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Preliminary Study on Wound Healing Activity of Propolis in Albino Rats

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Abstract:-

Background: *Infection can lead to delayed wound healing. Recently it has been shown that propolis which is used in complementary medicine has an antibacterial and anti-inflammatory effect. The aim of this study is to determine whether propolis may contribute to wound healing.*

Material and Methods: *Twenty-one male Wistar albino rats were randomly divided into three groups. Group1 and Group 2 were topically treated with propolis ointment and Thiocillin® oinment, respectively while Group 3 was the control group. On incision wound model, Thiocillin® and propolis ointments were applied on wound sites once daily for 30 days and the mean epidermal thickness (MET) at the 30th day was compared while antimicrobial activity of propolis was studied against different pathogens as well.*

Results: *Propolis exhibited in vitro antibacterial activity against Staphylococcus aureus, Escherichia coli, Streptococcus sp. and Pseudomonas sp. It is observed that the MET in the groups of Propolis ointment and Thiocillin® ointment were significantly greater than that of the control group, while the MET in the group of propolis ointment was significantly greater than that of Thiocillin® ointment treated group. Conclusion: Propolis is effective in wound healing. Further study in-depth is necessary to probe into clinical correlation.*

Keywords: ***propolis, integrative, complementary medicine, wound healing, antibacterial activity.***

1. Introduction

Wound healing is a process which consists of an orderly progression of events that re-establish the integrity of the damaged tissue. Normally that process can be broadly categorized into three stages which contain highly integrated and overlapping phases of cellular and biochemical activities including; inflammatory stage (consisting the establishment of homeostasis and inflammation); proliferate stage (consisting of granulation, contraction and epithelisation) and finally the maturation or remodeling stage which ultimately determines the strength and appearance of the healed tissue **[1]**. Wound healing is a complex series of reactions and interactions among cells and mediators: inflammatory mediators (cytokines, growth factors, proteases, eicosanoids, kinins, and more), nitric oxide, and the cellular elements **[2]**.

Research on wound healing agents is one of the developing areas in modern biomedical sciences and it is shown that the process of wound healing can be promoted by several natural products **[3]**. Some of these agents are composed of active principles like triterpenes, alkaloids, flavonoids and biomolecules that usually influence one or more phases of the healing process **[4]**.

Propolis is a resinous material that honeybees (A.mellifera L.) collect from living plants, mix with wax and use in construction and adaptation of their nests **[5]**. In recent years, propolis has been the object of extensive research for its biological activities and therapeutic properties, and therefore, it has gained popularity as a complementary medicine for health amelioration and disease prevention. The chemical composition of propolis is quite complicated and many components such as polyphenols, phenolic aldehydes, sequiterpene quinones, coumarins, amino acids, steroids and inorganic compounds have been identified in propolis samples and the ethanolic extract of propolis has been reported to possess various biological activities such as antibacterial **[6-8]**, antifungal **[9-10]**, antioxidant **[11-12]**, anticarcinogenic **[13]**, anti-inflammatory **[14]**, antinociceptive **[15]**, and immune-stimulating activities **[13,16]**. The chemical composition and biological activities

of propolis depends on many different factors such as the geographical region, collecting time, and plant source **[7]**.

Propolis has a long history of medicinal use for various purposes, and especially because of its antimicrobial properties topical application of propolis to wounds has been found to be effective in controlling infection and producing a clean granulating bed **[17]**. It is suggested that biocellulose /propolis membrane may favor tissue repair in less time and more effectively in contaminated wounds **[18]**.

Propolis is also known to accelerate the burned tissue repair by stimulation of the wound bed glycosaminoglycan accumulation needed for granulation, tissue growth, and wound closure whereas it also accelerates chondroitin/dermatan sulfates structure modification responsible for binding growth factors playing the crucial role in the tissue repair **[19]**. Topical application of propolis can accelerate wound healing in diabetes. As neutrophil infiltration is normalized, its mechanism of action may be through anti-inflammatory pathways **[20]**. Moreover, the anti-inflammatory action of propolis mediated by mast cells may be more effective than dexamethasone in the inflammatory phase of healing **[21]**. It is widely acknowledged that propolis down-regulates type I allergy and inflammation by affecting mast cells, but the effecting component of propolis, which cause these effects, still remain unknown **[22]**.

