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Apitherapy of Septic Metacarpal and Metatarsal Wounds (An Experimental Study on Donkeys)

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Abstract: Non-healed wounds are a significant problem in the healthcare system of the distal portions of the limbs. Nine types of honey were tested for wound healing judged by clinical gross healing parameters, rate of wound contraction and sings of epithelization histopathologically. Rounded full thickness skin wounds (1.5cm diameter) were performed at the distal portion of limbs. The wounds were infected by standard dose of MRSA (methicillin resistance *Staph. aureus*) for 72 hours. The tested types of honey were tested for MIC & MBC against clinical MRSA strain. Different types of honey were applied unprocessed as daily topical wound dressing for the infected wounds. Propolis with black seed honey followed by Hadramout sidr honey, then Egyptian sidr honey proved to have the most antimicrobial activity. Clinical healing appearance revealed that propolis with black seed honey, Egyptian sidr honey and Hadramout sidr honey showed the most preferable clinical parameters (gross appearance and rate of wound contraction), and histopathologically achieved the best healing epithelization 28 days after treatment. The study concluded that among different nine honey types retailed in Egyptian markets, all tested honey types had wound healing activities but with different potencies. Propolis with black seed, Egyptian sidr honey and Hadramout honey were the most potent with highly significance differences ($P < 0.01$), since achieving the most wound contraction rates, rapid epithelization post 28 days rather than the least MICs & MBCs values. While the marjoram and clover honey were the least effective.

Keywords: Wound Healing, Honey Apitherapy, MIC & MBC of Honeys, Histopathology of Wound Healing

1. Introduction

Honey is a traditional remedy in ancient folk medicine and owing to widespread multidrug resistant pathogens emerging, there is a renaissance in honey apitherapy nowadays [1] especially for wound healing either as it is [2-5] or pharmaceutically formulated pectin membrane [6, 7], chitosan hydrogel based [8] or burdock salve [9]. Horses often suffer cutaneous wounds on the distal portion of limbs difficulty to be closed primarily [10], associated with severe tissue avulsion and excessive contamination [11, 12]. Recently, the renewed wound healing activity of honey is widely documented [9, 13, 14], even with infected [3, 15], or non-healing wounds resulting in faster healing process, wound size reduction and lower pain intensity [16]. The present study aimed to investigate the potency of different types of honey as wound dressing for septic skin wounds at limb distal regions in donkeys. Healing was judged by infection clearance, healthy wound contraction or closure and histopathological repair post honey dressing as well as its synergistic activity with bee propolis.

2. Materials and Methods

Animals: Twenty four apparently healthy adult donkeys of (both sexes, 3-5 years of age weighing 150 – 200 Kg body weight) were used in the study. They were categorized randomly into six groups each of four donkeys. Seven days prior to surgery, the donkeys were preoperative treated with I/M administration of anti-tetanic serum oral anthelmintic (ivermectin) and housed in grass yards all over the study, feeder hay and green fodders with free access to water.

Honey Samples: Nine different types of honey collected from retailed markets in Egypt; wild herbs, marjoram, blackseed, clover, citrus,, Egyptian sidr, Hadramout sidr, flowers honeys and 10% propils in blackseed honey (Chinese propolis powder was added to honey as 10% weight to volume) were tried as a therapeutic dressing material of the standard induced septic wounds.

Wounds: Animals were tranquilizer by Diazepam (Valpam, Amoun pharmaceutical Co., El-obour city, cairo, Egypt) in a dose rate 0.2mg / kg body weight and anaesthetized by barbiturate Thiopental sodium (Egyptian Int. Pharmaceutical industries Co. 10th of Ramadan city Egypt.) intravenously in a dose rate of 1g / 90 kg body weight. Then local infiltration analgesia using lidocaine Hcl* 2% (Debocaine, Sigma-Tec Pharmaceutical Indust. Co., Packed by Al-Debeky pharmaceutical industeries Co., A. R. E.). A rounded 1.5 cm diameter full thickness skin wound was made at lateral distal part of both metacarpal and metatarsal regions. The full thickness skin was excised off and the hemorrhage was controlled by sterile gauze compresses [10].

