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ORIGINAL ARTICLE

Application of Propolis Ethanol Extract and Propolis Nanoemulsion in Treatment of Cutaneous Infection in Rabbit

Talieh Archin[1], Abdolgaffar Ownagh ✉[1], Ali Asghar Tehrani[2], Sajjad Keshipour[3]

[1] Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. [2] Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran. [3] Department of Nanochemistry, Nanotechnology Research Institute, Urmia University, Urmia, Iran.

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ABSTRACT

Pseudomonas aeruginosa is one of the most important opportunistic pathogens in humans and animals. Natural antibacterial compounds like propolis are the best substitute for antibiotics. The aim of this study was to evaluate the antibacterial effect of ethanolic extract of propolis, propolis nanoemulsion, and their combinations with ciprofloxacin in the treatment of experimental wound infection contaminated with *P. aeruginosa* in rabbits. Propolis was obtained from a different region of Western Azerbaijan in the year 2018. Then the ethanol extract of propolis was prepared. And so, nanoemulsion of propolis was prepared. The broth microdilution method was used to determine the MIC of propolis and propolis nanoemulsion on *P. aeruginosa*. 28 rabbits used in 7 groups: Negative control (CO), Tween 20 (T), extract of propolis (P), nanoemulsion of propolis (NP), ciprofloxacin (C), ciprofloxacin+ extract of propolis (C + P), ciprofloxacin + nanoemulsion of propolis (C + NP). Full-thickness skin wound was created under general anesthesia and bacterial suspension (108 CFU/ml) was inoculated to each wound site. Macroscopic and microscopic characteristics and Superficial bacterial load of wounds were studied on days 7, 14, and 21. The number of bacteria in treatment groups was significantly lower than in negative control groups ($p < 0.05$). The macroscopic evaluation of wounds showed that C+P and C+NP enhanced wound closure in comparison with the negative control group and ciprofloxacin ($p < 0.05$). Histopathology assessment of the wound showed that the combination of C+P, and C+NP had a better and faster healing effect than the other groups, however, its difference was significant only when compared to CO, C, and T groups ($p < 0.05$). The results of the present study showed that a combination of ciprofloxacin + ethanolic extract of propolis and ciprofloxacin + propolis nanoemulsion had better therapeutic effects than either agent alone.

Introduction

One of the main problems of the global scientific community is to find alternative antibiotics or substances against resistant bacteria. *Pseudomonas aeruginosa* is a Gram-negative and opportunistic bacterium that is separated from the land, water, and the environment. This organism as the second most common pathogenic bacteria, play an important role in surgery contaminations. Elderly patients with lymphoma, AIDS, undergoing chemotherapy and burned are those who are prone to severe infections caused by *P. aeruginosa* such as endocarditis, meningitis, and septicemia.[1].

P. aeruginosa has been shown to possess a high level of intrinsic resistance to most antibiotics through restricted outer membrane permeability, efflux systems that pump antibiotics out of the cell and production

✉ Corresponding author. Email: a.ownagh@urmia.ac.ir

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of antibiotic-inactivating enzymes such as β-lactamases. This bacterium can gain antibiotic resistance through mutational changes or acquisition of resistance genes via horizontal gene transfer. In addition to the high level of intrinsic antibiotic resistance of *P. aeruginosa*, the acquired resistance greatly contributes to development of multidrug-resistant strains, which increases the difficulty in eradicating this microorganism and leads to more cases of persistent infections. Adaptive resistance increases the ability of this bacterium to survive antibiotic attack due to transient alterations in gene and/or protein expression in response to an environmental stimulus, and it is reversible when the stimulus is removed. In *P. aeruginosa*, the best characterized mechanisms of adaptive resistance are the formation of biofilm and the generation of persister cells, which result in persistent infection and poor prognosis in CF patients.[2]

The main objective of wound healing is a restoration in the shortest time with minimal side effects. Patients with these infections suffer from pain, loss of functional ability, decreased quality of life, and risk of death.[3] Wound infections are complicated with pathogenic bacteria, and it is important to note that constantly rates of Multi-Drug Resistance among these bacteria are increasing today.[4] For these reasons, wound site infections have become a problem for patients and health services. Consequently, immediate monitoring of wound infections and preventive control and therapeutic policy have been proposed.[5] The negative effect of certain types of microorganisms on wound healing has been widely published; The microorganisms have been usually noted as the cause of delayed wound healing. One of the most common pathogens causing these infections is *P. aeruginosa*.[6] Antimicrobial therapy that controls colonization and proliferation of microbial pathogens is the most vital aspects of skin wound care.[7]

Propolis is a substance that is made by honey bees to protect the hive which has a good effect against fungi, bacteria, and viruses. Many studies have been done on the effect of propolis. Also, now it is being reviewed by many researchers.[7]

The rapid emergence of antibiotic-resistant microorganisms triggered the search for alternatives such as natural products with antimicrobial activity worldwide, the urgent need of research into natural alternatives to antimicrobials is emphasized also by the current farm animal health and welfare policies Propolis may represent a valid choice, in the view of bioavailability and complex therapeutic potential conferred by its rich content in biologically active compounds. Defined as a natural product derived from plant resins collected by honeybees, propolis is well-known as a highly valuable natural remedy with a multitude of biological and pharmacological properties, including antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, immunomodulation, wound healing, hepatoprotective anti-ulcer, and anti-tumor activities. Not only the biological properties are complex, but also the chemical composition features, with more than 300 identified compounds such as polyphenols, phenolic aldehydes, sesquiterpenes quinines, coumarone, amino acids, steroids, and inorganic compounds. Its content variations depending on the collecting location, time, and plant source. Few studies investigated the synergistic effects between propolis and antibiotics and no studies regarding the synergistic effect of Iranian propolis with other drugs have been done. Therefore, taking into consideration the importance of new scientific research relating to Iranian propolis.[8]

Nanoemulsions are dispersions of nano-scale droplets formed by shear-induced rupturing. Nanoemulsions are defined as O/W (oil in water) or W/O (water in oil) emulsion producing a transparent product that has a droplet size from 20-200 nm and does not have the propensity to coalesce. Nanoemulsions have many interesting physical properties that are different from or are micro-scale emulsions. Nanoemulsions appear visibly different from micro-scale emulsions since the droplets can be much smaller than optical wave lengths of the visible spectrum. So nanoemulsions can appear nearly transparent in the visible spectrum and exhibit very little scattering.[9]

Due to the antimicrobial properties of bee bean, this substance can be used as a suitable substitute for nano-metals in nano scale. Therefore, after commercialization, this product can be used to control and treat human, livestock, and honey diseases. Therefore, this study aimed to assess antimicrobial activity of ethanolic extract of propolis and propolis nanoemulsion on experimental cutaneous infection contaminated with *P. aeruginosa* in rabbit.