Based on its use in wound healing in traditional practices and research literature, the present study was undertaken to evaluate wound healing activity of ethanolic extract of propolis(EEP) in Wistar albino rats.

2. Materials and Methods

2.1. Propolis sample and ointment formulation

Propolis sample was collected from Bursa in Turkey (West Anatolia). Hand collected propolis was kept desiccated and in the dark up to their processing. Voucher specimen is deposited in the Department of Microbiology, Faculty of Medicine, University of Erciyes, Kayseri. An aliquot of crude propolis (7g) was dissolved in 80% ethanol by shaking at 50^{o}C for 3 days and protected from light. The aqueous-ethanol extract was filtered through a Whatman 1 paper and concentrated at 50^{o}C. The resin obtained was dissolved in 80% ethanol to a final concentration of 100 mg/ml. This final solution was employed for the antibacterial assays. Petroleum jelly obtained from Merck Chem was used as a base material in the 30% (w/w) propolis ointment formulation.

2.2. Chemical analysis of propolis sample

30g propolis sample was extracted for a week with 100 ml of 70% ethanol, at room temperature. After filtration the extract was evaporated to dryness at 50^{o}C under vacuum conditions. In this study, one mg of dry extract was reacted with 50 µl pyridine and 100 µl bis (trimethylsilyl) trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) in a sealed glass tube at 100oC for 30 min and sample was prepared for gas chromatography and one µl of a sample was injected and analyzed by GC-MS **[23]**.

Gas chromatography-mass spectrometry was carried out on an Agilent GC 6890 gas chromatograph coupled to an Agilent MSD 5973 mass detector under electron impact ionization. The chromatographic column for the analysis was Zebron (ZB-1) methyl polysiloxane column (30 m x 0.25 mm ID x 0.25 µm df). Helium was used as a carrier gas at a flow rate of 10 ml/min. Propolis sample was analyzed with the column held initially at 100^{o}C for 5 min and then increased to 150^{o}C and then kept at 150^{o}C for 2 min. Finally, temperature was increased to 280^{o}C with a ramp rate 2^{o}C/min and it was kept constant at 280^{o}C for 60 min. The injection was performed in split mode at 250^{o}C. Peaks were identified by computer searches in commercial reference libraries. The components of ethanol extract of propolis were determined by considering their areas as percentage of the total ion current.

2.3. Antimicrobial activity

Antimicrobial activity of propolis was studied against pathogens namely *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Streptococcus sp*. These organisms were acquired from infected wound sites of patients in Erciyes University, Gevher Nesibe Hospital in Kayseri, Turkey. Antimicrobial activity of propolis was determined by the agar dilution method, following the National Committee of Clinical Laboratory Standard guidelines (NCCLS, 1997). Bacterial strains were grown on Mueller Hinton agar (Oxoid) at 37^{o}C/ 24h. The turbidity of the bacteria suspension was adjusted to an equivalent 0.5 McFarland standard.

Each antimicrobial test also included plates containing the culture medium plus ethanol, in order to obtain a control of the solvent antimicrobial effect. Propolis extracts were added in arithmetical progression. The concentration of propolis in the media was expressed in µg per ml. After the inoculation procedures, plates were incubated at 37^{o}C/24h and MIC endpoints were read as the lowest concentration of propolis that resulted in no visible growth on the surface of the culture medium.

2.4. Animals and grouping

Twenty-one male Wistar albino rats (150-180 g) were used for the study. The rats were randomly divided into three groups consisting of seven in each group, where Group1 animals were topically treated with propolis ointment and Group 2 animals were topically treated with Thiocillin® (Bacitracin 15000 IU / Neomycin Sulphate 150 mg) while Group 3 as the control group was topically treated with

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petroleum jelly that was used as a base material in the propolis ointment formulation. The rats were kept individually in standardized environmental conditions, provided with pellet rodent diet and water *ad libitum*. The protocol of this study was approved by the Ethics Committee of Erciyes University.