Standard Bacterial Strain: A standard inoculum was prepared from overnight incubated nutrient broth of methicillin resistant Staphylococcus aureus (Field MRSA strain) originated from clinical septic cutaneous equine wounds which adjusted to 0.5 McFarland opacity standard and diluted up to 5 X 10^5 CFU/ml [17].

Determination of Minimal Inhibitory Concentration (MIC) & Minimal Bactericidal Concentration (MBC): All Honey Types Were Tested Against the Above Mentioned MRSA Standard Bacterial Strain to Determine both MICs & MBCs as follows; for MIC, tube serial dilution was freshly prepared from 50% up to 1.56% for each honey type where each tube received 0.1 ml from the previously mentioned standard bacterial strain which be incubated aerobically at 37^{o}C for 24 hour. The least dilution showed no visible bacterial growth was the MIC value. For MBC, from the MIC incubated tubes and least dilutions up to 50% a loopful was inoculated onto nutrient agar plates to be overnight incubation. The least dilution showed no bacterial growth was the MBC value [17].

Induced Infection and Sepsis Developed: A sterile gauze piece was dipped in the standard inoculum broth to be full saturated and then it was fitted on the induced wound for 72 hours till sepsis developed exuding purulent exudates. The process was adopted daily on the induced wound. This was the time (0) and apitherapeutic application would begin as follows: each animal had three wounds which were dressed using three different types of honey and the fourth one was left as control (saline dressed bandage).

Clinical findings were recorded at time (0), then on 7, 14, 21 and 28 post the 1st surgical dressing interference. The wounds were examined grossly for presence of swelling, odour, edema, marked accumulation of exudates, scab formation and exuberant granulation tissue as well as wound dimensions.

Histopathological Specimens: From the wounds treated with every honey type, two biopsy specimens were collected on the 14 & 28 days postoperatively. The specimens included the whole wound and 3 mm of the surrounding skin circumferentially and deeply to the subcutaneous tissue. The specimens were fixed in 10% neutral buffered formalin, dehydrated in a graded alcohol series, cleared in methyl benzoate and embedded in paraffin wax. Five-micron thick sections were cut and stained with hematoxylin and eosin [18].

3. Results & Discussion

Noteworthy is the use of honey as a wound-healing agent since it promotes a multifaceted and safe healing activity for all skin wounds [13]. Moreover, either ethanolic [19] or aqueous [20] extract of propolis accelerates wound healing and have synergistic action with honey [21, 22].

Since, honey potency and activity differ greatly according to botanical, geographical and seasonal conditions up to more than 100 fold in between different honeys [23], the present study tested the activity of different types of honey either *in vitro* to assess both MIC & MBC values or *in vivo* through the investigated different types of honey as unique septic wound dressing. The *in vitro* testing of different types of honey against MRSA revealed that all tested honey types had antimicrobial activity with different potencies where propolis with black seed honey followed by Hadramout honey showed the lowest MIC, Figure 1 and MBC values (the best activity) Figure 2, while both black seed and citrus honeys revealed the highest values (the lowest activity) as shown in Figure 1.

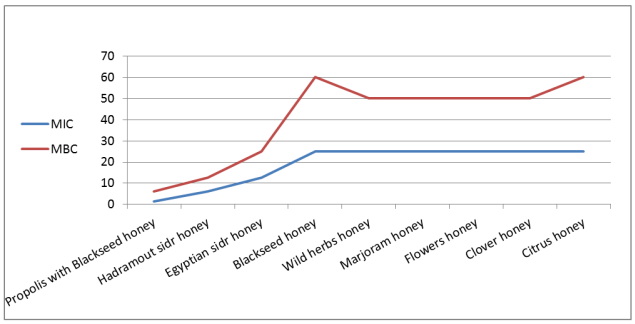


Figure 1. *MIC and MBC values of different types of honeys and propolis against MRSA.*

