anti-inflammatory, antibacterial, antifungal, and antiviral effects. Due to its tissue-regenerating properties, propolis can aid in wound healing and promote tissue repair. It can be applied to minor cuts, scrapes, and wounds to support the healing process (Przybyłek and Karpiński, 2019)

The aim of this study is to investigate the effects of propolis-incorporated nanofibers produced by the electrospinning method on skin wounds in rats and to explore the potential benefits of propolis in wound healing. The study aims to compare the effects of propolis and nanofiber-based propolis on experimentally created wounds to identify any differences in their impact on wound healing.

MATERIALS AND METHODS

Animals

For the study, a total of 24 male Wistar albino rats weighing 230-250 g were used, with 8 animals in each group. The Bursa Uludag University Experimental Animals Application and Research Center provided the rats. The study was conducted with the approval of the Bursa Uludag University Animal Experiments Local Ethics Committee under permit number 2019-06/02. The rats were kept in a 22°C, 12-hour cycle of light and darkness, with unlimited access to food and water.

The Prepation Propolis

The propolis utilized for this investigation was propolis number 30, collected using a propolis trap in summer from the village of Edirne-Uzunköprü-Aslıhan village. Propolis that had been collected was reddish in hue. Prior to being ground into a powder using a mill (Lavion grain mill), the entire propolis was first frozen at a temperature of -20°C. Propolis was extracted using ethanol (70%) and water (30%). 400 grams of propolis were combined with one liter of a 70% ethanolic solvent, and the mixture was then allowed to stand in an orbital shaker for ten days. It was submerged in an ultrasonic bath twice daily for 30 minutes each during its time in the orbital shaker. To obtain the extract, the obtained mixture was filtered through Whatman No. 1 filter paper (Sorucu and Oruc, 2019). The extract was sent to the Department of Textile Engineering at Bursa Uludag University order to produce nano-tissue**.**

Techniques for Electrospinning and Nanopropolis

In this work, polyethylene oxide (PEO) (Mw: 900 kDa, Sigma Aldrich) was used as the polymer and propolis was used as the active ingredient to be added to the polymer solution. There was no pre-treatment applied to the materials used in the investigation. A polymer solution was first created, and 3% PEO by weight was added to it. For this, the PEO polymer was thoroughly dissolved in a 1:1 mixture of ethanol and water using a magnetic stirrer. The prepared solution was then combined with a propolis: ethanol solution, with propolis constituting 1% of the total weight of the PEO solution. On a magnetic stirrer, it was mixed for roughly 24 hours to produce the final product.

Measurements and Model for Wounds

Rats were anesthetized with 10 mg/kg of Xylazine hydrochloride (Rompun, Bayer 23.32 mg/mL) before receiving injections of 70 mg/kg of Ketamin hydrochloride (Ketalar, Parke-Davis, 50 mg/mL) intramuscularly to create a skin wound model. On the dorsal side, a 3 cm line of shaving encircled the intrascapular area. After cleaning the shaving area with povidone-iodine, a full-thickness skin wound model was created using a 5mm punch trephine. Up until the day of sacrifice, the wound site was measured and photographed after the procedure. The images were imported into a computer environment and loaded into the "Image j" (Wayne Rasband National Institutes of Health, USA) program to calculate the wound surface areas. The wound areas was calculated in square millimetres.

Experimental Design

The animals were divided into three groups of eight each. The applications were filled out in the groups in the manner described below; Control Group: Until the day of the sacrifice, no application was made after the wound had formed. Nanopropolis Group: Following the development of wounds, nanopropolis was applied just once every day up until the day of sacrifice. Ethanol extracted propolis: From the time the wound first appeared, it was applied once daily until the day of the sacrifice.

