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ORIGINAL ARTICLE

Comparative Efficacy of Manuka Honey and its Combination with Fusidic Acid in Surgically Inflicted Methicillin Resistance *Staphylococcus aureus* Infected Wounds in Rabbits

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Abstract
Wound healing is a complex mechanism that involves recruitment and pragmatic proliferation of cells at the site of injury and it may be delayed due to many reasons including wound infection. Topical use of antimicrobials/antibiotics is one of rationale for wound care. However, development of resistance to antimicrobials is an emerging and serious problem for the clinicians over the globe. The present study was, therefore, been planned to evaluate the comparative efficacy of Manuka honey (MH) alone and in combination with fusidic acid (FA) in the treatment of surgically inflicted full thickness MRSA infected wounds in rabbits. Adult male rabbits (n=30) were randomly divided into 3 groups (A to C) of equal size. A set of full-thickness skin wound (3 on each rabbit) were inflicted on the dorsolateral aspects of lumber region of each rabbit. Then infection was induced with 10 µl of 0.5 McFarland turbidity standard suspension of MRSA and waited for 24 hours to let the infection develop. Wounds in groups A and B were respectively treated with MH and FA, whereas, group C received a combined therapy comprising a mixture of MH and FA in equal proportions. The wound healing was evaluated in terms of rate of wound contraction, healing time and histopathological evaluations. The data was then subjected to statistical analyses using analysis of variance (ANOVA) considering it significant at $p<0.05$ and highly significant at $p<0.01$.

Keywords
Fusidic Acid, Manuka Honey, MRSA (Methicillin Resistant Staphylococcus aureus), McFarland

1. Introduction
Wound refers to any discontinuity of skin or superficial layer of any organ. Cutaneous wounds are most commonly encountered in animals and man throughout the world. Depending on type and intensity of trauma and surgical procedure, two types of wounds viz., open and close are seen.

Open wound are more serious and life threatening owing to risk of bacterial infection and fluid loss **(Bowler, 2003; Gardner et al., 2006).** In response to wound, the competent host (body) mediates a process of healing which encompasses various complex physiological phases of hemostasis, inflammation, proliferation (development of granulation tissue and remodeling). Among the normal healthy individuals, all these events of healing occur at an optimum rate **(Kwon et al., 2007).** Within a few hours after the injury, granulation tissue is formed at the site of injury and lining of the residual tissue starts the re-epithelialization and subsequently covers the granulation tissue over the site of injury **(Rieger et al., 2014)**

Emergence of Multi drug resistant (MDR) strains have reduced the antimicrobial options. Combined antimicrobial therapy is recommended because two antimicrobials with different mechanism of action prevent the development of resistance **(Rahal, 2006).** This strategy also reduces cost and side effects and provide augmented efficacy due to synergistic. Similarly, natural products (honey for one) have shown potential for use with antimicrobials to increase the antimicrobial effects and to reduce the emergence of resistance **(Wagner, 2011).** Unattended open wounds invariably harbor bacteria, which can be lethal when the pathogens are MDR. Skin infections are mostly caused by Staphylococci (resident flora of skin) and Enterobacter (E. coli, an environmental pathogens). However, nosocomial bacterial pathogens (Methicillin resistant *Staphylococcus aureus* 'MRSA' and *Pseudomonas aeroginosa*) are highly dangerous as they survive under extreme antimicrobial pressure and invariably resistant to several antimicrobials. Both pathogens cause serious wound infections especially in immunocompromised patients **(Jenkins and Cooper, 2012a).**

Manuka honey is a type of mono- floral honey which is obtained from *Leptospermum scoparium* in Australia and New Zealand and is an effective treatment for the healing of wounds in animals. Honey used for this purpose must not contain any sort of residues or particles from pesticides or herbicides **(Bosma and Creemers, 2006).** Honey is a material of high nutritive value; comprising of 20 types of different sugars, eight different kinds of vitamins, 11 different minerals, 16 kinds of amino acids, and also contains many enzymes **(Fattahi-Bafghi et al, 2006).** Among the medicinal properties, honey carries angiogenic, antibacterial and anti-inflammatory activities and is reported to enhance the generation of granulation tissue, speed up the process of epithelialization, prevents and reduces the formation of scars, and help in alleviating the pain.