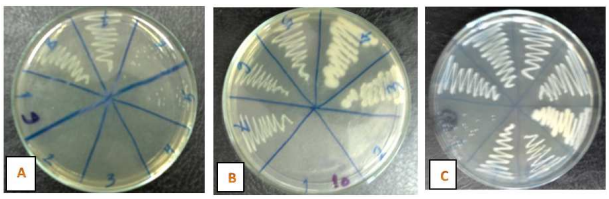


Figure 2. *In vitro MBC of different honey types against MRSA: (A) Propolis with Blackseed honey (6.25%), (B) Egyptian sidr honey (25%), (C) Blackseed honey and Citrus honey (>50%).*

It is widely documented that against MRSA, honey [24-31] or propolis [32-34] has potent antimicrobial activities with synergistic action of both together [28, 30, 35].

The referred activity of honey is attributed to its acidity (pH between 3.2 and 4.5), osmotic effect of its high sugar content [3], and also derived from H_2O_2 generated which is involved in oxidative damage causing bacterial growth inhibition by DNA degradation [36]. Although H_2O_2 is an important factor in the bacterial growth inhibition, polyphenolic compounds and their interaction with H_2O_2 are the key factors responsible for honey high antibacterial activity [30].

There is great variety of nonperoxide factors of plant origin such flavonoids mainly Chrysin [37] and polyphenolic mainly caffiec acid [38]. Honey-induced extracellular Ca^{2+} entry results in wound healing due to H_2O_2 production and redox regulation which prefer utilization of the honey as a beneficial tool [39]. Propolis is rich in these flavonoids and phenolic components [40]. Honey antimicrobial action will be maximized and synergized by propolis [28, 30].

In the present study, clinical gross appearance of septic wounds revealed that topical application of all honey types cleared sepsis comparing with control group and enhanced for healing with different rates of wound closure, where black seed with propolis, Egyptian sidr and Hadramout sidr honeys showed the most accelerated healing appearance, while flowers honey and marjoram honey had low wound healing rates (Figure 3).