Histopathologic Analyses

The rats were put to death by cervical dislocation under anesthesia 15 days after the corneal wound was made. Each euthanized rat had a sample of tissue from a wound removed. The panniculus layer and the surrounding full-thickness skin were also removed from the body for this reason. For histopathological analysis, the collected samples were fixed in a 10% formaldehyde solution. Following fixation, the samples were dehydrated using a series of alcohol and xylene before being placed on paraffin blocks. Hematoxylin-eosin was used to stain 4 m sections from these paraffin blocks, which were then examined under a microscope.

Statistical analysis

Statistical evaluation of the data was performed using SPSS 26 statistical package programme (Inc., Chicago, II, USA). The conformity of the variables to normal distribution was analysed by analytical methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). One-way ANOVA test was used for comparison between groups for wound area. In cases where there was a significant

difference between groups in variables, pairwise post-hoc comparisons were made using the Tukey test. Kruskal-Wallis test was used for histo-pathological examination. Paired comparisons of variables were performed using the Mann-Whitney U test and evaluated using Bonferroni correction. P-values below 0.05 were considered as statistically significant results.

RESULTS

The Content of Propolis

The LC-MS/MS method was used to analyze 15 phenolic compounds in propolis. The phenolic compounds which ware contained in propolis; apigenin 8419 µg/ml, caffeic acid phenethyl ester 5731 µg/ml, trans-ferulic acid 4380 µg/ml, gallic acid (not detected), caffeic acid 6967 µg/ml, kaemferol 443 µg/ml, quercetin 5956 µg/ml, lutein 590 µg/ml, p-coumaric acid 945 µg/ml, naringenin 1333 µg/ml, pinosembrin 10736 µg/ml, p-coumaric acid 2869 µg/ml, rutin (not detected), trans-cinnamic acid 2300 µg/ml (Sorucu and Oruc, 2019).

Wound Sizes

The wounds were followed macroscopically throughout the study. All rats were photographed daily throughout the study (15 days) and the wound area was calculated in square millimetres (Figure 1).

When compared macroscopically, a gradual healing was observed in 3 groups. On the 11th day, it was observed that the wounds of the rats in the ethanol extracted propolis and nanapropolis group healed completely, while the wounds in the control group healed on the 14th day (Table 1).

When the wound sizes were analysed, there was no significant difference between the groups on day 0 ($p>0,05$). Wound sizes in the nanopropolis group decreased more than the control group in the first 10 days. It also decreased more than the propolis group on days 1 ($p<0.01$), 2 ($p<0.001$), 3 ($p<0.001$), 4 ($p<0.01$), 5 ($p<0.001$) and 7 ($p<0.01$) (Table 1).

The average values of the wound sizes of the groups were taken and the percentage healing amounts were calculated. According to the percentage healing amounts, the nanopropolis group consistently healed more than the other groups. When the control and propolis groups were compared within themselves, the propolis group healed more in percentage after the 3rd day (Figure 2).

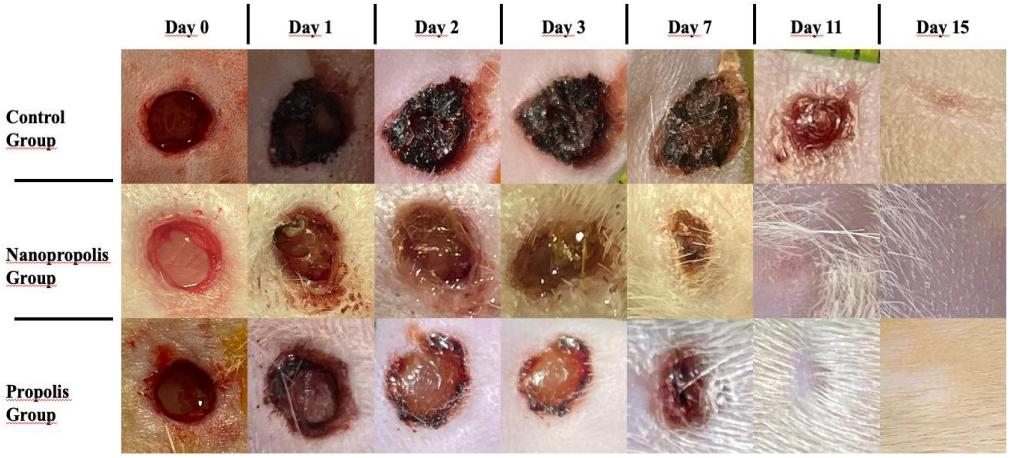

Figure 1. Macroscopic wound size.

