ISSN NO: 2771-8840
Date of Publication: 26-06-2023

The effect of propolis ethanolic extract on negative and positive bacteria *in vitro* and *in vivo*

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Abstract: The study aimed to investigate the bioactivity effect of different concentration of ethanol Iraqi propolis against two species of bacteria. *Staph.aureus and pseudomonas* that were infected wound in skin of mice ,Results revealed that the concentration of 4000 and 5000 µg/ml of Iraqi propolis extracted by 75% ethanol was the most effective in growth inhibition of *Staph.aureus and pseudomonas* in agar diffusion method and the diameter of inhibition *in Staph.aureus* reached (22.30, 22.50), mm. respectively, while the diameter of inhibition of *pseudomonas* in a concentration of 4000 and 5000 µg/ml was (21.12,23 .60) mm. by the agar diffusion method as shown propolis was highly effective against *Staph.aureus and pseudomonas* in agar diffusion method with comparing the antibiotic Amoxicillin 30µg with different concentration of ethanol extract of the Iraqi propolis showed no inhibition of the Ampicillin30µg, Methoprim 25 and gentamycin 100 µg against *Staph.aureus* and *pseudomonas*. The previous concentration (4000,5000) was also superior in comparing with Ordinary ointment in terms of its effect on the healing of contaminated and non-contaminated wounds, as well as the return of the tissue to its normal position in a shorter time. where The propolis extract showed a high potency in treating wounds infected with Gram-negative (*pseudomonas*) and Gram-positive bacteria(*Staph.aureus*).

Key word: Propolis, Gram Negative, Gram Positive, Ethanolic Extract**.**

Introduction

The honey has bacteriostatic and bactericidal effects on a variety of infections. ethanol extracts from honey produced a wide range of antibacterial activities. According to the study, honey is similar to antibiotics in that it can treat some bacteria and has organisms that are sensitive to it. Therefore, it is necessary to identify the active ingredients in honey extracts and to promote research into the advantages of using honey as one of several medicines for the treatment of bacterial infections. More than 50% of all medications used in clinical practice worldwide are natural compounds and their derivatives, which includes antibiotics. Around 80% of people in underdeveloped countries, according to estimates from the World Health Organization, rely on wild plants that have been gathered for some aspect of their basic healthcare **(Elisabetsky *et al*., 1996).** Numerous studies have been conducted on the antibacterial properties of various herbal extracts in various parts of the world **(Hammer *et al*., 1999; Gulluce *et al*., 2003)**. Recently, a lot of focus has been placed on extracts and physiologically active chemicals obtained from natural species used in herbal therapy due to the adverse effects and the resistance that pathogenic germs have evolved against antibiotics.In 1892, Dustmann became the first to notice honey's antibacterial properties **(Dustmann, 1989)**.

Methodology

Propolis collection and preparation for the purpose of the study

Propolis was obtained from a private apiary in Baghdad (Al-Mahmoudia) and then classified in the College of Biotechnology, Al-Nahrain University. Propolis was cleaned from dust and suspended wood pieces manually, and then grinded using an electric mill until it became soft. The product was kept in clean, sterile and dark containers. Airtight sealed at room temperature until use

Preparation of ethanolic extract of propolis .

The ethanolic extract was prepared according to the method of **Krell, (1996)**, in which 20 gm of propolis A powder was mixed with 200 ml of ethyl alcohol (75%), placed in a volumetric flask, and left for five days at room temperature with daily shaking for a few minutes, and then shaken using a magnetic shaking device. (magnetic stirrer) for 15 minutes, and after completing the dissolution process, the solution was filtered

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through a clean cloth to get rid of large undissolved particles, and then the solution was filtered using filter paper (Whatman No.1), then the solution was extracted and dried by the oven at a temperature of 45 degrees. Then the extract was weighed and placed in clean sterilized containers and kept at a refrigerator temperature of 4 °C until use.