Materials and Methods

Preparation of Ethanolic Extract of Propolis (EEP)

In this study, extracts of propolis were performed according to Li-Chang Lu method.[10] Crude samples of *Apis mellifera* propolis were obtained from a different region of Western Azerbaijan, Iran. Propolis samples were cut into small pieces and stored in 4°C. Thirty grams of propolis were extracted by 300 ml of 50% (v/v) ethanol by orbital shaking at 150 rpm at room temperature for 3 days. Then ethanolic extract of propolis was filtered with Watman paper No.1. Various concentrations of propolis extract were prepared (8000, 4000, 2000, 1000, 500, 250, 125, 62.5, 31.75, 15.62 µg/ml). Prepared samples were stored in the dark condition at 4 °C till used in this study.

Preparation of Propolis Nanoemulsion (NP)

Preparation of nanoemulsion of propolis was carried out using high energy emulsifying method. To prepare 10% w/w nanoemulsion of propolis, mixing 8 ml of tween 20, 0.6 gram of ethanolic extract of propolis, 10 ml of distilled water, and 2 ml of 96% ethanol. The mixture homogenized by using 75 W ultrasonic waveform generator and frequency 30 kHz for 15 minutes and the final solution was stored at room temperature.[11]

The stability of obtained nanoemulsion was determined measuring the size and distribution of particles in dynamic light scattering (DLS) method with the HORIBA Jobin Yvon machine. For confirmation of nanoemulsion size and taking a general image, the TEM electron microscope (Philips EM208) specification with an acceleration voltage of 100 kV was used.

Bacterial Suspensions

P. aeruginosa (ATCC 19582) was provided from Microbial Collection in Department of Microbiology, Faculty of Veterinary Medicine, Urmia University. To prepare a bacterial suspension, bacteria cultured in Mueller Hinton Broth (Merck, Germany) and incubated for 18 h at 37 °C. The bacterial suspension centrifuged at the 1000 g for 10 min at 4 °C. The supernatant discarded and pallet bacteria washed twice with phosphate-buffered saline (PBS) solution and finally were dissolved in PBS solution. The bacterial suspension (108 CFU/ml) prepared in OD 600nm based on the turbidity of 0.5 McFarland.[12]

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The broth micro dilution method was used to determine Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of ethanolic extract of propolis, propolis nanoemulsion, and ciprofloxacin. MIC values were calculated according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Broth micro dilution method was used for testing in vitro the inhibitory concentration of the antimicrobial agent against specific bacterium.[13] To determine the MBC, 10 µl of each well was transferred to Mueller Hinton agar plates (Merck, Germany) and incubated at 37 °C for 24 h. The MBC was considered as the lowest concentration of propolis associated with no visible growth of bacteria on the agar plates.[14]

In vitro Synergistic Nature of Propolis

Synergistic effect of propolis and nanoemulsion of propolis (NP) with standard antibiotic ciprofloxacin evaluated with Checkerboard method.

The combination interaction of propolis extract with standard antibiotic ciprofloxacin (Sigma-Aldrich) was determined in 96-well plates by checkerboard microdilution method.[15] Propolis extract was diluted horizontally and ciprofloxacin was diluted vertically to get a matrix of a different combination of the two. Plates were incubated at 37 °C for 24 h after the addition of 1×10^8 CFU/ml of *P. aeruginosa*. After 24 h, with the help of MIC of the drug alone and in combination, the fractional inhibitory concentration (FIC) and the FIC index (FICI) were calculated.

FIC was calculated for ciprofloxacin as well as for propolis and propolis nanoemulsion according to the following formulae:

$$\text{FIC of ciprofloxacin} = \frac{\text{MIC of drug ciprofloxacin in combination}}{\text{MIC of drug ciprofloxacin alone}}$$

$$\text{FIC of EEP and NP} = \frac{\text{MIC of EEPor NP in combination}}{\text{MICof EEPor NP alone}}$$

FIC index (FICI) = FIC of ciprofloxacin +FIC of EEP or NP.

Synergy is defined as a FIC index of ≤ 0.5. Additivity is defined as an FIC index of ≥ 0.5 to ≤ 1. In difference is considered when FIC ≥ 1 but ≤ 4.0. When the FIC index is ≥ 4.0 then it is antagonist.

Animals and Animal Care

The experimental protocols were approved by the Animal Research Ethics Committee of the veterinary faculty of Urmia University, Iran (Approval number AECVU -183-2018/Dec.18, 2018).

Twenty-Eight rabbits, each weighing 2.5–3 kg were used for experimental infections. Animals were fed food and water *ad libitum*. The animals were randomly divided into seven groups (n = 28): A treated group with ciprofloxacin (positive control) and normal saline (negative control), the ethanolic extract of propolis (EEP) group, nanoemulsion of propolis group (NP), combination of ciprofloxacin and EEP, combination of ciprofloxacin and NP, Tween20 group. Rabbits were kept under specific pathogenic-free conditions, housed, fed, and treated in accordance with the international guidelines' principles of laboratory animal use and care.[16] They were maintained for 2 weeks to be acclimatized prior to the investigation.