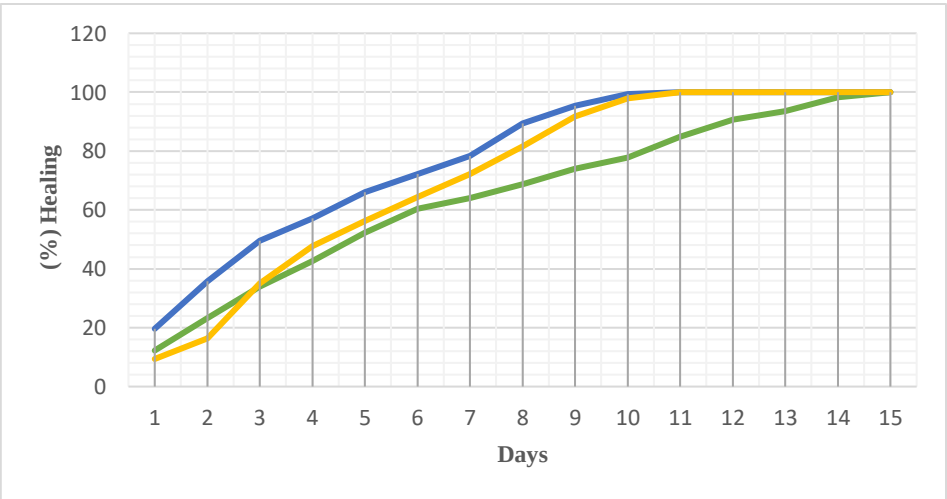

Figure 2. Healing percentages of wound sizes.

Table 1. Wound areas (wound areas was calculated in square millimetres).

Days	n	Control		Nanopropolis		Propolis		P-value
		Mean	S.E	Mean	S.E	Mean	S.E	
Day 0	8	20.71	0.46	20.42	0.68	20.48	0.61	0.935
Day 1	8	18.17[a]	0.45	16.41[b]	0.45	18.59[a]	0.09	0.001
Day 2	8	15.90[a]	0.31	13.12[b]	0.50	17.14[a]	0.25	<0.0001
Day 3	8	13.66[a]	0.41	10.31[b]	0.48	13.32[a]	0.70	<0.0001
Day 4	8	11.88[a]	0.60	8.75[b]	0.63	10.70[a]	0.33	0.002
Day 5	8	9.88[a]	0.43	6.93[b]	0.46	8.96[a]	0.31	<0.0001
Day 6	8	8.19[a]	0.60	5.69[b]	0.47	7.31[ab]	0.26	0.003
Day 7	8	7.45[a]	0.59	4.42[b]	0.53	5.70[a]	0.27	0.001
Day 8	8	6.38[a]	0.68	2.15[b]	0.73	3.78[b]	0.36	<0.0001
Day 9	8	5.38[a]	0.72	0.95[b]	0.50	1.69[b]	0.45	<0.0001
Day 10	8	4.58[a]	0.68	0.13[b]	0.13	0.43[b]	0.30	<0.0001
Day 11	8	3.13	0.68	0.00	0.00	0.00	0.00	-
Day 12	8	1.93	0.64	0.00	0.00	0.00	0.00	-
Day 13	8	1.34	0.48	0.00	0.00	0.00	0.00	-
Day 14	8	0.35	0.23	0.00	0.00	0.00	0.00	-
Day 15	8	0.00	0.00	0.00	0.00	0.00	0.00	-

[a,b]: Different letters in the same line are statistically significant (p<0.05).