Prepare the concentrations used in the study

1gram of propolis extract was taken and dissolved in 10 ml of dimethyl sulfoxide as a solvent for each of the ethanolic extracts on a standard stock solution at a concentration of 10%, and then the concentrations were (prepared 5000, 4000, 3000, 2000, 1000 µg / ml

The required amount for the test is 1g/10ml = 100mg/ml

gram = 1000 milligrams 1 mg = 1000 micrograms 1

And using the law of first concentration x

first volume = second concentration x second volume100 x the first volume = 1000 x 1 ml

µL + 990 µL (dimethyl sulfoxide) and so on for the rest of the concentrations. 10 = 100 ÷ 1 × 1000

Diagnosis of pathogenic bacteria

:Pathogenic bacteria were identified according to(**Sneath *et al.*, 1986**), by the following steps: By culturing the bacteria on nutrient agar media, blood agar media, and meconkey agar to observe the phenotypic characteristics of the colonies, the pattern of hemolysis on blood agar media, and lactose fermentation on meconkey agar media. After the bacterial culture, the dishes were incubated at a temperature of 37ºC for 24-48 hours, then the growing colonies on these culture media were examined to know the shapes and sizes of the bacterial colonies, and then swabs of these bacterial colonies were made on the Selective Media and the Differential Media for each type of isolated bacteria. And incubated at a temperature of 37 ºC for a period of 24-48 hours after that

Preparation of culture dishes to test the concentrations of the extract:

The following steps were followed in order to prepare the culture dishes to test the concentrations of the extract in vitro.

1-The nutrient agar medium was prepared after autoclaving it for 15 minutes, at a temperature of 121 ºC and under a pressure of 15 p / inch2.

2-Leave the culture medium to cool down to a temperature of 48ºC using the water bath, then add 0.1 ml of the bacterial suspension for Staph. aurous andpseudomonas with a sterile automatic syringe.

3-Add 20 ml of bacteriostatic medium to each sterilized Petri dish and place in the refrigerator to help harden the culture medium. .

4-Five holes with a diameter of 6 mm were made in each dish, so that the distances are equal between one hole and another, as well as equal distances from the wall of the agricultural dish. It was used for this purpose with a plan on which the agricultural dish is placed, after which the perforation is done. .

5-the extracts were added to the holes at a rate of 0.1 ml for each hole and incubated at 37 °C for 24 hours. The results were read by measuring growth inhibition zones around the holes.

Comparison of propolis extracts with an antibiotic:

The test was conducted and compared between the inhibitory effect of each of the propolis extracts on the one hand and the antibiotic Amoxicillin, Metheprim, Gentamycin on the other hand, in pathogens hat contaminated wounds By the method of well diffusion where the concentrations worked out 5000, 4000, 3000, 2000, 1000 for propolis extract, and ready-made disck were used in the antibiotic Gentamycin loaded with 100 gµ, Metheprim, 25, gµ Aoxicillin30 gµ for the purpose of comparison in inhibiting bacterial pathogens 0.1 ml of bacterial suspensions for each type of bacteria was added to the food medium for each type of bacteria. It was spread in a glass diffuser, and three replications were made for each concentration. The plates were incubated in the incubator, then the diameter of growth inhibition around the disc was calculated using the ruler.

Determine the minimum inhibitory concentration (MIC)

A series of graduated concentrations of ethanolic propolis extract were prepared in test tubes using the nutrient broth, where their values ranged from 500 - 5000 µg / ml. The tubes were inoculated with 0.1 ml of bacterial culture at the age of 24 hours, containing 1.6 x 810 cells / ml, with the preparation of a tube containing the nutrient broth. Inoculated with each type of germs, the experiment is represented by control 1

ISSN NO: 2771-8840
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and another tube containing the nutritious broth only, representing control 2, as the experiment is repeated in the event that no turbidity appears in control 1 or when it appears in control 2. The tubes were incubated at a temperature of 37 °C for a period of 48 hours, then they were removed from the incubator and from. The extract's minimum inhibitory concentration (MIC), which is the lowest concentration at which the culture medium does not look clear to the naked eye, was then calculated.

Distribution of laboratory animal groups

The experiment was carried out practically inside the animal house, with 70 mice divided into seven groups in each gage, each group consisting of 10 mice, and they were divided as follows:

1- Group No. (1) natural skin without a wound to know the texture of the skin naturally.
2- Group No. (2) a wound with bacterial contamination...negative for a gram stain and without treatment.
3- Group No. (3) a wound with bacterial contamination which is positive for a gram stain and without treatment.
4- Group No. (4) Wound with infection with negative bacteria , with treatment with propolis ointment.
5- Group No. (5) a wound with infection with negative bacteria with treatment with a regular ointment.
6- Group No. (6) a wound contaminated with gram-positive bacteria with treatment with propolis ointment.
7- Group No. (7) a positive bacteria wound with treatment with a regular ointment.