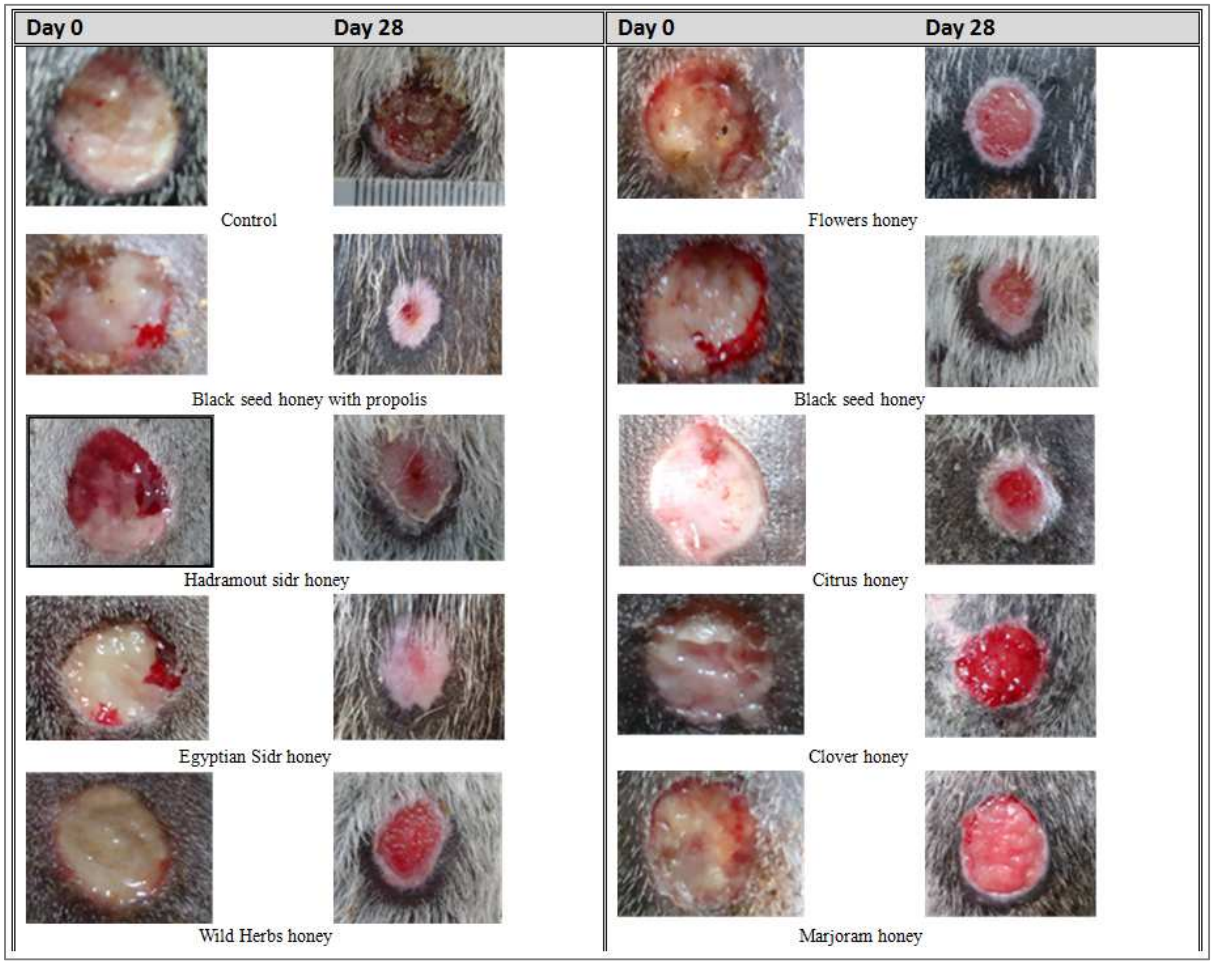
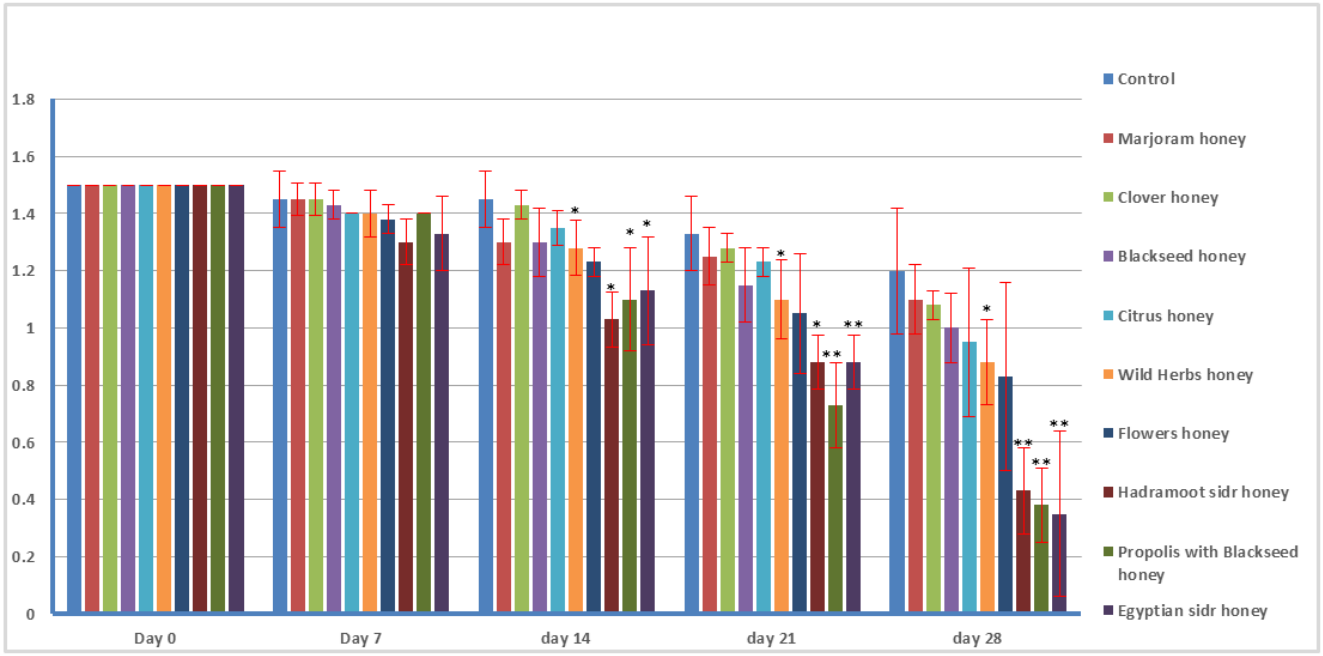