Anesthesia and Wounding

For the induction of anesthesia, ketamine hydrochloride (35 mg/kg, Alfasan, Woerden, Netherland) and xylazine hydrochloride (3.5 mg/kg Alfasan, Woerden, Netherland) was administered intramuscularly.[17] The back of the rabbits were disinfected with 70% ethanol and hair shaved and 3 full thickness skin wounds (5 mm in diameter) were created by a sterile punch biopsy equipment (Revolving punch pliers, Germany). The wounds were left open without any dressing material during the study.[18]

Infected Wound Model

The *P. aeruginosa* PAO1 was obtained from Faculty of Veterinary Medicine, Urmia University. The bacteria were grown in Muller-Hinton broth (Merck, Germany). When bacteria were in the log phase of growth, the suspension centrifuged at 1000 g for 10 min, the supernatant was discarded and the bacteria were diluted to 10^8 CFU/ml in sterile phosphate-buffered Saline. 40 µl of the bacterial suspension (10^6 CFU) added to each wound bed immediately after wound surgery.[19]

Grouping and Treatment

As mentioned before the rabbit were randomly divided into seven groups. In all groups, treatments applied topically in the wound bed and all groups were treated with 40 µl of MIC concentration. Also, normal saline and tween 20 (10 µL per wound bed) in control group were used.[20]

Measurement of Wound Infection

Wound infection was assessed using the measurement of the bacterial load at the wound site on days 7, 14, and 21 by swab test. A swab test was performed from the wound surface for analyzing bacterial superficial load on days 7 and 14. The sample transferred to an appropriate diluent. Samples were transferred to a suitable transport medium. Serial dilutions of the suspension ($1:10^2$ to $1:10^{10}$) were made with sterile broth media and were cultured on Mueller-Hinton agar for numbering the bacterial load.[21,22]

Measurement of Wound Size

Wound healing was monitored by taking digital photographs on days 0, 7, 14, and 21 post-treatments. In order to evaluate healing performance, the relic wound size was measured using Digimizer (v5.4.4) software. Wound size was expressed as the percentage of the wound area determined on every post treating day, compared with the original wound area. The following formula was used to determine wound contraction percentage:

$\%$ of wound contraction $= (A_0 - A_t)/A_0 \times 100$.[23]

Where A_0 is the original wound area and at is the area of the wound at the time of bacterial counting and taking images (on day 0, 7, 14, and 21 accordingly). The area was measured by the images of the wounds using image analysis software after calibration in same days.

Histologic Evaluations

To evaluate the microscopic changes of healing wound tissue, the area of wounds with normal tissue surrounding them was removed on 7, 14, and 21 days after the beginning of treatment time in each group using scissor and scalpel in a way that the sample included 2 mm of normal tissue. The samples were fixed in 10% formalin solution they embedded in paraffin and the paraffin blocks were cut into sections of five µm thick. The sections were stained with hematoxylin-eosin. The sections were examined with light microscopy to evaluate the microscopic changes of healing wound tissue.[24,25]

To evaluate the healing process between different groups, the histological analysis, and a quantitative scoring method performed for different histopathologic parameters including angiogenesis, inflammation, fibroplasia, and restoration of the connective tissue matrix, wound contraction and remodeling and epithelialization, based on 0-3 scoring in the following ways: (0) absent, (1) scanty, (2) moderate, and (3) profound.[26]

Statistical Analysis

All statistical data were analyzed by a one-way ANOVA with Tukey-Kramer post-test using SPSS 17.0 (Chicago, IL, USA). The results of variable analysis were expressed as Means. Semi-quantitative data also used a Kruskal-Wallis test. Values of $p < 0.05$ were considered statistically significant.

Results

Particles

In this study, Tween 20 was used to stabilize nanoemulsion. Nanoemulsion of the propolis provided by ultrasonic waves and Tween prevents their congestion. To determine the size of the nanoemulsions prepared from the samples, DLS analysis was performed. This chart shows the distribution of particles were in the range of 100 to 500 nm, that the highest distribution of particles was between 100 and 200 nm. Also, the highest particle size was 156.8 nm (Figure 1).

To illustrate the shape and size of the particles, the image of the TEM micrograph is depicted. This image was well illustrated by the formation of nanoparticles. The

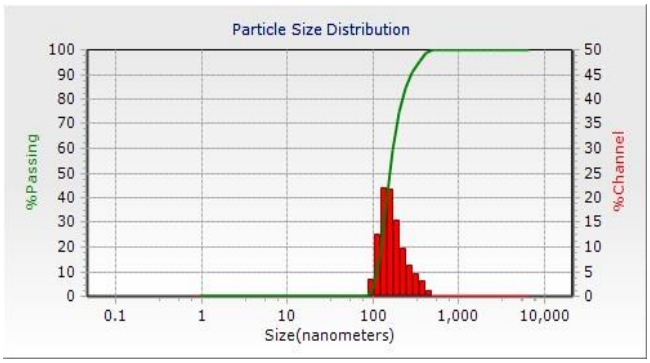

Figure 1. Particle size distribution chart obtained from Dynamic Light Scattering (DLS) for propolis nanoemulsion sample.

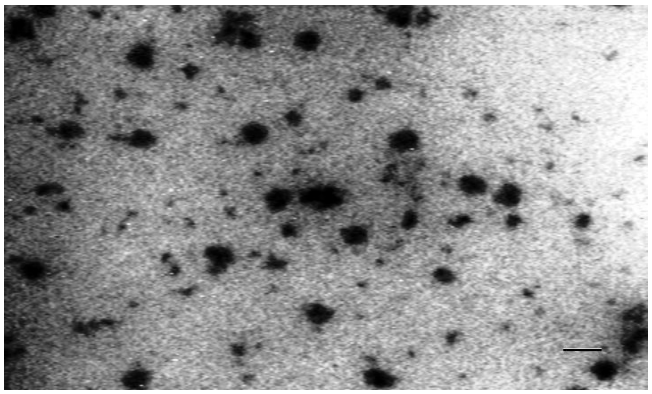

Figure 2. TEM micrograph related to propolis nanoemulsion (rod 200 nm).