Result and discussion:

Incorrect treatment methods have encouraged the emergence of antibiotic resistance in many diseases, Alternative means must be found to limit the occurrence of this phenomenon. Among these means is the use of bee by-products, which include honey, beeswax, royal jelly, bee venom, and propolis. The latter is considered one of the most important bee by-products from a medical and therapeutic point of view, as it contains effective chemical components such as flavonoids and antioxidant compounds (**Hegazi and Abd El-Hady, 2002B; Kumazawa et al. al. 2004, Alencar et al., 2007,** also The variation in the chemical components depends on the vegetation of that region, which works to supply propolis compounds with materials that have a dampening effect on many microorganisms. **Al-Musafer, (2005)** explained that the common vegetation in the central region of Iraq is Sidr, citrus and eucalyptus **(Tom' as–Barberan et al., 1993; Kumazawa et al., 2004.**

The results of measuring the minimum inhibitory concentration (MIC)

For this purpose, 10 graduated concentrations of the ethanolic extract of propolis were used, dissolved in the nutrient broth, starting from 500 µg/ml up to 5000 µg/ml. The concentration of 500 µg/ml did not show any effect on the tested bacterial species(negative and positive), while the concentration of 600 µg/ml recorded the lowest concentration that inhibited growth. *Staph.aureus* the concentration of 700 µg / ml is the lowest concentration that inhibits the growth of *pseudomonas* As shown in **Table No. (1)**

Table (1). The effect of different concentrations of the ethanolic extract of propolis on inhibiting the growth of pathogenic microorganisms in the tubes.

µg / ml conc	***Staph.aureus***	***pseudomonas***
500	-	-
600	-	+
700	+	+
800	+	+
900	+	+
1000	+	++
2000	++	++
3000	++	++
4000	+++	+++
5000	+++	+++

Non-influencing (+) inhibits the microbe (++) kills the microbe(-)

The results showed that the concentrations of 4000 and 5000 mg/µg of the ethanolic extract have greator inhibition of the above two types of bacteria whose inhibition circles were (22.30, 22.50) mm and (21.12,23 .60) mm in diameter over the concentrations of 1000, 2000 and 3000 mg/µg, whose inhibition circles were

ISSN NO: 2771-8840
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10.30, 11.30 and 11.30 mm in diameter for the extract. ethanol by the same method, and the concentration of 4000 mg/µg of the ethanolic extract using the drilling method is the best from an economic point of view **figure(1,2,3,4).**

figure(1) The inhibitory effect of different concentrations of propolis on Staphylococcus aureus bacteria and pseudomans in concentrations of 4000

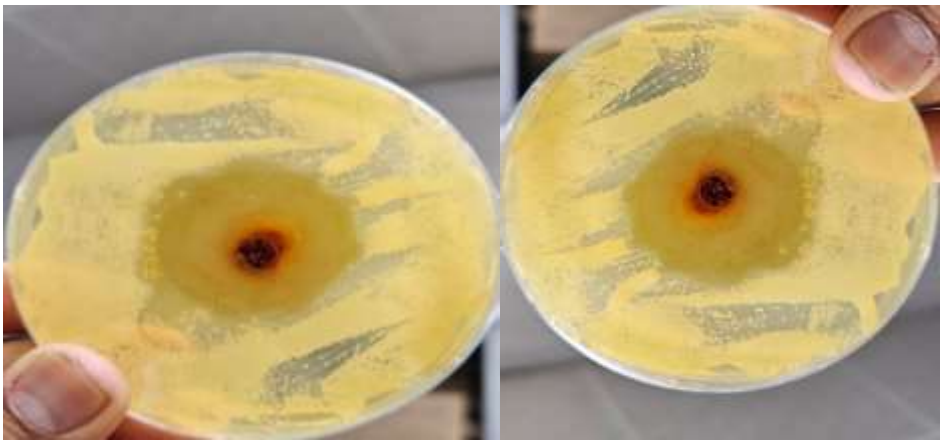

Figure (2) The inhibitory effect of different concentrations of propolis on Staphylococcus aureus bacteria and pseudomans in concentrations of 5000 mg/µg

Figure (3) The inhibitory effect of different concentrations of propolis on Staphylococcus aureus bacteria and pseudomans in concentrations of 1000 mg/µg