Such properties are because of the generation of hydrogen peroxide, very high osmolarity of honey and the presence of certain phytochemical substances in nectar of plants **(Khan et al., 2007).** Honey has both the medicinal as well as nutritive value. Apart from being highly nutritious, it enhances the process of wound healing and produces soothing effect particularly on burn wounds by reducing microbial activity and enhances the process of re- epithelialization **(Khiati et al., 2014).** The antibacterial activity of Manuka honey is parallel to the scale value of 18 on the scale of phenol acid strength and makes this honey as the strongest currently available medical honey as far as antibacterial activity is concerned **(Sare, 2008).**

2. Materials and Methods

The present study was conducted on surgically inflicted full thickness skin wounds on the torso (lumber region) in adult male rabbits. Experiments were conducted under the guidance of the rules of the Institutional Review Board of the Ethical Committee of Animal Care.

2.1 Experimental Animals

Thirty locally bred, clinically healthy male adult rabbits, weighing between 1.5–2.5kg were randomly divided into 3 groups, labelled A, B and C having 10 animals each. Following randomization, all animals were housed and acclimatized for 2-weeks in Laboratory Animal Facility of the Department. Animals were endowed with light and dark cycle in a well ventilated, temperature maintained (~ 22-28°C) room. During course of acclimatization each rabbit were dewormed with ivermectin (400microgram/kg) and received a 3-day prophylactic course of Amoxicillin (15mg/kg subcutaneously) for pasturellosis. All these prophylactic tactics were completed a week before the commencement of experimental trial **(Awais, 2013).**

2.1.2. Preparation of the Surgical Site

The skin and hair follicles are the prime medium to harbor bacteria, which in real sense cannot be sterilized completely. Bacterial flora on the skin surface is responsible for the causation of wound associated infections in animals. The chances of acquiring such infections during the infliction of surgical wounds can be reduced transiently to a comparatively safer level by the removal of hair, mechanical scrubbing and using some germicidal solution. Considering all these things in mind, hairs from the surgical site and liberal surrounding area were clipped in order to reduce the chances of infection as much as possible without harming skin which might later-on interfere with wound healing.

Close careful clipping of hair within the operative field is an absolute necessity in surgery **(Awais, 2013).** Therefore, an electric clipper was preferred over the ordinary razor for this purpose as abrasions caused by the conventional razor disrupts the top most layers of skin and dents the body's first line of defense against bacterial invasion. A general guideline was supervened in each case while clipping the hairs properly on the torso (trunk; thoracolumbar) region. The clipped area was then given a general cleaning scrub, with soap and water followed by thorough rinsing. After completing the washing process, the entire clipped area was dried with an autoclaved clean towel. The operation field was then fashioned for aseptic surgery by spraying methylated spirit twice and tincture of iodine once after the other.

2.1.3. Pre-medication and Anesthesia

Each rabbit from all groups was pre-medicated by administering atropine sulphate @ 0.035mg/kg body weight subcutaneously about half an hour before surgical procedure. The animals were anesthetized by parenteral anesthesia via intramuscular route using a mixture of ketamine (35mg/kg) and xylazine (5mg/kg) **(Lipman et al., 1990).**

2.1.4. Positioning of Rabbit and Draping of Surgical Site

The rabbits were secured in sternal recumbency. Before surgical intervention, area was cleaned and sanitized completely with iodine followed by alcohol swabbing. An effective draping system is considered mandatory and is an important barrier for contaminants from surrounding skin and environment. Wet cloth drapes allow penetration of bacteria when dry serve as a potent barrier. Keeping this fact in mind, a single square drape having a central square cut was used in a separately for each rabbit in this pursuit of research.

2.1.5. Surgical Infliction of Wounds and Identification Marks

Wounding area was marked with permanent black marker using Vernier calipers. Three, 1×1 cm ($1cm^2$) full-thickness skin wounds were created on the trunk comprising on each rabbit in a triangular symmetry with an anterior wound on dorsal midline and two rear wounds, one on the either flank. Inter-wound distance was kept 3cm on either side. Full thickness wound was created on the trunk with the help of #15 blade **(Carbon Steel, Feather Safety Razor Co, Ltd. Japan)** and the piece of skin from wound site was removed by undermining the area with a sharp-sharp scissors that exposed subcutaneous area. The subcutaneous fat and fascia was also removed and trunk.

