The effectiveness of ethanolic extract of propolis on wound healing in albino rats

Ahmad Hassan Sahib ¹, Maan Abdul Azeez Shafeeq ¹, Salah Mahdi Mohsen ²

¹Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.
²Biotechnology Research Center-AL-Nahrian University, Bagdad, Iraq.

*Corresponding author: ahmadhassansahib@gmail.com (Sahib)

Abstract

Background: Wound healing is a complex process. So, the use of natural products such as propolis, which has anti-inflammatory and antioxidant properties to accelerate wound healing, was an important target. Aim of work: The aim of this study was to assess the possible wound healing effect of ethanolic extract of propolis (EEP) on full-thickness wound model in albino rats. Materials and Methods: Healthy adult 72 albino rats were used in this study. Excision wound with 1.5 cm length was done on the posterior of the neck. Rats were randomly divided into equal four groups (negative control, positive control, ethanolic extract of propolis (EEP) ointment and tetracycline ointment 1%). Each group was divided into three subgroups that received treatment for 4, 7 and 14 days. Wound area in the experimental group was covered twice daily with a fixed amount of EEP ointment and tetracycline ointment (1%), the negative control group did not receive any treatment, while positive control group treated with physiological saline solution (0.9 % Na Cl). Histological analysis was performed and counting fibroblast, neutrophils, macrophages and new blood vessels in the wound bed was done followed by statistical analysis. Results: The findings show that healing process of excision wound in albino rats treated with the EEP ointment started earlier and had a faster course than the standard tetracycline therapy. Conclusion: The powerful effect of EEP ointment on accelerating wound healing was revealed. This could be an effective strategy for managing delayed wound healing.

Keywords: Excision wound, ethanolic extract of propolis (EEP), experimental rats.

How to cite this article: Sahib AH, Azeez Shafeq MA, Mohsen SM (2020): The effectiveness of ethanolic extract of propolis on wound healing in albino rats, Ann Trop & Public Health; 23 (7): 1107-1118. DOI: http://doi.org/10.36295/ASRO.2020.23735

Introduction

Wound healing is a highly complex process characterized by a perfect coordinated sequence of cellular events that happens in response to a lesion. This process includes three main steps: inflammation, proliferative and remodeling (maturation) (Dzoboet al., 2016). Use of natural products for wound healing was a very ancient practice in human history. In the last decades, it was replaced by the application of chemicals and synthetic drugs, which however, have been shown to present shortcomings, limitations and side effects. Currently, natural products have been investigated as an alternative source of remedies which modulate the inflammatory response in a shorter time period.
and with minimal complications (Jacob et al., 2015). Propolis or bee glue is a resinous material produced by *Apis mellifera*, which honey bees gather from buds, leaves and exudates of the plants. They mix it with bee enzymes, pollen and wax. Over 300 chemical components that make up propolis, flavonoids, terpenes, and phenolic acids have been identified and the biological effects of propolis were attributed to these components (Martinotti and Ranzato, 2015). Propolis-based products have been the object of research due to their immunomodulatory, antimicrobial, anti-inflammatory and wound-healing properties (Hashemi, 2016). Propolis has been showed to be effective in both experimental and in clinical wounds (McLennan et al., 2008; Henshaw et al., 2014). Thus, the current study was aimed to determine the healing potential of ethanolic extract of propolis (EEP) on excision wounds induced in albino rat.

**Materials and methods**

**Animals**

Seventy two adult male-albino rats with an average weight of 250-300 ± 50 g and ages were varying between three to four months were used in the study. Experimental rats were acquired from the animal house of Biotechnology Research Center of AL-Nahrain University-Baghdad and singly housed in cages in a room with an artificial 12-h light/dark cycle at a constant temperature range (24±2 °C) and relative humidity (55±10%). The animals were acclimated for one week before the experiment and had free access to standard laboratory chow and water. This study has been done according to the ethics committee for animal research of the animal house of Biotechnology Research Center of AL-Nahrain University-Baghdad following international ethics and regulations for animal research in laboratory applications (Gluck et al., 2002). Animals were treated in accordance with Guide for the Care and Use of Laboratory animals by National Research Council, (2010).