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Figure (**4**) The inhibitory effect of different concentrations of propolis on Staphylococcus aureus bacteria and pseudomans in concentrations of 2000 mg/µg

And when comparing the level of inhibition of the two propolis extracts of the tested bacteria with the level of inhibition of antibiotics, the antibiotic amoxcilin30 µg did not lead to any significant inhibition, as all concentrations of propolis were superior to it in µg by the drilling method. While the inhibition circle diameter of each of Methiprim g 25 µ and Gentamicin 100 g µ were did not lead to any significant inhibition for both of them exceeded the diameter of the inhibition circles of the ethanol extract by etching method and for all concentrations. **Figure**(5) However, the diameter of the inhibition circles of these two antibiotics did not differ significantly from the diameter of the inhibition circle of the ethanolic extract at a concentration of 4000 mg/µg using the etching method, as well as the diameter of the inhibition circle of the ethanolic extract at a concentration of 5000 mg/µg using the etching method, which was 22.30 mm, exceeded the diameter of inhibition of the two antibiotics methiprim and gentamicin. The inhepetary zone of propoles agenst gram posetive and negative its may contributed to that propoles had the fallowing ingredient and acid that have greter antibacterial action (isoferiolic acid, caffeic acid, and chrysin. as described by **(Al-Waili and K. Y. Saloom., 1999; Radwan et al., 1984; Boukraa et al., 2008) Molan, (2001); Schepartz and M. H. Subers, (1964)**, demonstrated the power full antibacterial effect of propoles agents negative and positive bacteria and promote wound healing in short time.

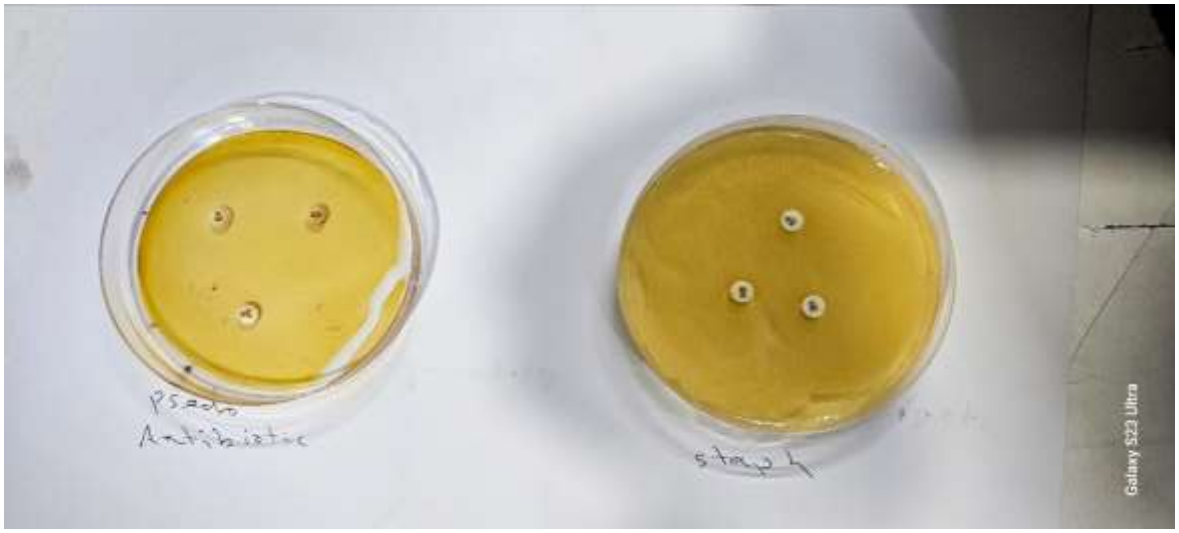

Figure (5) The effect of two antibiotics, gentamicin and methiprim and Amoxicillin , on Staphylococcus aureus and pseudomonas

Regarding the healing of wounds using the alcoholic extract of propolis, the results show It had an effective and rapid effect on wound healing in groups infected with positive and negative bacteria **figure**(6,7). compared to groups treated with normal ointment and infected with the same bacteria it's may be contributed to that propels had greter effect on Enhancing the structural stability of the cell, preventing and blooking the infetinal bacteria and fungi from entering the cell, as menatied by **(A. Hannan *et al.*, 2004).**