Table 1. MIC, MBC results in different dilutions of propolis, nanoemulsion of propolis and ciprofloxacin against *Pseudomonas aeruginosa PAO1*.

Sample	MIC µg/ml	MBC µg/ml
Propolis	1000	2000
Propolis nanoemulsion	468.8	937.6
Ciprofloxacin	4.9	9.8

$p < 0.05$.

Table 2. Evaluation of the combined effects of propolis and nanoemulsion of propolis (NP) with standard antibiotic ciprofloxacin (Checkerboard method: CB method).

Sample	MIC µg/ml	MBC µg/ml	FICI
Propolis + ciprofloxacin	9.76 + 2.44	19.53 + 4.9	0.5 S
Propolis nanoemulsion + ciprofloxacin	7.3 + 1.22	14.64 + 2.44	0.26 S

S: synergy, FIC: Fractional inhibitory concentration, $p < 0.05$.

maximum particle size distribution was between 140 and 160 nm (Figure 2).

MIC, MBC, and FIC Indices

In this study, the antibacterial properties of propolis ethanol extract, propolis nanoemulsion of and their combination with ciprofloxacin against *P. aeruginosa PAO1* were investigated by microdilution method. The results of broth microdilution test, namely Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) and Fractional Inhibitory Concentration (FIC), are shown in the Tables 1 and 2. Based on this, the value of FIC index was found to be ≤ 0.5 indicating synergy.

Microbial Loads in Wounds

Results of bacterial count in wound surface indicated that except control group, on day 0 to 21, the bacterial loads dropped in all groups. The primary inoculation of bacteria was approximately 10^6 that it reaches to 0 CFU/ml in the treated groups on day 21. The C + P, C + NP groups showed the quick decline on day 0 to day 21. The greatest amounts of bacteria were determined in the negative control group. Reduction of the bacterial load were significant in all treatment groups on days 7, 14, and 21 compared with the negative control group ($p < 0.05$). So that no bacterial growth was observed on day 21 (Table 3). The surface infection evaluation showed significant decrease ($p < 0.000$) in bacterial load in all treatment groups.

Wound Closure

A rabbit wound healing model with a 5 mm diameter of full-thickness cutaneous wound used to evaluate the effect of treatment groups propolis, ciprofloxacin, propolis with ciprofloxacin, propolis nanoemulsion, propolis nanoemulsion with ciprofloxacin and normal saline (control on the wound treatment process). Quantitative measurements of wound size are routinely used to assess initial wound size as well as progress toward wound closure. The wound contraction rate measured as the reduction in wound size on days 7, 14, and 21 post-treatment. Significant progress in the wound contraction was observed in the treated excision wounds to the control group. Among all groups, the NP+C group demonstrated the best wound healing properties than other groups in (Figure 3, Table 4).

Histopathology Results

Inflammatory response. The inflammatory response in tissue sections on day 7 in all treatment groups was lower than the negative control, and this difference was statistically significant ($p < 0.5$). Moreover, the cellular response in all the groups were

Table 3. Bacterial load average in wounds area of experimental groups post-treatment

Treatment	Day	Bacterial count
Control	7	4.1×10^{10} CFU/ml
	14	4.4×10^{5} CFU/ml
	21	467 CFU/mL
ciprofloxacin	7	3.2×10^{5} CFU/ml
	14	4.3×10^{3} CFU/ml
	21	-
propolis	7	3.4×10^{4} CFU/ml
	14	2.6×10^{3} CFU/ml
	21	-
Propolis nanoemulsion	7	2.8×10^{3} CFU/ml
	14	305 CFU/ml
	21	-
Propolis + ciprofloxacin	7	683 CFU/ml
	14	143 CFU/ml
	21	-
Propolis Nanoemulsion + ciprofloxacin	7	310 CFU/ml
	14	50 CFU/ml
	21	-
Tween 20	7	3.8×10^{10} CFU/ml
	14	3.9×10^{5} CFU/ml
	21	395 CFU/ml

* Result of Tukey's analysis indicate significant different ($p < 0.05$) between the groups of C, P, NP, C + P, C + NP, and control Tween20 on days 7, 14, and 21.