Histopathologic Results

At the end of the 15th day, wound samples were collected and microscopically analysed. The samples evaluated in terms of epithelialisation, inflammatory cell infiltration and granulation cell formation were given numbers from 1 to 3 (0: none, 1: mild, 2: moderate, 3: severe).

Microscopically, it was observed that the epidermis layer was more regular in propolis and nanopropolis groups compared to the control group. In the control and propolis groups, there are ulcerated areas close to the epidermis layer. Granulation cells are observed in the dermis layer in the nanopropolis group (Figure 3).

Histopathological parameters such as epithelialisation ($p<0.001$), inflammatory cell infiltration and granulation tissue formation increased in nanopropolis and propolis groups ($p<0.01$), whereas there was no difference between the experimental groups ($p>0.05$) (Table 2).

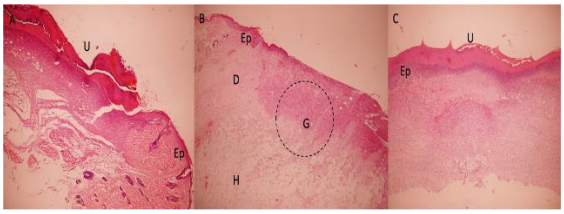

Figure 3. Histopathological images (A) control group, U: ulceration, Ep: epidermis. (B) nanopropolis group, Ep: epidermis, D: dermis, G: granulation cells, H: hpoderma. (C) propolis group, U: ulceration, Ep: epidermis

Table 2. Histopathologic evaluaiton. The findings were evaluated as follows: 0; none, 1; mild, 2; moderate, 3; severe.

Histopathologic Parameters	n	Control		Nanopropolis		Propolis		P-value
		Mean	S.E	Mean	S.E	Mean	S.E	
Epithelization	8	0.88[b]	0.23	2.75[a]	0.16	2.38[a]	0.18	<0.0001
Inflammatory cell infiltration	8	0.75[b]	0.25	2.13[a]	0.23	1.88[a]	0.23	0.004
Granulation tissue formation	8	0.88[b]	0.29	2.25[a]	0.16	2.00[a]	0.27	0.007

[a,b]: Different letters in the same line are statistically significant (p<0.05).
S.E: Standart error.

DISCUSSION AND CONCLUSION

Wound healing is the body's physiological response to tissue damage, which causes living tissue to replace dead tissue and restore the integrity of the injured tissue. It entails a carefully orchestrated series of cellular reactions that involve the interaction of numerous cell types over extended periods (Pillai et al., 2010; Anjum et al., 2019). The degree of tissue damage, the tissue's capacity for repair, and the overall health of the tissue are the main determining wound contracture, which develops during the healing process (Balata et al., 2018). Debridement, irrigation, antibiotics, tissue grafting, and protein hydrolases are some of the current methods used to treat wounds; however, all of these methods have significant disadvantages and these treatments have limitations in terms of cost, treatment time, and toxicity (side effects) (Ahmad et al., 2021). Due to its accessibility and low price, traditional medicine is growing in popularity worldwide (Dwita et al., 2019). Natural products are crucial to this process. Natural product therapy could provide a fresh approach to treating skin wounds (Dong et al., 2018). With the advancement of technology, new approaches in wound healing have emerged in the field of medicine. Nanotechnological products produced through various methods have been increasingly used in wound care, especially in recent years. The combination of natural and technological products can eliminate the disadvantages of current treatment methods. Since ancient times, propolis has been employed in traditional medicine (Gavanji and Larki, 2017). Propolis is a suitable component of

biomaterials because it has been demonstrated to have wound healing and antimicrobial properties, making it useful for use in wound and skin dressings (Lesmana et al., 2022). The purpose of this study was to assess the efficacy of propolis and nanopropolis in treating wounds at the sites of experimental rats' wounds.