**Preparation of ethanolic extract of propolis (EEP)**

The crude propolis was bought in September 2019 from the mountainous region Isfahan /Iran from beekeeper who gathers these resinous materials from bee hives by scraping. They were kept desiccated and in the dark until their processing. Propolis sample crushed to make a fine powder using a grinder and then 30 gram was extracted in 250 mL ethanol (95% v/v) by stirring for two days and centrifuged at 27,000 g for 15 min. The alcoholic extract obtained was filtered through a Whatman paper 42 scores, extraction was done twice then concentrated in a rotary evaporator under reduced pressure 450 mmHg at 40°C and the residue was kept in the dark at room temperature until use (Berretta et al., 2012).

**Phytochemical analysis of propolis**

Phytochemical analysis of propolis sample was carried out at Ministry of Science and Technology, Baghdad ,Ira as earlier described by Ejikeme et al. (2014).

**Preparation of propolis ointment**
The ethanol extract of propolis (EEP) was prepared as ointment using petroleum jelly (melting point 65°C) at a concentration of 10% (w/w) once a few days before the beginning of the experiments. The ointment was kept in sterile and properly sealed container at 4°C (Pillai et al., 2010).

**Rat excision wound model**

All experimental animals were anesthetized with halothane via inhalation, then the general anesthesia of the rats was done by utilizing intraperitoneal administration of Ketamine 5% at a dose (50 mg/kg) and Xylazine 2% at a dose (4.5 mg/kg), which was carried out as reported by (Eyarefe and Amid, 2010). The fur on the back of the anesthetized rats was shaved with hair removal cream and cleaned with 70% ethanol to maintain aseptic conditions (Yesuf and Asres, 2013). Full-thickness 1.5 cm diameter excision wounds were made on the dorsum of each rats using toothed forceps, sterile pointed scissors and a scalpel blade (Okada et al., 1997). Rats were closely observed for any infection.

**Experimental groups and administration of substances**

Experimental rats were divided randomly into four equal groups, with 18 rats in each group. After that, each group was divided into three subgroups each of 6 rats corresponding to 4th, 7th, 14th days as follows:

- **Group I:** Negative control (normal rat, without any surgical procedure).
- **Group II:** Positive control excision wound-induced rats treated with normal saline solution (0.9 % Na Cl).
- **Group III:** Excision wound-induced rats topically treated with EEP ointment.
- **Group VI:** Excision wound-induced rats topically treated with tetracycline (standard drug ointment 1%).

The topical application of the EEP formulation as well as the standard ointment was done twice daily throughout the experiment.

**Quantitative assessment of wound healing**

The degree of wound repair was measured on the 3rd, 5th, 7th, 9th, 11th and 13th days of experiment. The wounds were photographed, and diameters were measured using Vernier callipers, where it was calculated based on the original area (measured on day zero) and expressed as follows: 

\[
\% \text{Wound repair} = \frac{\text{initial wound size-specific day}}{\text{initial wound size}} \times 100
\]

(Martins et al., 2006).

**Sample collection**

The rats were anesthetized using an intraperitoneal injection of thiopental sodium 50 mg/ kg. Sacrification was done on the 4th, 7th and 14th days from the start of the application of different treatments in the different groups. The wounds sites were excised with a rim of 5 mm of normal surrounding skin. Tissue specimens were fixed in 10% neutral buffered formalin solution and processed for paraffin sections. Five micrometer sections were cut and stained with H&E. Subsequently, sections were photographed at high power magnification (x40) with an Olympus BX41 America light microscope equipped with an Olympus DP70 camera (Masson-Meyers et al., 2013).

**Morphometric study**

Mean number of neutrophil, macrophage, fibroblasts and blood vessels were counted in 10 non overlapping high

power fields in a magnification of (x40) using software 3 tools.

Statistical analysis
The Statistical Analysis System-SAS. (2012) program was utilized to detect the effect of difference factors (groups and days) in experiment parameters. Least significant difference–LSD test (Analysis of Variation-ANOVA) was utilized to significant compare between means in this study after checking for distribution of normality.

Results
Physicochemical Parameters of propolis
The phytochemical contents reported for propolis sample in this study as shown in (Table1) contained numerous essential biological constituents that may exert potential therapeutic effects.