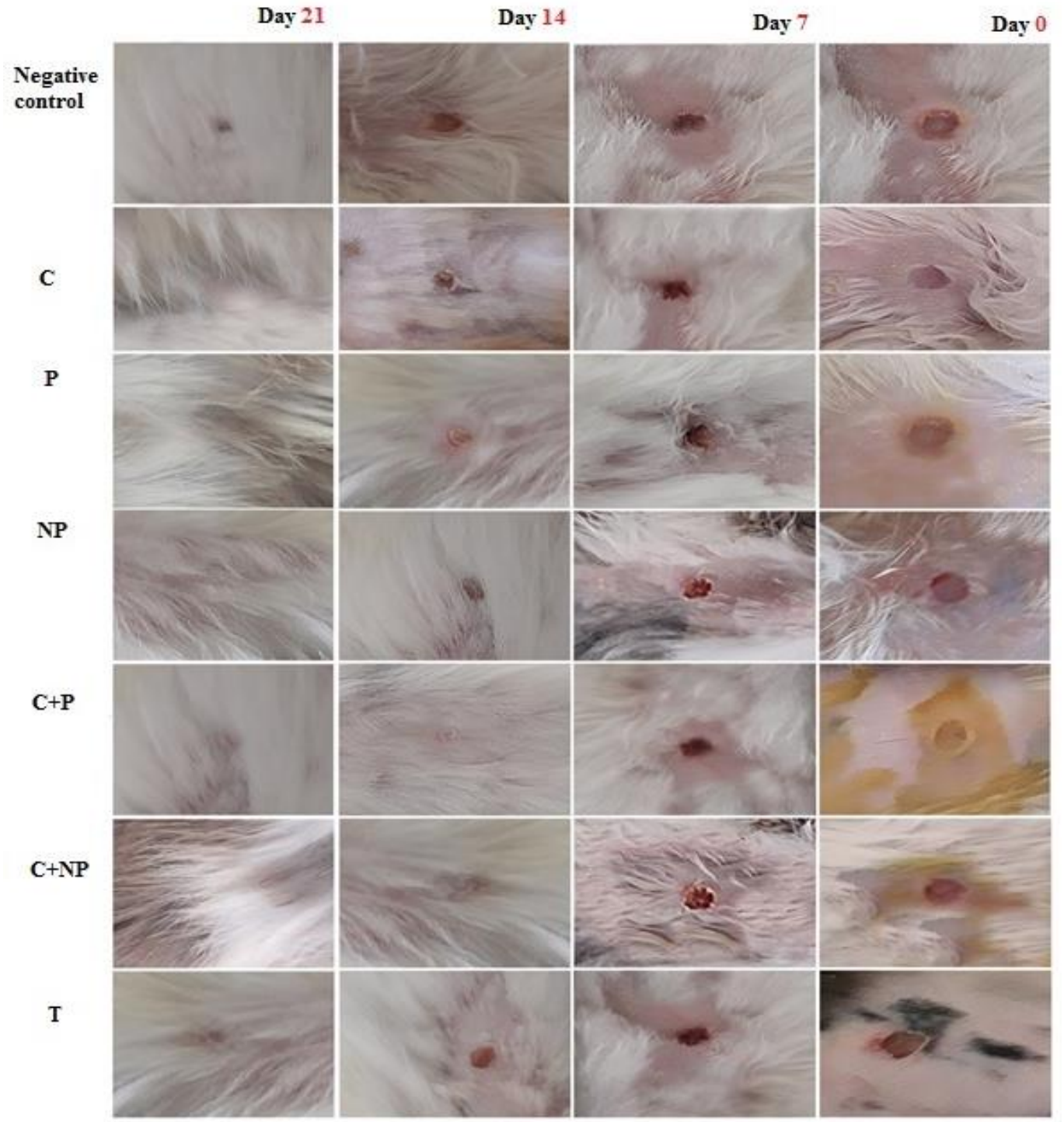

Figure 3. Macroscopic findings in wound healing of rabbits in all treatment groups and all studied days.
C = ciprofloxacin, P = propolis, NP = Propolis nanoemulsion, T = Tween 20.

predominantly neutrophils. In all the other experimental group compared with ciprofloxacin and ethanolic extract of propolis group, the inflammatory response reduced significantly ($p < 0.5$). The number of polymorph nuclear cells in the ethanolic extract of Propolis group and nanoemulsion of propolis in combination with ciprofloxacin was significantly lower compared to the all the other groups ($p < 0.5$). Although, in comparison with each other the difference was not significant ($p > 0.5$). On day 14, the percentage of inflammatory cells reduced in all groups.

Angiogenesis. The highest number of blood vessels was observed on the day 7th and 14th respectively, in ethanolic extract of propolis group and nanoemulsion of propolis in combination with ciprofloxacin. On the other hand, a much reduced number of blood vessels belonged to negative control and Tween 20 groups. The increased rate of angiogenesis in treatment groups compare to negative control and Tween 20 was significant ($p < 0.5$). The high rate of angiogenesis in propolis nanoemulsion compare to ciprofloxacin group was significant ($p < 0.5$).

Fibroblastic density. On day 7 the highest rate of

Table 4. Mean of the wound area on 7, 14, and 21 days in P, C, NP, P + C, NP + C, control (normal saline), and Tween20 (solvent) groups.

	Mean of the wound area (mm²) and (% of wound contraction)			
Treatment	0	7	14	21
Control	19.96	13.06 ± 0.880 (34.5)	9.03 ± 0.033 (54.8)	2.1 ± 0.023 (89.5)
ciprofloxacin		9.2 ± 0.057 (53.9)	5.16 ± 0.088 (74)	0 (100)
propolis		9.04 ± 0.030 (54.7)	5.13 ± 0.033 (74.2)	0 (100)
Propolis nanoemulsion		6.7 ± 0.057 (66.4)	3.35 ± 0.012 (83.2)	0 (100)
Propolis + ciprofloxacin		3.3 ± 0.057 (83.5)	1.61 ± 0.580 (91.9)	0 (100)
Propolis nanoemulsion + ciprofloxacin		2.3 ± 0.054 (88.5)	0 (100)	0 (100)
Tween 20		13.11 ± 0.060 (34.3)	9.1 ± 0.047 (54.4)	2.2±0.076 (89)

$p < 0.05$

fibroblast proliferation was in propolis nanoemulsion with ciprofloxacin and after that to ethanolic extract of propolis and ciprofloxacin ($p < 0.5$). There was no significant difference in fibroblast migration in ethanolic extract of propolis group and ciprofloxacin ($p > 0.5$)

Granulation tissue. In all treatment groups and negative control group, on day 7 the whole wound bed was covered with mature granulation tissue containing the fibroblasts, and blood vessels were at their highest level. On day 14, the highest amount of granulation tissue was observed in non-emulsion propolis with ciprofloxacin and ethanolic extract of propolis with ciprofloxacin.

Epithelialization. The re-epithelialization was higher in the group with non-emulsion propolis with ciprofloxacin, ethanolic extract of propolis with ciprofloxacin on day 7 compared with another group. The difference in all treatment group compared to negative control and Tween was significant ($p < 0.5$). In all treatment groups the formation of epidermis on day14 was higher than day 7 and was statistically significant ($p < 0.5$). The process of epithelial regeneration epithelialization in groups of ciprofloxacin and ethanolic extract of propolis was equal.