Bees gather propolis from tree buds or other plant sources such as poplar, willow, birch, elm, alder, beech, conifer, and horse chestnut in order to create a mixture of natural resinous substances, pollen, waxes, and enzymes (Sforcin, 2016). In terms of pharmacological effects, various biological components have been shown to have antibacterial (Afrouzan et al., 2018), antioxidant (Cao et al., 2017), anti-inflammatory (Moura et al., 2020), accelerated tissue repair (Olczyk et al., 2013), antitumor (Alserbiny et al., 2021), liver protection (Badr et al., 2023), oral health (Sakaba et al., 2013), anti-radiation (Anjaly and Tiku, 2022, anti-ulcer effects (Paulino et al., 2015) and hypoglycemic (Rivera-Yanez et al., 2018) as well as gastrointestinal disorders (Song et al., 2020) properties. Propolis samples from various regions have yielded more than 300 chemical components, the majority of which are rich in flavonoids, terpenes, phenolic acids, amino acids, as well as different hydrocarbons, minerals, trace elements, vitamins, and enzymes (Huang et al., 2014). In addition to preventing or delaying the onset of cell necrosis, flavonoids are known to improve vascularity and lower lipid peroxidation. Therefore, it is thought that any medication that inhibits lipid peroxidation will increase the viability of collagen fibrils by increasing their strength, circulation, preventing cell damage, and promoting DNA synthesis (Getie et al., 2002). Triterpenoids and flavonoids are also known to hasten the healing of wounds primarily because of their astringent and antimicrobial properties that appear to be responsible for wound contraction and accelerated epithelization (Carvalho et al., 2021). According to our results propolis and nanoprolis showed an improvement skin wound, for the first 5 days after the injury. This effect comes from the flavonoids contained in propolis.

A natural nano-material called nano-propolis can be helpful for veterinary medicine in terms of performance, health, and dependable food production. Because of their smaller size, nanoparticles are more readily absorbed by the body (Sahlan et al., 2017), whereas nano-propolis has greater antibacterial and antifungal activity than propolis (Afrouzan et al., 2012). In their study, Patil and Ark, (2015) reported that silver nanopropolis loaded with propolis could be used as a potential product for burn wounds in rats. Adomaviciute et al., (2016) stated in their in vitro study that silver and propolis-loaded wound dressings demonstrated antibacterial and antifungal properties and supported wound healing. Cavalu and Ark, (2019) stated in their in vitro study that encapsulated propolis in nano formulation supports the healing of cutaneous wounds. Saharaf and El Naggar, (2018) reported in their in vitro study that propolis and cellulose acetate loaded nanofibers are effective in wound healing and have antibacterial properties. Eskandarinia et al., (2020) demonstrated the antibacterial properties of biodegradable gelatin-based nanofibers with polyurethane and propolis additives in their in vitro and in vivo study. They also stated that the biomaterial they produced created a moderate exudate and low microbial load in the wound area during the in vivo study.

Within a few hours of injury, inflammatory cells begin to invade the wound site. Monocytes, macrophages, fibroblasts, lymphocytes, and neutrophils are the next to arrive (Yang et al., 2023). This phase can last up to 4 days after an injury. The blood vessels become leaky as a result of the inflammatory response, releasing plasma and polymorphonucleocytes into the surrounding tissue. The neutrophils act as the body's first line of defense against infection by phagocytizing debris and microorganisms with the help of mast cells. Bacteria can be phagocytosed by macrophages, which act as an additional line of defense. Additionally, they secrete a variety of chemotactic and growth factors, including granulation tissue formation-initiating interleukin-1 (IL-1), transforming growth factor beta (TGF-), fibroblast growth factor (FGF), and epidermal growth factor (EGF) (Broughton et al., 2006). In our study, an increase in the level of cell infiltration was observed in the propolis and nanopropolis groups compared to the control group, which was statistically significant. This finding occurred at the expected level during a healthy wound healing phase and contributed to wound healing.