2.5. Wound model

All the groups were studied respectively on incision wound model in rats. Animals in each group were anesthetized and a paravertebral-long incision was made through the skin and cutaneous muscles at a distance of about 1.5 cm from the midline of the depilated back of the rats as described by Yesuf & Asres [**24**]. Full aseptic measures were not taken and no local or systemic antimicrobials were used throughout the study. The Propolis extract ointment and Thiocillin® (Bacitracin 500IU/g, Neomycin sulfate 5mg/g) ointment were administered once daily for 30 days and the mean thickness values of epidermis at the 30th day were used to compare wound healing in the groups. From the healed wound of each rat, three tissue specimens were isolated for calculating the mean values of epidermal thicknesses in each group.

2.6. Histochemical analysis

Application of ointments was done daily and treated wound tissues were collected for histological study. The healing tissues obtained on the 30th day from all groups of animals of the incision wound model were processed for histological study. A specimen sample of the tissue was isolated from the healed skin of each group of rats for the histopathological examination. The cross sectional full thickness epidermis specimens from each group were collected at the end of the study. Granulation tissue specimens were collected at regular intervals of 2 days. All samples were fixed in 10% buffered formalin, processed and blocked with paraffin and then sectioned into 5-7 µm thick sections and stained with haemotoxylin and eosin [**25**].

2.7. Statistical analysis

Results were expressed as Mean±SD. Differences between group means were calculated by one-way analysis of variance (ANOVA) and the results were considered statistically significant at $p<0.05$.

3. Results

The main compounds of propolis are flavonoids (*pinobanxin* and *galangin*), aromatic and fatty acids and their esters, alcohols, esters and ketones.(Table 1.)

The EEP sample exhibited in vitro antibacterial activity. All of the assayed bacterium species were susceptible to propolis extract. *S.aureus* was the most sensitive strain and it was susceptible to low propolis concentration (MIC_{90}: <0.1µg /ml). On the other hand Gram-negative bacteria growth was only inhibited in higher propolis concentrations. The MIC_{90} of propolis against *P. aeruginosa* and *E.coli* strains was 3.5µg/ml. The MIC range of propolis against *Streptococcus spp*. was <0.1-1.75 µg (Table 2).

In the groups treated by propolis ointment and Thiocillin® ointment the wounds showed epithelisation and the wound margins had no oedema by the 7th day. Histologically, early attenuation of acute inflammatory changes, control of infection and early reparative activities were observed in the propolis treated group in comparison with the control group (Figure 1and 2). The progress of healing on incision wound method induced by the propolis ointment and Thiocillin® ointment as well as the respective control group is measured by using the mean values of epidermal thickness values. It is observed that the epidermal thickness values in the groups of propolis ointment and Thiocillin® ointment were significantly greater than that of the control group, while the epidermal thickness values of propolis ointment group was significantly greater than that of both Thiocillin® ointment treated group and the control group ($P<0.05$) (Table 3).

4. Discussion

The EEP sample displayed the typical pattern of "poplar type" propolis. It contains the combination of secondary metabolites characteristics for the buds of *Populus spp*. of the section of *Aigeiros* [**5**]. The bud exudates of poplar trees (*Populus spp*.) are the main source of European and North American propolis [**26**]. It was confirmed that the propolis sample obtained from Western Anatolia contained typical poplar flavonoid aglycones [**27-28**]. Also recent studies on Turkish propolis have shown that the main source of the propolis was poplar exudates [**29-30**].

Infection is one of the most common complications causing delay in wound healing, therefore infection control is important in wound healing. *Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa)*, and *β-hemolytic streptococci* are common bacteria in infected wounds **[31-32].** Antibacterial effect of propolis has been shown by numerous studies [**33-34**]. In the present study, the wounds treated with propolis ointment did not exhibit any sign of infection and propolis exhibited in vitro antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus sp*. and *Pseudomonas sp*. presumably propolis acted as an antibacterial medium and prevented bacterial growth.