Figure 3. *Clinical gross appearance of septic wound treated with topical application of different honey types.*

Among the promising previously mentioned results, propolis with black seed honey and Egyptian sidr honey showed highly significant ($P < 0.01$) decrease of wound dimensions by the 21th day while Hadramout sidr honey reached to the same results by the 28th day post topical honey application (Figure 4). These three honey types had high rates of wound contraction. The flowers honey followed by marjoram honey had the lowest wound contraction rates (Figure 4) 28 days post topical honey application.



* Significant at P < 0.05 ** Highly significant at P < 0.01

Figure 4. *Rate of wound contraction treated with different honeys application.*

Although the cutaneous wounds on the distal portion of horse's limbs are difficult or impossible to be closed primarily and must heal by 2nd intention [10], the present study revealed good healing parameters by the day 28th post honey topical application. Wound area reduction up to complete healing (wound closure) was achieved with topical application of honey in surgical induced non septic wounds by the 10th day [41], 12th day [42], 14th day [43], 16th day [44], 21th day [45], 28th [46] or by the day 28th in clinical septic wounds [4, 30]. Also, topical application of propolis powder by the 21th day [47, 48], its ethyl extract by the 25th day [15], its ethyl extract in association with bacterial cellulose by the 14th day [49] or addition of its aqueous extract to rubber membrane by the 10th day [20].

Honey dressings improve wound healing as it makes it sterile in short duration and have good outcomes in terms of incidence of hypertrophic scars [50], since it significantly enhances wound closure and accelerates the rate of re-epithelialization [46], subsequently wound debridement [51].

The mechanism responsible for this positive final effect includes its ability to reduce free radical oxygen spices (ROS) production and inflammation through suppression of pro-inflammatory cytokines production (tumor necrosis factor-α, interleukin-1, and interleukin-6) [52], from monocytes and macrophages [53] or epidermal keratinocyte [54] or by the action of apigenin and kaempferol, identified as honey flavonoids. Moreover, honey stimulates angiogenesis, promoting an efficacious skin-remodeling phase [13, 55] and tensile strength [56].