The granulation phase is identified by the presence of pebbled red tissue at the wound base and involves replacement of dermal tissues, and in deeper wounds, subcutaneous tissues as well as wound contraction. Reparatory cells, such as fibroblasts, endothelial cells, pericytes, and keratinocytes, become more prevalent in the wound site as the granulation phase develops. These cells are in charge of creating the new matrix required for the structure and function repair of injured tissue (Pillai et al., 2010). In our study, statistically significant differences were found in terms of granulation tissue formation between the propolis and nanopropolis groups compared to the control group. The wounds that had been treated with propolis and nanopropolis appication were tidy and had strong granulation tissue. The data gathered for this study are consistent with the conclusions made above.

Through regulation of skin extracellular matrix components, expression of transforming growth factor, and improved skin cell growth and remodeling, propolis promotes re-epithelialization (Toreti et al., 2013). This process involves fibroblast adhesion, migration, proliferation of keratinocytes and epidermal cells, as well as contraction of extracellular matrix components in the skin (Olczyk et al., 2014). Reduced granulation tissue maturation time and wound contraction due to the presence of biofilm in propolis suggest that it enhances re-epithelialization and has a significant impact on inflammatory infiltration and fibroblast population in a time-dependent manner. In rats with excisional wounds, nanopropolis significantly accelerates full-thickness wound healing (Abbaszadeh et al., 2019). In our study, it was determined with histopathological data that the level of epithelialization in the wound area was statistically significantly higher in the propolis and nanopropolis groups compared to the control group. The obtained data were found to be consistent with the literature data.

An important parameter to consider when evaluating the wound healing process is the amount of time needed for the excision wound to completely epithelialize (Behyari et al., 2021). Yang et al., (2023) stated in their study on mice that nanopropolis applications provided the fastest wound healing, with 48% of the wound healed on the first day. They also mentioned that this rate was 40% in the propolis group. Another study suggested that topical administration of a nano-emulsion containing nanovitamins C, E, and propolis gel in the mouth cavity during surgical procedures under local anesthesia might

aid in wound healing, especially in the first three days following the procedure. It provided efficient healing and anti-inflammatory effects in addition to safeguarding patients undergoing oral surgical procedures from the early complications of wound healing (such as bleeding) (Furukawa et al., 2021). Yang et al., (2022) have stated in their study on wounds in mice that nanopropolis provides faster wound healing compared to propolis and other experimental groups. The healing parameters examined changed significantly, clearly demonstrating the pro-healing activity of nanopropolis and propolis. Epithelial reorganization occurred very slowly in control wounds. According to the findings of the study, both propolis groups had wound contraction rates close to 100% within 10 days, accelerating wound closure from the early stages. In the nanopropolis group, the wound healing rate was found to be higher compared to other groups, and on the third day, this rate was detected at a level of 49.51%. The topical application of propolis and nanopropolis at the wound site elicited significant wound healing activity due to their angiogenic and mitogenic potential.

This study was conducted to investigate the effects and potential benefits of propolis-incorporated nanofibers produced by the electrospinning method and ethanol-extracted propolis on skin wounds in rats. At the end of the study, it was revealed that the application of propolis and nanopropolis had a positive contribution to wound healing at a statistically significant level in terms of histopathology. It was determined that nanopropolis exhibited a faster healing effect on the wounds compared to propolis and the control group in terms of wound healing rate. As a result of the study, it was concluded that further research is needed to determine the effects of propolis and nanopropolis obtained with different extraction methods and materials, and that new and more effective wound healing methods and materials could be developed based on the obtained data. It was also concluded that more clinical studies are needed for conclusive results.

Conflict of Interest
The authors declare that they have no competing interests.

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Authorship contributions
Concept: O.B., Design: O.B., T.M.D., Data Collection or Processing: O.B., A.S., T.M.D., Z.A., Analysis or Interpretation: O.B., A.S., T.M.D., Z.A., Literature Search: O.B., T.M.D., Writing:O.B

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Ethical Approval
The study was conducted with the approval of the Bursa Uludag University Animal Experiments Local Ethics Committee under permit number 2019-06/02.

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