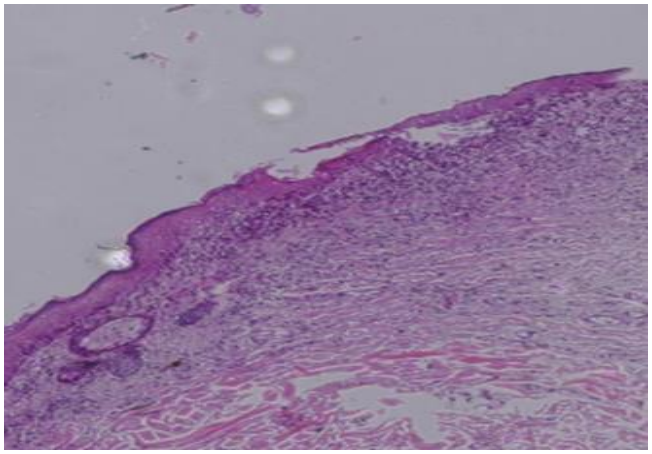

Figure 4. Photomicrograph of skin wound section of rabbit, there is a disruption in the re- epithelialization of wound tissue in the ciprofloxacin group on day 7 of treatment. There are leukocyte infiltration and granulation tissue formation (H&E staining, ×100).

The histopathologic slides are demonstrated in Figures 4-11.

Discussion

Nanomaterials with antimicrobial activity that elevate the effectiveness and safety of antimicrobial administration are called Nano-antibiotics.[27] Their capability in control of infection has been explored and demonstrated in vitro and *in vivo.*[28] Due to the prompt

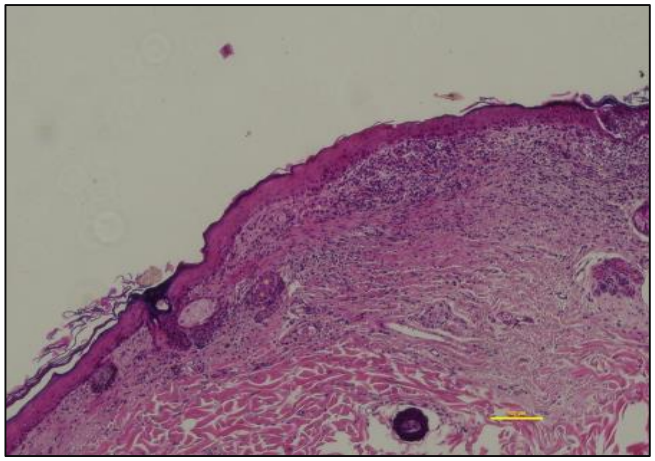

Figure 5. Granulation tissue is formed and re-epithelialization is complete in ethanolic extract of propolis group on day 7 of treatment (H&E staining, ×100).

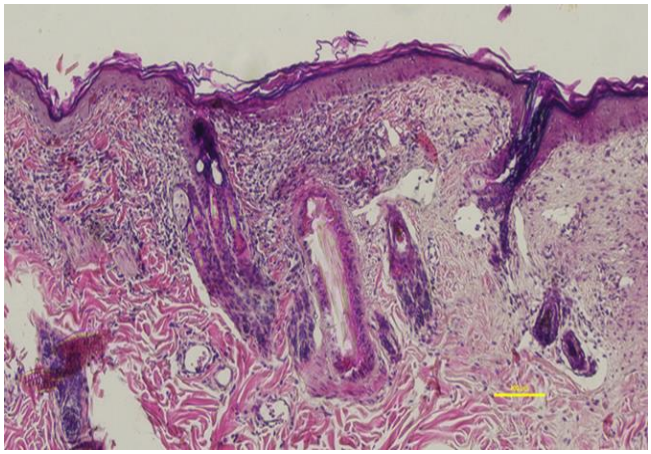

Figure 6. The wound tissue section of propolis nanoemulsion group on day 7 of treatment. The re-epithelialization is high with granulation tissue (H&E staining, ×100).

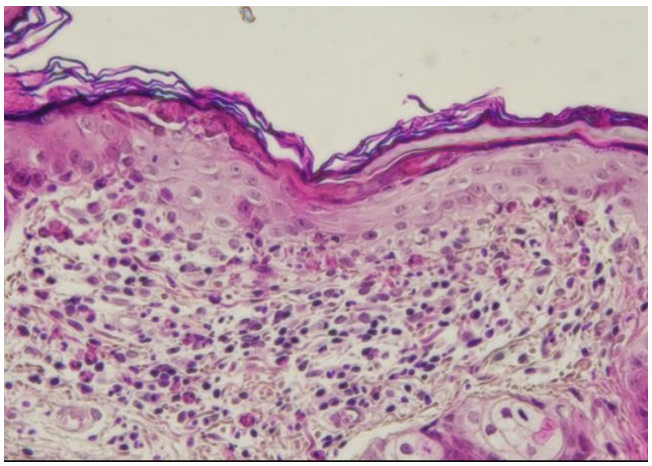

Figure 7. The epithelium is formed in ciprofloxacin group on day14 of treatment. There are leukocyte infiltration and granulation tissue (H&E staining, ×100).

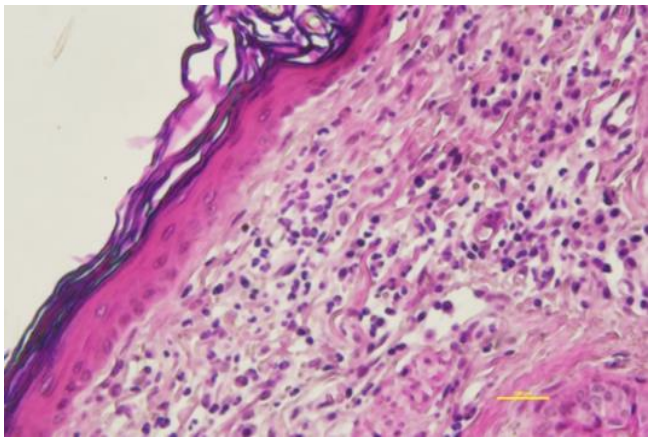

Figure 8. The wound tissue sections of the propolis group on day 14 of treatment. The re-epithelialization is complete. There is leukocyte infiltration with an irregular pattern of collagen formation (H&E staining, ×100).