<table>
<thead>
<tr>
<th>No.</th>
<th>Constituents of propolis</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Terpenoids</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Steroids</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Alkaloids</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Resins</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Coumarins</td>
<td>+</td>
</tr>
</tbody>
</table>

Neutrophils number
A significant increase of neutrophils were observed in the wound bed of control positive and other treated groups as compared with the negative control through all the experiment periods. Moreover, a significant decrease in number of neutrophils per mm 2 in all experimental groups was noticed on day 14 as compared with the previous periods (4th and 7th days). There was also a significant difference in the number of neutrophils per mm2 in the wound area of EEP group as compared with positive control and tetracycline groups on the 4th day. While on 7th and 14th days, there was no significant difference between EEP and tetracycline groups. Despite the nearly normalizing effect of EEP on neutrophil number, there was still significant difference with that of normal control rats (Table 2).

Table 2: Comparing the number of neutrophils in the wound bed of groups on the 4th, 7th and 14th days of study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>LSD value</th>
</tr>
</thead>
</table>

Macrophage number

A significant increase in macrophages was revealed in the wound area of control positive and other treated groups as compared with the negative control through all the experiment periods. On the 4th and 7th days during the inflammatory and early proliferative phases, the number of macrophages per mm2 in the wound area of the EEP-treated group was significantly higher than that of other experimental groups (P<0.01); nevertheless, on the 14th day, EEP treated group showed to have fewer macrophage than the other groups (P<0.01) (Table 3).

Table 3: Comparing the number of macrophages in the wound bed of groups on the 4th, 7th and 14th days of study.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>LSD value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Day</td>
<td>7 Day</td>
</tr>
<tr>
<td>Negative control</td>
<td>2.33 ± 0.42</td>
<td>1.83 ± 0.31</td>
</tr>
<tr>
<td>Positive control</td>
<td>7.83 ± 0.31</td>
<td>7.67 ± 0.71</td>
</tr>
<tr>
<td>Ethanol extract of propolis (EEP)</td>
<td>10.12 ± 1.31</td>
<td>9.83 ± 1.07</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>9.16 ± 0.40</td>
<td>8.50 ± 0.42</td>
</tr>
<tr>
<td>LSD value</td>
<td>2.247 **</td>
<td>2.202 **</td>
</tr>
</tbody>
</table>

Means having with the different big letters in same column and small letters in same row differed significantly, ** (P<0.01).

Fibroblasts number

The number of fibroblasts as shown in (Table 4) showed a significant increase in all experimental groups compared to normal rats during all the experimental periods. The number of fibroblasts per mm2 in EEP-treated group significantly peaked on 7th day. While on 14th day, there was a significant reduction compared with the peak period. Whereas, tetracycline-treated group and positive control groups showed significant gradual increase from 4th day to
14th day. There was a significant difference between EEP treated group as compared with tetracycline and positive control groups.

**Table 4: Comparing the number of fibroblast in the wound bed of groups on the 4th, 7th and 14th days of study.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>LSD value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Day</td>
<td>7 Day</td>
</tr>
<tr>
<td>Negative control</td>
<td>5.40 ± 0.87</td>
<td>6.60 ± 0.60</td>
</tr>
<tr>
<td>Positive control</td>
<td>64.80 ± 1.71</td>
<td>81.80 ± 9.49</td>
</tr>
<tr>
<td>Ethanolic extract of propolis (EEP)</td>
<td>82.40 ± 3.96</td>
<td>139.60 ± 0.75</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>70.20 ± 1.42</td>
<td>86.80 ± 1.35</td>
</tr>
<tr>
<td><strong>LSD value</strong></td>
<td>6.148 **</td>
<td>12.034 **</td>
</tr>
</tbody>
</table>

Means having with the different big letters in same column and small letters in same row differed significantly, * (P<0.05), ** (P<0.01).

**Blood vessels number**

There was a significant increase in number of blood vessels in all experimental groups as compared with normal controls. The number of new blood vessels (Table 5) per mm² in the wound area of the EEP-treated group significantly increased from day 4 to day 7 (each P<0.05) and it peaked on day 14. While those of the tetracycline treatment groups showed non-significant change through the different periods of the experiment. Moreover, there was a significant difference between EEP-treated group compared with other experimental groups.