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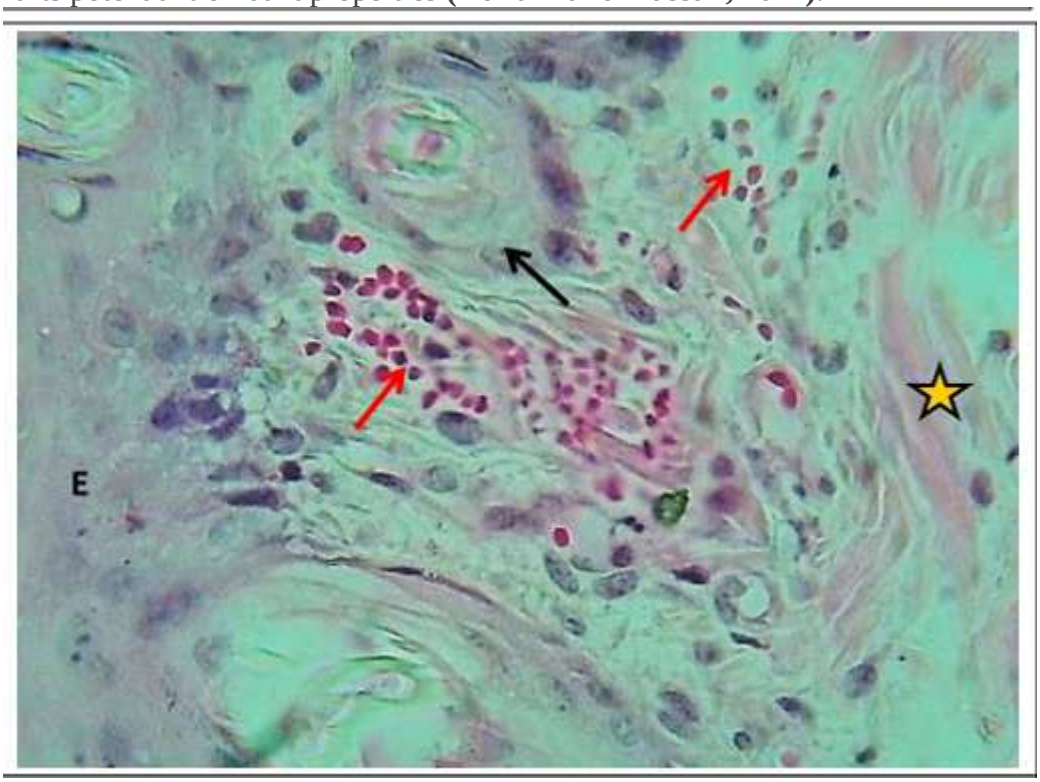
Figuer (8,9). And may duo to the propoles contains active components ingredient such as balasm (50%), waxes (30%), primary oils (10%) and pollen (5%). That prevent insect and flies contaminated the wound with bacteria **(Patton *et al*., 2006; Radwan *et al*., 1984).** Biofilm development is a significant contributor to poor wound healing; propolis, an anti-microbial substance, can decrease biofilm production and speed up healing. Propolis contains antioxidant qualities, which is the third characteristic that makes it useful for wound treatment. Flavonoids and phenolic acids, which make up a large portion of the polyphenols in propolis, give it its potent antioxidant properties **(Adham and Hassan, 2022).**



Figure(6): The histopathological figures of the skin exhibited normal thickening of epidermis, and normal epithelium, infected with negative bacteria after treatment of propolus the dermis revealed normal composite of collagen bundle, hair follicles, the sebaceous glands with little infiltration of mononuclear cells.