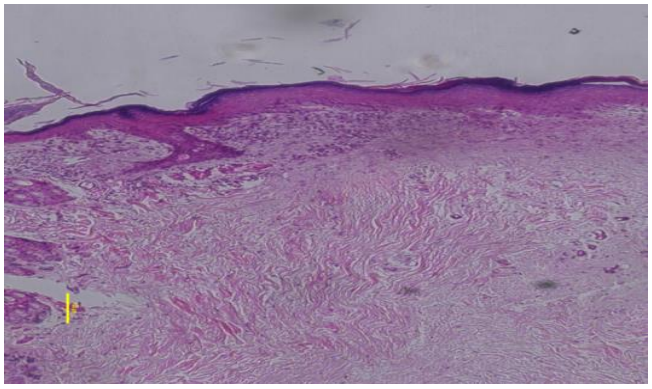

Figure 9. The wound tissue sections of propolis nanoemulsion group on day 14 of treatment. The re-epithelialization is high. There are leukocyte infiltration and granulation tissue (H&E staining, ×100).

prevalence of multidrug -resistant pathogens and insufficient research regarding antibiotic production, the propolis could be useful alternatives for routine antibiotic therapy. Attention to the wound tissue remodeling and its infection is critical for quick repair with no side effects. Wound healing is a treatment priority especially for diabetic or infected wounds suffering patients.[29]

In the United States, 4 to 6 million people affect by chronic wounds each year, consuming over 25 billion

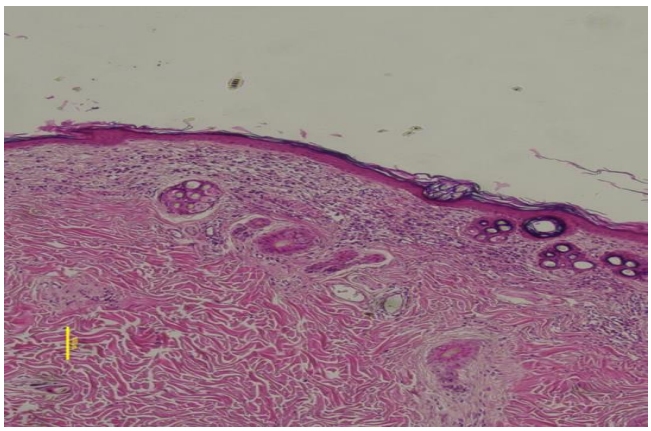

Figure 10. Wound tissue sections of the combination of ciprofloxacin and propolis group on day 14 of treatment. The epithelium is incomplete. There are leukocyte infiltration and granulation tissue (H&E staining, ×100).

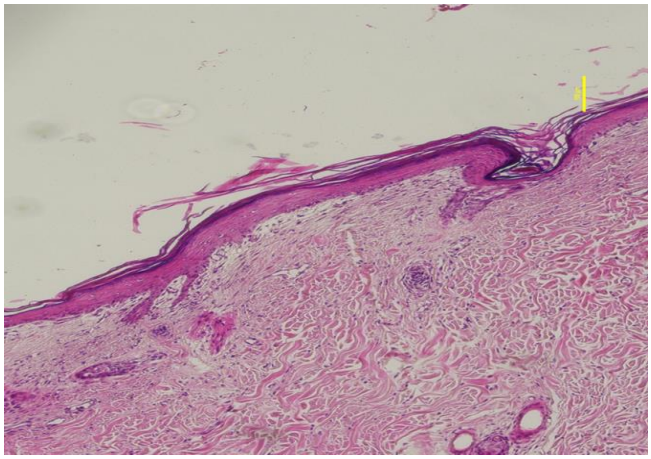

Figure 11. Wound tissue sections of the combination of ciprofloxacin and NP on day 14 of treatment. The epithelium is incomplete. There are leukocyte infiltration and granulation tissue (H&E staining, ×100).

dollars of health-care spending.[21] These startling statistics and the healthcare risks inherent in the setting of this chronic disease serve as the driving force behind the development of novel wound therapies. Nanomaterials, which either show antimicrobial activity by themselves or elevate the effectiveness and safety of antibiotics administration are called "Nano antibiotics" and their capability of controlling infections *in vitro* and *in vivo* has been explored and demonstrated.[30,31]

Chemical or drug resistance is a consequence of evolution and is a response due to pressures imposed on any living organism. Increasing antibiotic resistance among microbes urgently necessitates the development of novel antimicrobial agents. The alternative therapeutics incorporating natural products with standard medication is a promising approach in disease remediation. Plant and animal sources offer good potential for exploitation and bee products because of their documented application in home remedies are of great interest for such researches.[19] The present study was planned to focus on the antibacterial role of propolis and propolis nanoemulsion against *P. aeruginosa PAO1.* So as to analyze if it could help in reducing the antibiotic

clinical doses. The effectiveness of propolis and propolis nanoemulsion was checked in combination with the antibiotic ciprofloxacin during *in vitro* experiments. It was observed that the sub MIC of EEP (9.76 µg/ml) and MIC of propolis nanoemulsion (7.3 µg/ml) in combination displayed synergistic effect with sub MIC of ciprofloxacin (2.44 and 1.22µg/ml).

It is the first report of propolis nanoemulsion that was used synergistically with ciprofloxacin against *P. aeruginosa*. Earlier studies reported the synergistic effect of Brazilian propolis and some antibiotics against *S. typhi*.[32,33] Synergistic combinations to fight MDR have been experimented earlier also and were quite successful like propolis with clarithromycin against *H. pylori* had synergistic or additive activity.[34] The results of present study further supported the outcome of previous experiments. The possible reason for the effectiveness of propolis could be the active components, which were confirmed by phytochemical analysis as well as GC-MS studies performed by the authors earlier.[35,36] The results showed the presence of flavonoid 4,5,7trihydroxyflavone (galangin), 4H-1-benzopyran-4-one (pinocembrin), cinnamic acid, tannins, alkaloids, terpenoids, fructofuranans, fructopyranose, tagatofuranose. The phytochemicals detected in the present study have previously been shown to exhibit biological activities, such as antibacterial, antitumor, and anthelminthic.[37,38]

Studies have proved the effectiveness of propolis against both Gram-positive and Gram-negative bacteria. This might be due to components of propolis like flavonoids, benzoic acid, cinnamic acid, and caffeic acid that may have acted on the membrane or cell wall of microorganism and caused structural as well as functional deformities.[39,40] The data obtained from the present study revealed a significant reduction in the *P. aeruginosa* bacterial count in combination groups. These results point to the synergistic effect of propolis and ciprofloxacin.