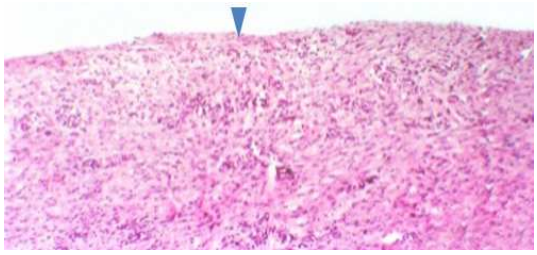


Figure 5. *Control.*

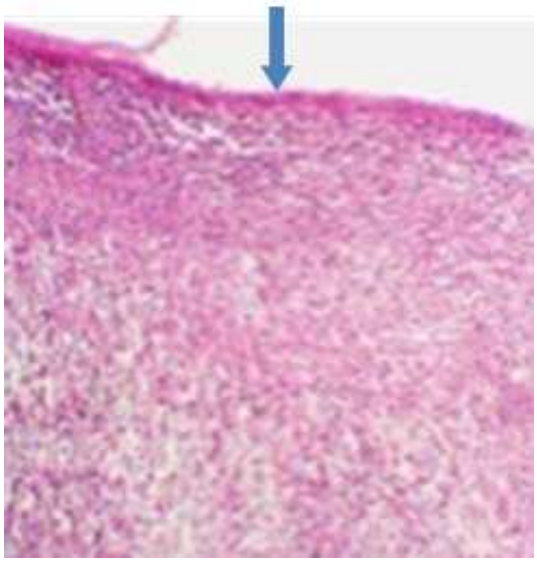


Figure 6. *Black seed honey with propolis.*

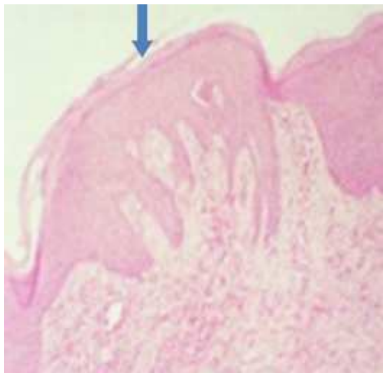


Figure 7. *Hadramout Sidr honey.*

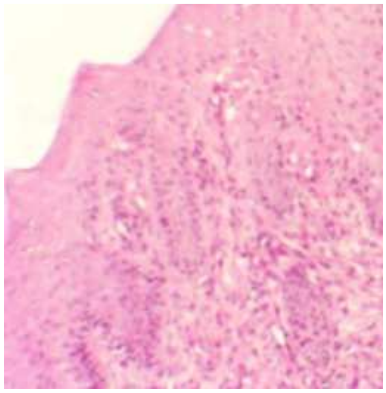


Figure 8. *Egyptian sidr honey.*

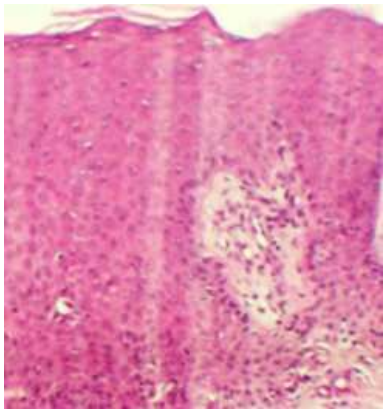


Figure 9. *Wild Herbs honey.*

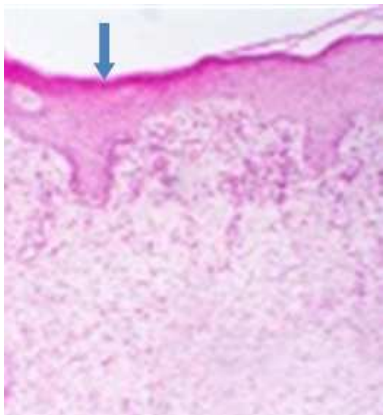


Figure 10. *Flowers honey.*

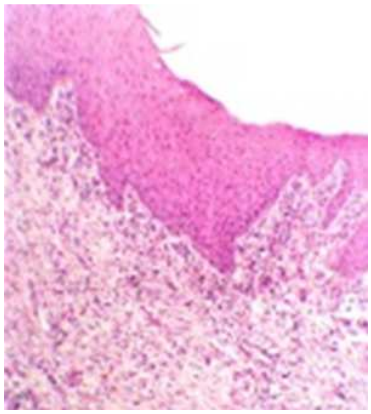


Figure 11. *Black seed honey.*

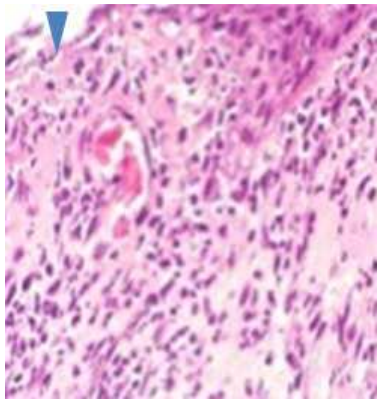


Figure 12. *Citrus honey.*

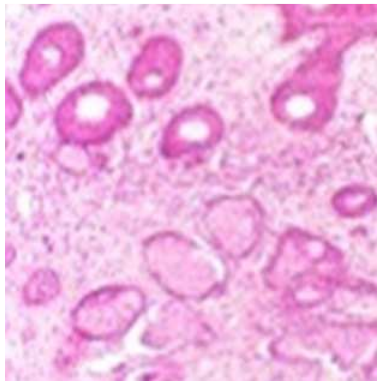


Figure 13. *Clover honey.*

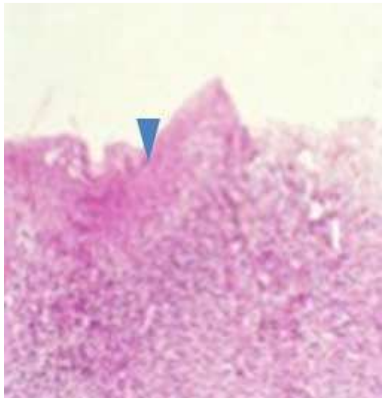


Figure 14. *Marjoram honey.*

Propolis induces wound healing with collagen production, angiogenesis, re-epithelization accelerating the healing process [49] and tissue repair without the curative switch [20]. Even in bone marrow mesenchymal stromal cells, propolis enhances cell proliferation and tissue regeneration [57].

The present histopathological results revealed that all honey treated septic wounds had different re-epithelization process indicating variant healing potencies (figures 5-14). Black seed honey with propolis, Egyptian sidr honey and Hadramout sidr honey (Figures 6, 8 & 7 respectively) induced the best re-epithelization degree, while citrus and marjoram honey show incomplete epithelization up to 28 days (Figures 12 & 14 respectively).

Wound healing is a complex and systematic process, in terms of histopathology it includes three phases including inflammation, proliferation, and remodeling [58]. Honey increases activity of fibroblasts [29] and collagen formation [20, 59]. Honey contains high levels of glycine, methionine, arginine, and proline, which are all necessary for collagen formation and fibroblast deposition, which are essential factors needed for healing [6].

Collagen films with propolis were able to improve wound healing by modulating the collagen deposition process and the inflammatory evolution [60] as it decreases the inflammatory severity, induces earlier replacement of type-III for type-I collagen, improving the epithelization rates and the myofibroblastic count which were significantly increased in 14 and 21 days [61]. As honey increases keratinocyte closure rate and fibroblast migration [62] and propolis stimulates the ability of keratinocytes to close the wound [29, 55], mixing both apitherapeutic products explain the synergistic activity of wound closure.

4. Conclusion

The study concluded that among different nine honey types retailed in Egyptian markets, all tested honey types had wound healing activities but with different potencies. Propolis with black seed, Egyptian sidr honey and Hadramout honey were the most potent with highly significance differences ($P < 0.01$), since achieving the most wound contraction rates, rapid epithelization post 28 days rather than the least MICs & MBCs values. While the marjoram and clover honey were the least effective.

5. Recommendations

It is highly recommended to manage the septic incurable equine wounds with propolis ethanolic extract solution combined with honey as the main dressing antimicrobial and anti-inflammatory agent. Further investigations must be adopted to examine the efficacy of the crude propolis powder combined with honey since the crude powder rather than it has the effective active principles, it aids for wound dryness with clean aseptic surface.

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