We found that *S.aureus* is susceptible to low propolis concentration. Several authors also showed an efficient propolis antibacterial action on *S.aureus* strains [**35-36**]. On the other hand Gram–negative bacteria growth was inhibited only in higher propolis concentrations.

These results are in agreement with those of Grange and Davey [**36**], who observed a marked action of propolis against Gram - positive bacteria and limited activity against

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Gram - negative ones. Epidermal regeneration of a wound is a complex process in which residual epithelial cells proliferate in an integrated manner to form an intact epidermis. Infection is one of the most common complications causing delay in wound healing. Therefore prevention of infection is the primary aim of treatment of wounds so that there is an optimal regeneration of the cells. Recently topical application of propolis has been recognized to be effective in controlling infection and producing a clean granulating wound bed [**17, 37]**.

In addition to this, early dermal and epidermal regeneration in the propolis treated rats also confirmed that the propolis extract had a positive effect toward cellular proliferation, granulation tissue formation and epithelisation. The histological study of the tissues of the wound area treated with propolis ointment, Thiocillin® ointment and the control group revealed that in the case of the skin wounds treated with propolis ointment, fibrosis was relatively less and the original tissue regeneration was much better. The skin adrenal structures such as the pilosabecaeous glands, sweat glands etc. were better presented in the wounds treated with propolis ointment compared to Thiocillin® treated wounds. Previously, Morales and Garbarino [**38]** stated that the usage of propolis in the treatment of different cutaneous lesions of such as burns, wounds and ulcers is positive in the reparation process, shortening the healing of wounds and reducing the risk of infections. By minimizing acute inflammatory exudate and stimulating macrophage and CD4 T lymphocyte activity, propolis may play a positive effect in wound healing [**39]**.

Besides its antimicrobial activity, the wound healing property of propolis may probably be due to the presence of bioflavonoids which are plant derived substances with strong antioxidant activity. It is supposed that bioflavonoids may also help to relieve pain by inhibiting prostaglandin cyclooxygenase, lipooxygenase and phospholipase [**40]**. In addition to this apparent enzyme inhibition, bioflavonoids have demonstrated enzyme activation -namely that of praline hydroxylase, an enzyme necessary for collagen crosslinking [**40]**. Additionally, some bioflavonoids bind to skin elastic fibres, preventing its degradation by elastases markedly affect the rate of degradation by elastases released as a result of inflammation [**41]**.

Better epithelisation suggests prohealing activity and propolis is able to enhance the reepithelization process [**20]**. Propolis ointment shows a significant prohealing activity when topically applied on rats. Better epithelisation seen under the influence of propolis extract may be because of the presence of flavonoids, which is responsible for the free radical scavenging activity, that is supposed to be an important component of wound healing also [**42]**.

Restoration of the epithelial thickness is a proliferative stage in wound healing. Epidermal healing is characterized by a reepithelialization process and better epithelisation suggests prohealing activity. Therefore, an optimal increase in epithelial thickness can be used as a marker of improved healing. In contrast to this, the lack of reepithelialization is an indicator of decline of wound healing

while an extreme increase can be an indicator of delayed remodeling process. In this preliminary study, it was attempted to compare the epidermal thickness in the control and the treated wounds (by Thiocillin® ointment and Propolis ointment), just to determine whether propolis ointment may be effective in closing the wounds or not. Further study in depth is necessary to probe into the exact mechanism.

5. Conclusion:

Wound healing is a complex biological process that consists of precisely and highly programmed phases. Infection can lead to elongate the inflammatory phase and then the wound in a chronic state may fail to heal. Many chronic wounds do not heal because of the failure of antibiotics.

Propolis is an antibacterial, antioxidant and anti-inflammatory agent. It can be used as a topical agent, which does not adhere to surface and it has been found to be effective in controlling infection and producing a clean granulating bed. Propolis ointment may be an efficient topical therapeutic agent for wound treatment because it appears to promote reepithelisation and decrease prolonged inflammatory reaction. In addition, propolis treatment of superficial wounds is cost effective because it shortens the duration of treatment.