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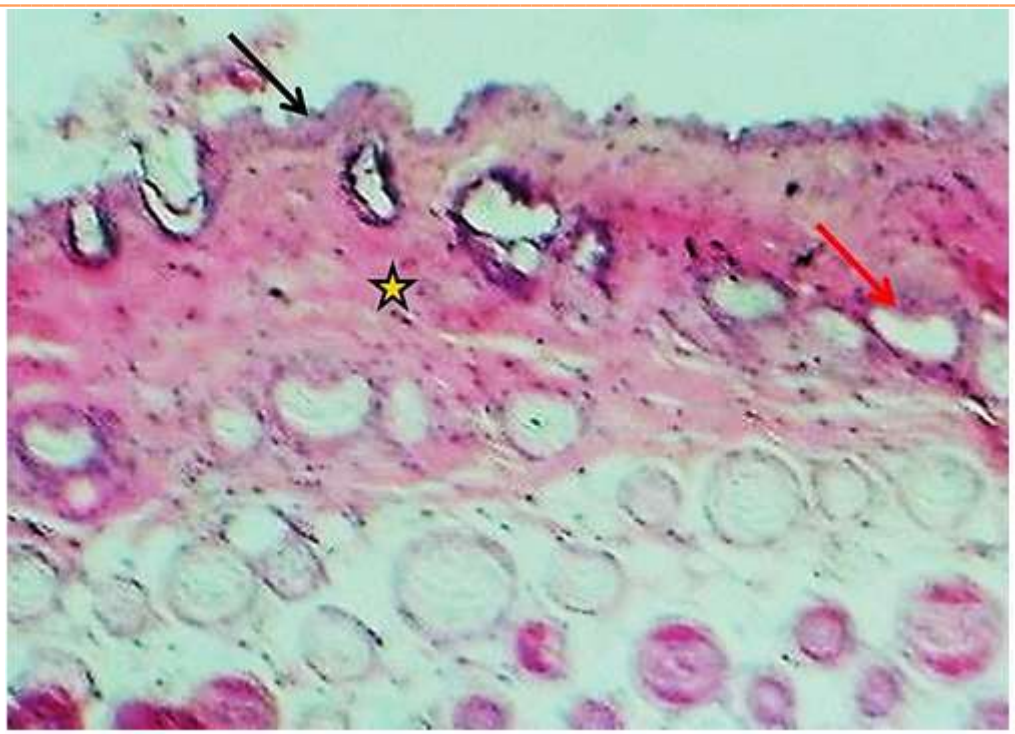
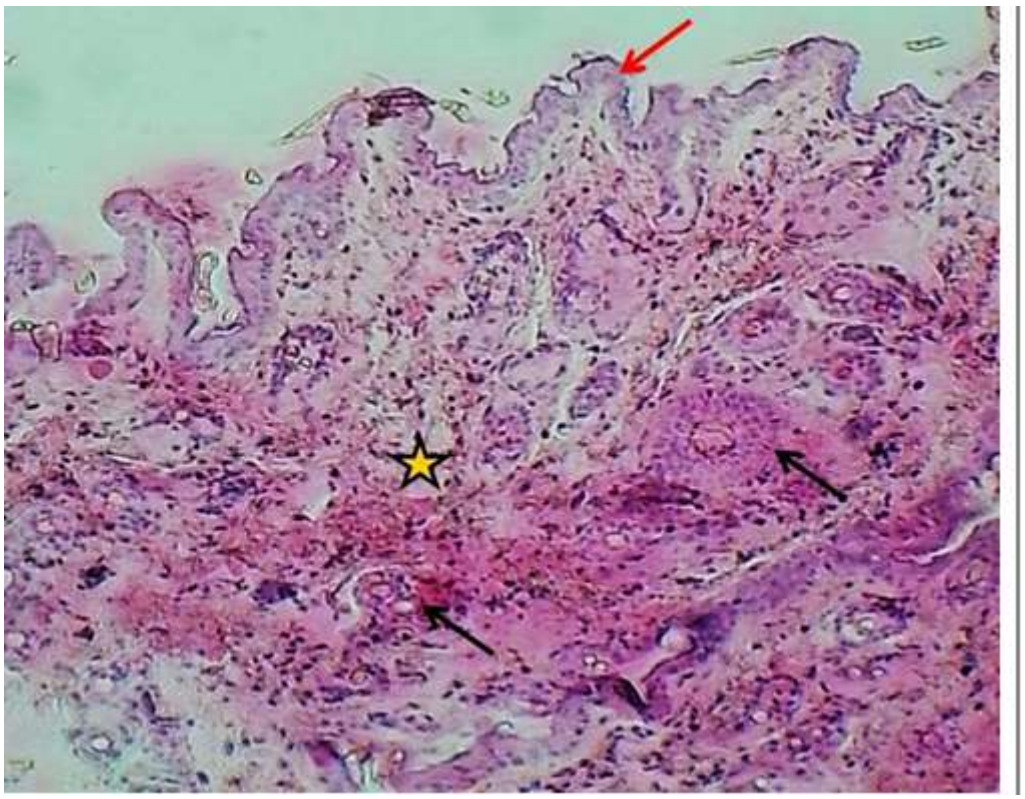


Figure(7): The histopathological figures of the skin exhibited normal thickening of epidermis, and normal epithelium, infected with positive bacteria after treatment of propolus the dermis revealed normal composite of collagen bundle, hair follicles, the sebaceous glands with little infiltration of mononuclear cells.