A total of three wounds of similar dimensions were inflicted on the torso of each rabbit's dorsum. The wounds of group A designated as follows for the proper identification and avoid repetition of treatment:

A-wF= wound created on the midline of thoracolumbar area on front of the body

A-wRR= wound created lateral to the thoracolumbar area on right rear of the body

A-wLR= wound created lateral to the thoracolumbar area on left rear of the body

A similar naming was assigned to the wound on subject of the group B the wounds were designated as follows:

B-wF= wound created on the midline of thoracolumbar area on front of the body

B-**wRR=** wound created lateral to the thoracolumbar area on right rear of the body

B-wLR= wound created lateral to the thoracolumbar area on left rear of the body

Similarly identifications for group C wounds were as was follows:

C-wF= wound created on the midline of thoracolumbar area on front of the body

C-wRR= wound created lateral to the thoracolumbar area on right rear of the body

C-wLR= wound created lateral to the thoracolumbar area on left rear of the body

2.1.6. Induction and Development of Infection

After the surgical infliction of wounds, the infection was given with strain-35 of methicilline-resistant *Staphylococcus aureus* (MRSA) (ATCC 33592) as described earlier **(Roche et al., 2012).** Each wound was given 10µl of 0.5 McFarland Turbidity Standard suspension of MRSA in the center of each wound and then spread out using a pipette tip to cover the entire wound area (McFarland is a standard unit used for estimating the number of bacteria in suspensions, for calculating the opsonic index and vaccine production on the basis of transmittance and absorbance of light, where 0.5 McFarland is equivalent of 10^5 bacteria/ml of suspension). After the induction of infection, we waited 24hrs for the development of infection before starting any treatment.

2.2 Experimental Protocol

The following treatment protocol was adopted for each of the groups:
Group A = wounds were treated with Manuka honey (MH)
Group B = wounds were treated with fusidic acid (FA)
Group C = wounds were treated with mixture of MH and FA

Wounds in group A were treated with topical application of Manuka honey alone right from the day next to the induction of infection. Similarly, wounds in groups B were treated with topical Fusidic acid **(Fusidin® 2% Fusidic acid by LEO Pharma Pvt. Ltd.)** alone which served as control group. The subjects in groups C received a combination of MH and FA mixed in equal proportion, also applied topically on the wounds in relevant group.

After the application of treatments, the wounds were left open. The treatments were applied once a day until the day of complete healing. In order to prevent licking by the other animals, all the rabbits were kept in separate cages. During the whole course of treatment, all the rabbits were checked daily for general health condition, any cross infection into the wounds and other unspecified abnormalities.

2.3 Evaluation Parameters

2.3.1 Wound Contraction

Rate was estimated by tracing the edges of wounds on piece of trace paper. These sketches of wound sizes were measured on graph paper (mm^2) and wound contraction values were presented in percentile **(Awais, 2013).**

2.3.2 Healing Time

Refers to time (days) elapse between creation of a wound and the complete regeneration and epithelization. It was estimated by sum of daily observations until scar was fallen off **(Awais, 2013).** The experiment was conducted from February 20, 2016 to April 19, 2016.

2.4 Statistical Analysis

The data thus obtained was subjected to analysis of variance using SPSS program to discern the substantial effects of treatment considering level of significance at $P<0.05$.

3. Results

The present study was planned and conducted to evaluate the efficacy of manuka honey alone and in combination with fusidic acid in the treatment of surgically inflicted full thickness MRSA infected wounds in adult rabbits. One experiment was carried out on 90 full thickness skin wounds model in 30 rabbits and randomly divided into 3 equal groups with 30 in each. The study lasted over 60 days. All general physiological parameters (including body temperature, respiration rate and heart rate) were checked frequently and were found within normal ranges. None of the animal showed any signs of anorexia, however a remarkable increase in the water intake was observed in almost all rabbits that may also be co-related with the change in external weather, though the temperature of the animal house of maintained within comfortable zone.