**Table 5: Comparing the number of new blood vessels in the wound bed of groups on the 4th, 7th and 14th days of study.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>LSD value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 Day</td>
<td>7 Day</td>
</tr>
<tr>
<td>Negative control</td>
<td>2.40 ± 0.51</td>
<td>2.60 ± 0.40</td>
</tr>
<tr>
<td>Positive control</td>
<td>8.80 ± 1.02</td>
<td>10.80 ± 0.73</td>
</tr>
<tr>
<td>Ethanolic extract of propolis (EEP)</td>
<td>16.20 ± 1.28</td>
<td>18.60 ± 0.51</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12.80 ± 1.02</td>
<td>14.20 ± 0.80</td>
</tr>
<tr>
<td><strong>LSD value</strong></td>
<td>2.659 **</td>
<td>2.229 **</td>
</tr>
</tbody>
</table>

Means having with the different big letters in same column and small letters in same row differed significantly, * (P<0.05), ** (P<0.01).

**Wounds contraction assessment**
Wound healing process was assessed as shown in (Table 6). Wound contraction was gradual in all the groups from days 0-13. Epithelial closure rate was significantly increased in the EEP-treated wounds when compared with tetracycline-treated wound and saline-treated wounds.

### Table 6: Wound healing rate obtained with different treatments in all study groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SE</th>
<th>LSD value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 Day %</td>
<td>5 Day %</td>
</tr>
<tr>
<td>Positive control</td>
<td>20.16 ± 1.04</td>
<td>27.44 ± 2.45</td>
</tr>
<tr>
<td>C</td>
<td>D c</td>
<td>D b</td>
</tr>
<tr>
<td>Ethanolic extract of propolis (EEP)</td>
<td>30.69 ± 0.56</td>
<td>50.83 ± 3.35</td>
</tr>
<tr>
<td>B d</td>
<td>AB c</td>
<td>AB b</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>24.61 ± 1.54</td>
<td>40.16 ± 3.58</td>
</tr>
<tr>
<td>C d</td>
<td>C c</td>
<td>C b</td>
</tr>
<tr>
<td>LSD value</td>
<td>4.84 **</td>
<td>9.54 **</td>
</tr>
</tbody>
</table>

Means having with the different big letters in same column and small letters in same row differed significantly, ** (P<0.01).