Figure(8): The histopathological figures of the skin exhibited normal thickening of epidermis, and normal epithelium, infected with positive bacteria after treatment with normal ointment the dermis revealed normal composite of collagen bundle, hair follicles, the sebaceous glands with little infiltration of mononuclear cells.

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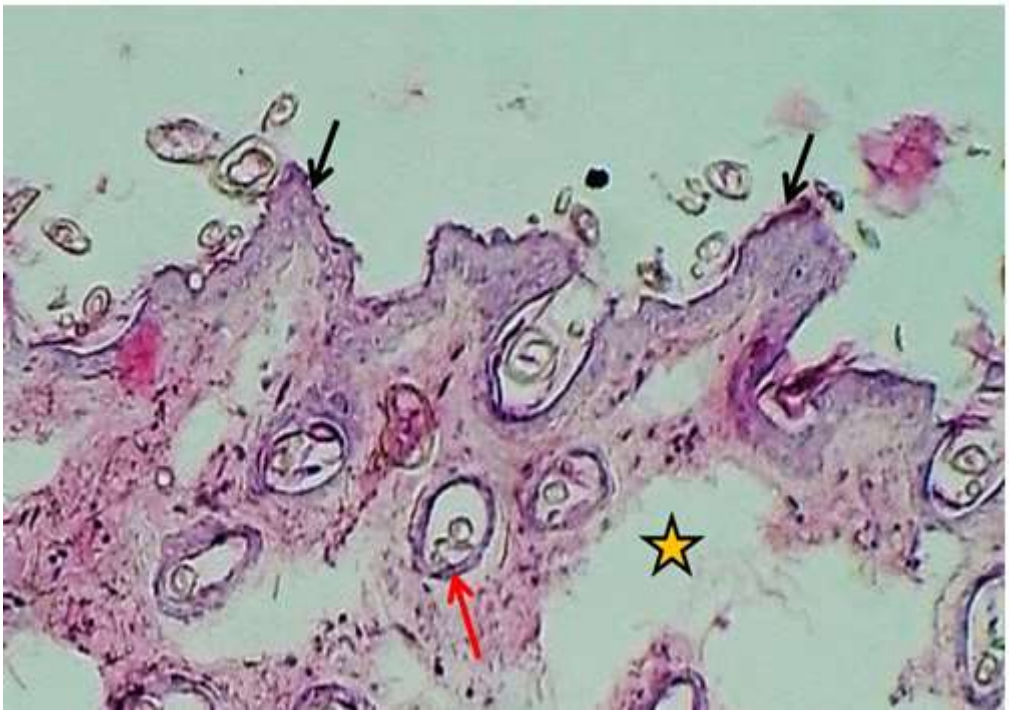
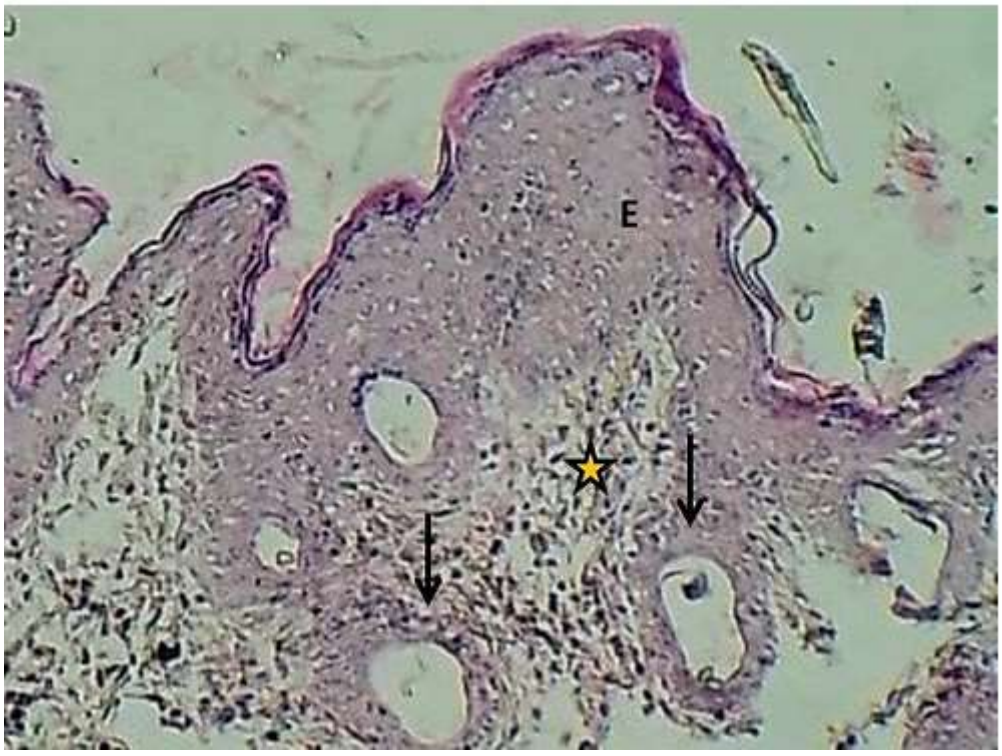