The result of the present study suggests that EEP posseses a potent wound healing activity, thereby justifying its use in indigenous medicine. Further pharmacological, biochemical investigation will clearly elucidate the mechanism of action and under internationally acceptable quality control propolis can constitute a potential evidence based complementary therapeutic agent for wound healing in integrative medicine.

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Table 1. Chemical composition of propolis

Compounds	RT	Compounds	RT
Organic acids		Flavonoids	
Octadecanoic acid	46.89	Pinobanxin	21.85
9,12,15 Octadecanoic acid	14.58	Galangin	24.44
Tetradecanoic acid	20.96	Esthers	
Undecanoic acid	51.21	4,3 acetyloxycaffeate	34.84
Oleic acid	30.92	2-propenoic acid esther	11.81
Aromatic acids		Cafeic acid TMS esther	36.21
Benzoic acid	5.87	Ketons	
Caffeic acid	29.19	Cyclohexanon	19.55
Palmitoleic acid	31.19	3-methyl antitricyclo undec-3-en-10-on	21.52
Alcohols		Hydrocarbons	
Pentitol	45.18	Cyclohexane	51.21
Ribitol	20.96	Cyclopentene	50.31
Vaniletanediol	38.78	5-n-propyl-1,3 dihydroxybenzene	42.19
Bicyclohept-3-en-2-ol	14.72		

RT: Retention time

(The peak numbers in the table are given according to the retention time only to the major peaks)

Table 2. Minimum inhibitory concentration (MIC) of propolis using agar dilution method

Microorganisms	MIC $_{50}$ (µg/ml)	MIC$_{90}$ (µg/ml)	MIC range (µg/ml)
Staphylococcus aureus (n=5)	<0.1	<0.1	<0.1
Escherichia coli (n=6)	3.50	3.50	1.75-3.50
Streptococcus spp. (n=4)	<0.1	0.2	<0.1-1.75
Pseudomonas spp. (n=4)	3.5	3.5	3.5

Table 3. Thickness of epidermis of treatment and control groups (p<0.05)

Groups	Thickness of epidermis at 30th day (mm) (Mean ±SD)	Min.	Max.
Propolis ointment	4.97±0.16 ^{c}	4.44	5.58
Thiocillin® ointment	3.96±0.41 ^{b}	2.66	5.42
Control	2.41±0.22 ^{a}	1.80	3.38

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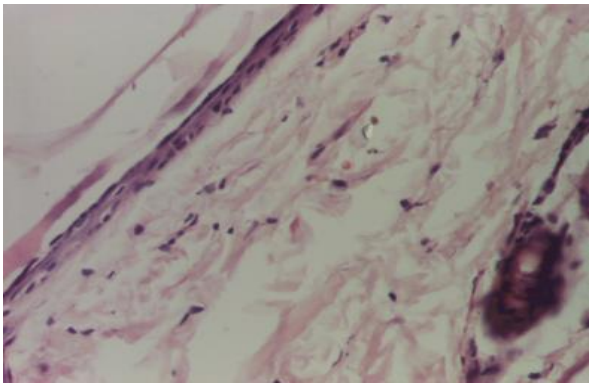

Figure 1. Dermis of the control group (HEx200).

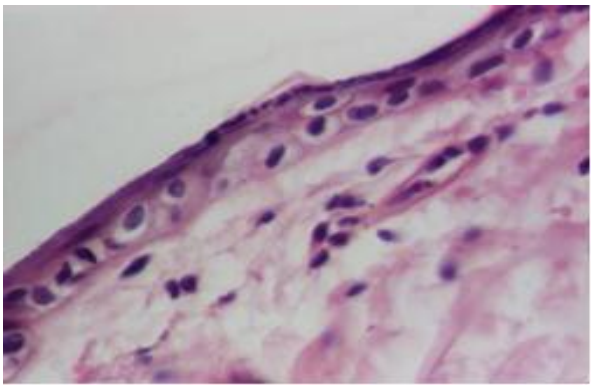

Figure 2 The regenerated epithelium and perivascular fibroblasts of propolis group (HEx400).

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