### Discussion

Cutaneous wound healing is a dynamic and complex process of epithelial repair that occurs in response to an injury. This process includes three main steps: inflammation, proliferation and maturation or remodeling (Gonzalez et al., 2016). In the inflammatory phase (which usually lasts up to four days post injury), a large number of neutrophils are typically found within (24-36 hours), followed by macrophages, fibroblasts and lymphocytes (Gantwerker and Hom, 2012). The main role of neutrophils is to phagocyte debris and microorganisms and provides the first line of defense against infection. Macrophages play essential roles in all phases of wound healing (Krzyszczyk et al., 2018), but its most important role is in the early period of healing processes. In addition to phagocytosis of wound debris and bacteria, they secrete cytokines such as a platelet derived growth factor (PDGF), which stimulates chemotaxis and proliferation of fibroblasts and smooth muscle cells (Delavary et al., 2011). They also release substances that attract endothelial cells to the wound and stimulate their proliferation to promote angiogenesis (Landen et al., 2016). The histological examination in this study showed that treatment with ethanolic extract of propolis (EEP) nearly normalized the count of neutrophils and macrophages compared to those in the other experimental groups (Tables 2 and 3). Accumulating evidence suggests that (CAPE, cinnamic acids and artempillin C) which are the main active ingredients in propolis, has an anti-inflammatory activity that is activated by mechanisms associated with the inhibition of inflammatory cell activity. Several research’s state that the anti-inflammatory effectiveness of these active constituents stems from the inhibition of nuclear factor kappa B (NF-κB), the reduction of prostaglandin E2, and inhibition of nitric oxide production (Khayyal et al., 1993; Paulino et al.,...
Current results were in accordance with those that were stated by de Lopes-Rocha et al. (2012). The proliferative phase begins after inflammatory phase. Histopathologically, fibroblast count were the highest in the EEP-treated group compared to the untreated group (positive control group) and standard treatment group (tetracycline group) on day 7, which may indicate that EEP accelerates the inflammatory reaction and initiates the healing process in the early phases (Table 4). This is in agreement with a previous report that propolis has an active substance that can increase fibroblast proliferation such as (flavonoids, phenolics, terpenoids, steroids, alkaloids, coumarins and saponins) (Tyszka-Czochara et al., 2014; Sabir et al., 2016). These active ingredients content were checked by the phytochemical test carried out on propolis sample in this study as shown in (Table 1). Significant increase of fibroblasts in EEP group on the 4th day as compared with other experimental groups indicated that EEP formulation had better effect and shortened the inflammatory phase. Increased count of fibroblasts resulted in an increase in collagen, which was the main cause for reducing the size of the wound (Table 6). Angiogenesis increases the supply of oxygen and other nutrients that are necessary for local collagen synthesis (Grossman and Grossman, 2019). Upon epithelial injury, injured endothelial cells release angiogenic mediators with growth factors to form new blood vessels in granulation tissue (Gonzalez et al., 2016). Further, wound generated hypoxia also induces angiogenic growth factors by hypoxia inducible factor alpha (HIFs) (Pugh et al., 2003). Our data showed that EEP formulation caused a gradual increase in rate of angiogenesis throughout the duration of the experiment days without having any repressing effect (Table 5). Owing to the fact that stimulation of angiogenesis is effectively seen with compounds including flavonoids and it is a determining factor in wound healing (Martinotti and Ranzato, 2015). The angiogenic effect of EEP seemed to be closely related to this characteristic. Wound contraction is a crucial process in healing that leads to wound closure. Consequently, measurements of wound contraction become reliable parameters in macroscopic evaluation for dermal repair (Wang et al., 2018). In the current study, the percentage of wound closure as shown in (Table 6) was gradually increased in treatments groups from the 3rd day and continued during the 5th, 7th, 9th, 11th days to reach the highest rate on the 13th day which indicating the development of granulation tissue. This was in line with literature findings of Mandelbaum et al. (2003). In this study, wounds of all experimental rats treated with EEP formulation were healed and had nearly 93 % wound closure on day 13, while the wounds were closed approximately by (76 % and 53 %) in the rats were treated with the tetracycline and normal saline solution respectively. The increased percentage of wound closure in the EEP-treated groups might be attributed to the capacity of EEP to influence the production of transforming growth factor-alpha and beta 1 (TGF-α and TGF-β1) via immune cells which stimulate cell growth, mobilization of fibroblasts and epithelial migration (Martinotti and Ranzato, 2015). Propolis contains also vitamin B complex, provitamin A, Arginine and various minerals and also Bioflavonoids, and it could contribute to the production of collagen, so wound healing would be more rapid (Kujumgiev et al., 1999). Moreover, the increased rate of wound contraction reported in EEP-treatment group, might be due to enhanced activity of fibroblasts which is mediated by specialized myofibroblasts located in the granulated tissues (Darby et al., 2014). Myofibroblast is critical for epithelial closure. This is because the stress fibrils α-SMA affect contractility, enabling it to close the wound faster and give a stronger...
cell adhesion to the extracellular matrix (Li et al., 2016). The presence of α-SMA in the myofibroblasts allows it to produce a stronger and faster contractile power than the ordinary fibroblasts (Myrna et al., 2009). The early re-epithelialization and quicker closure of wounds in EEP groups may also be associated with increased proliferation of keratinocytes and their migration to the wound surface. Wound shrinkage in untreated rats was slower. This may attributed to insufficient granulation tissue production as a result of extended inflammatory and debridement period (Molan, 2006). These results were in agreement with findings previously published by Khorasani et al. (2016) and Takzaree et al. (2017), who revealed the same findings. The results of this study showed that topical application of EEP ointment enhanced wound contraction and reduced the healing time. In addition to its safety and efficacy, propolis is an inexpensive topical wound treatment natural product. Therefore, propolis could be considered as a good alternative to several synthetic topical wound treatment products.

Conclusion

Based on the results presented, this short experimental study concludes that the ethanolic extract of propolis (EEP) can be considered a valuable formulation in accelerating healing of full thickness skin wounds in rats. This may pave the way for new formulation for treating delayed wound healing. However, further research is needed to understand the mechanisms of action of propolis and to investigate the best means for applying propolis to different types of wounds.

Acknowledgment

The authors express their sincere thanks to the technical support and infrastructure provided by Biotechnology Research Center of AL-Nahrain University-Baghdad.

References


---

*Sahib et al (2020): Effectiveness of ethanolic extract*  
*April 2020 Vol. 23 Issue 7*

*May 2020*