Figure(9): The histopathological figures of the skin exhibited normal thickening of epidermis, and normal epithelium, infected with negative bacteria after treatment with normal ointment the dermis revealed normal composite of collagen bundle, hair follicles, the sebaceous glands with little infiltration of mononuclear cells.



Figure(10): The histopathological figures of the skin revealed normal appearance of epidermal epithelial cells, dermis fibrous connective tissue and hair follicles (negative control).

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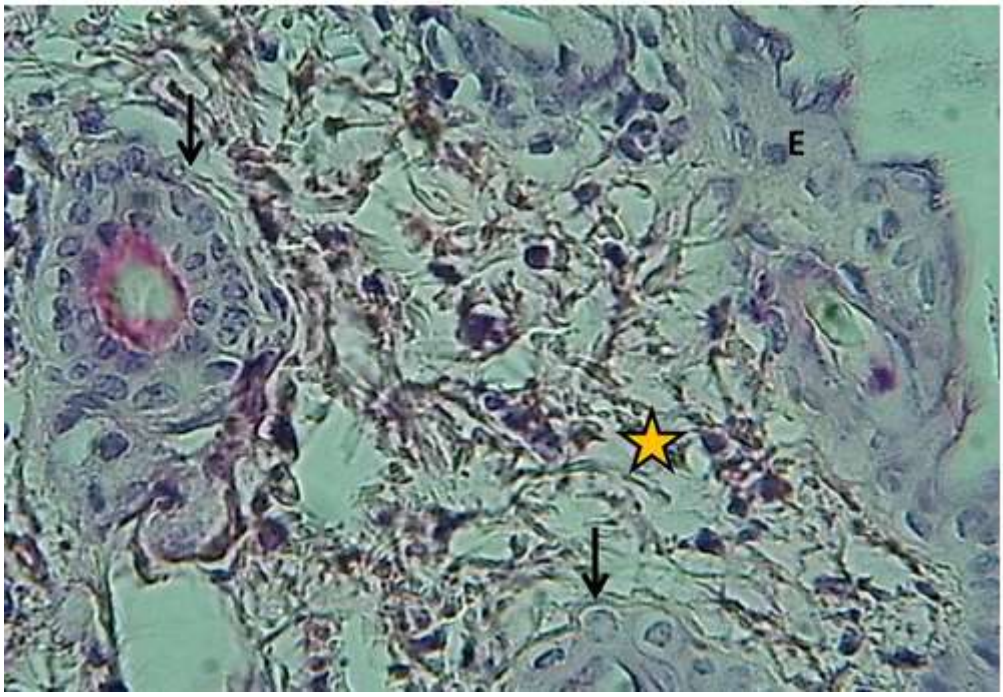

Figure(11): The histopathological figures of the skin revealed normal appearance of epidermal epithelial cells, dermis fibrous connective tissue and hair follicles (positive control).

Conclusion:

The outcomes demonstrated that the alcoholic propolis extract had a definite impact on the organism both externally and inside. It was more effective in treating and healing wounds infected with Gram-negative and Gram-positive bacteria than traditional ointment. When it was tested against both Gram-negative and Gram-positive bacteria in a lab setting, this was shown.

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