Topical application of propolis and nanoemulsion of propolis effectively enhanced the remodeling of wounds area and diameter and skin macroscopic appearance in rabbit. In this study, it was observed that 14 and 21 days after treatment, in groups treated with ciprofloxacin or propolis and nanoemulsion of propolis, total *P. aeruginosa* bacterial loads reduced significantly. There is no clinical study about the effects of propolis on bacterial load, however, the in vitro antibacterial effects of propolis are well documented.

In the present study, propolis nanoemulsion prepared 156.8 nm in size that measured by using a Dynamic Light Scattering (DLS) device, and its antimicrobial properties were investigated. This study showed that propolis nanoemulsion was better in antimicrobial activity than propolis extract.

The rapid growth of researches about antimicrobial activities *in-vitro* strengthens the need for closer evaluation of their potential activities *in-vivo*. However, most of the studies were only carried out *in vitro*, without follow-up of *in vivo* data support. Here, we describe the use of propolis and propolis nanoemulsion with and without antibiotic combination to improve interactions on infectious wound healing. Antimicrobial agents, such as antibiotics, silver compounds, iodine compounds, and others are often loaded in wound dressings to prevent or treat infection.[41] However, concerns about the using of antimicrobials on open wounds still exist because of their potential cytotoxicity that may delay healing.[7]

Bacteria are thought to play a critical role in delayed healing by altering host cell function, and lowering the level of endogenous growth factors.[42] Strategies to control the risk of infection, and the level of bacterial activity, are generally directed toward several variables number of bacteria, strength of their virulence, and the immune status of the host.[43]

According to a report by Bucknall in 1980, the rate of infection is directly related to the number of organisms inoculated. Inoculation at 10^6 bacteria/ml resulted to 100% of the wounds producing pus without mortality while at 10^{10} cells/ml all the test animals died with an overwhelming infection, at 10^4 cells/ml approximately 50% of the wounds are showed no sign of infection.[20] So, in the present study, the skin wound of the rabbit inoculated with 10^6 CFU of pathogen, and a good local infection was established on post-operative days without mortality. The treatment of infectious wounds in rabbit with nanoemulsion of propolis, ciprofloxacin, and nanoemulsion of propolis with ciprofloxacin resulted in a significant decrease in surface wound infection on days 7 and 14 post treatment. In all treatment groups, the bacterial count reduced from day 0 to 21. In this study, the presence of propolis nanoemulsion and propolis and in combination with ciprofloxacin improved wound appearance better than other groups (Tween 20 and control groups) and wound trace less remains (Table4).

The positive effect of ethanolic extract of propolis and nanoemulsion of propolis on the healing process of a wound infected with *P. aeruginosa* was evaluated macroscopically and microscopically. Microscopically, on day 7 of the healing process due to the presence of microorganism, the rate of purulent reaction and inflammation were the most predominant changes that were observed in all groups. Moreover, combination of ciprofloxacin and EEP, combination of ciprofloxacin and NP ethanolic extract of propolis and nanoemulsion of propolis groups compare with negative control Tween 20 caused better control of infection increased vascularization and fibroblast migration and consequently more collagen formation, and significantly

accelerated wound healing. On day 14 ethanolic extract of Propolis in comparison with negative control had a better repair process and with ciprofloxacin had a similar healing condition. On day 21 in all treated groups the wounds were fully healed, the epithelial cover restored, the purulent reaction was gone and the inflammation was limited and the healed wounds were matured. Also, the reduction in the number of neutrophils in the experimental groups on day 14 and 21 was statistically significant when compared to the negative control group ($p < 0.5$). There was less fibroblast proliferation and angiogenesis and re-epithelization was high compared to control group. Considering the wound contraction, the wound treated with ethanolic extract of propolis had a similar effect with ciprofloxacin and were significantly smaller than the sizes of negative control and control groups.

Ibrahim in 2013 in a study on diabetic mice model showed that the use of propolis significantly accelerate the wound healing.[44] A research in 2015 by Abu-Seida showed that the topical use of propolis paste on induced cutaneous wounds led to a faster wound contraction and had a significant impact in repair process.[45] Antibacterial properties of propolis on acne on the faces of 40 patients were investigated by Basma *et al*. and showed significant antibacterial effect of propolis both clinically and in bacterial culture.[46]

Ownagh and Adibhesami, 2012 in the treatment of experimental vaginal candidiasis showed that ethanolic extract of propolis has a therapeutic effect on vaginal candidiasis and dose of 1000 µg/ml of EEP was the most effective dose in the treatment of experimental vaginal candidiasis in rabbits in a shorter time in comparison with standard nystatin treatment.[47]

The result of current study showed that ethanolic extract of propolis and nanoemulsion of propolis combination with antibiotic ciprofloxacin in spite of its lowest therapeutic dose will result in a more robust wound healing response than either use of them alone or in contrast with other groups.

In conclusion, The results of this study showed that propolis nanoemulsion has very effective antibacterial properties against *P. aeruginosa* in *in vitro* conditions Also, the results of this study showed that the combination of propolis ethanol extract and propolis nanoemulsion in combination with ciprofloxacin leads to more drug effects on the organism. Therefore, combined use of propolis and nanoemulsion of propolis with ciprofloxacin has a synergistic effect. As a result, the dosage of each compounds and the time that it takes to eliminate the bacteria is reduced in contrast the use of them alone.

Due to the failure of antibiotic treatments, including the occurrence of antibiotic-resistant strains, against bacterial infections and the side effects of antibiotics, more serious and useful studies on the use of natural antibacterial compounds to treat this type of infection is important.

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Conflict of Interest

The authors declare no conflict of interest.

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