3.1 Wound Contraction Rate

The inward movement of the wound edges towards the center during the course of healing is inferred as contraction. Following the initial expansion during 2 days post-surgery, all wounds started to contract inward and this rate was measured (in mm) by tracing the edges of wounds on a tracing paper and then measuring it on a graph paper until the wound edges had completely closed **(Figs. 1, 3).** In order to counter test the presence of active infection / infectious agent, we took random swabs from different wounds in different groups and cultured them onto culture medium and incubated. After 24hrs, we observed presence of MRSA in group 2 swabs) and these results were in full accordance with the end results of healing time as well showing slowest healing in that very group **(Fig. 2).** The results of wound contraction rates in different groups and their statistical comparison through Analysis of Variance (ANOVA) have been presented in **Tables (1, 2, 3, 4, and 5).**

3.2. Healing Time

The time (days) elapse between the creation of wound and the complete regeneration and re-epithelialization of wound edges is termed as healing time. The shortest mean wound healing time (18.75 days ± 0.425) were observed in the wounds included in group C (treated with a combination of manuka honey and fusidic acid) and in group A (Manuka honey alone) mean healing time was 21.1 ± 0.459) and slowest healing (34.1 days ± 1.197) was recorded in group B (Fusidic acid alone) **(Table, 4).** Statistically, healing was significantly faster ($P< 0.05$) in group C than group A and B. The difference in healing time between group A and C was comparatively less significant. The ANOVA on the healing time is presented in **Table (6).**

3.3. Histopathological Evaluation

Biopsy specimens of full skin thickness were collected from the healed wounds on day 7 and 14 of the treatment and were preserved in 10% buffer formalin (v/v). After fixation, the biopsy specimens were subjected to blocking, cutting, sectioning by microtome, mounting on slide, fixing and slide staining processes. Then finally analyses were made for the deposition of collagen fibers, their arrangement, dermal and epidermal layers' thickness, fibroblast proliferation, re-epithelialization and formation of new vascular beds in the healed tissues **(Yadav et al, 2012).** The wounds treated with a combination of MH and FA showed highest collagen contents and maximum angiogenesis **(Fig. 6)** which were markedly better than the other two groups but the development of epidermis was observed best in the results of wounds treated with MH alone **(Fig. 4)** than group B and C. Whereas, thin epidermis with some collagen fiber of blood vessels were observed in histopathological findings of wound tissue being treated with Fusidic Acid alone **(Fig. 5).**

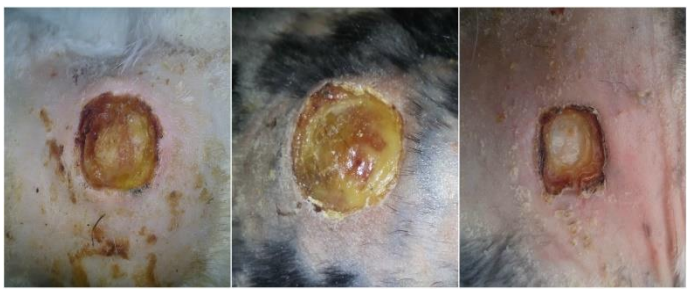

Fig 1. Wounds on 5th day of treatment (Group A, B and C from Left to Right).

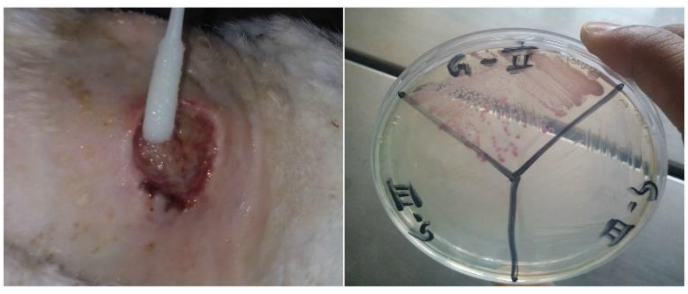

Fig. 2. Taking swab from the wound (Left) Petri plate showing growth of MRSA on Nutrient Agar in group-II (Right).

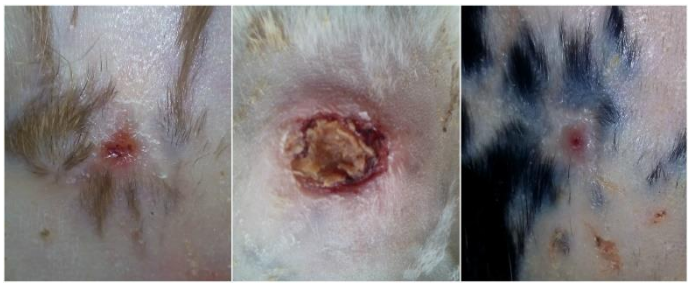

Fig. 3. Wounds on 18th day of treatment (Group A, B and C from Left to Right).

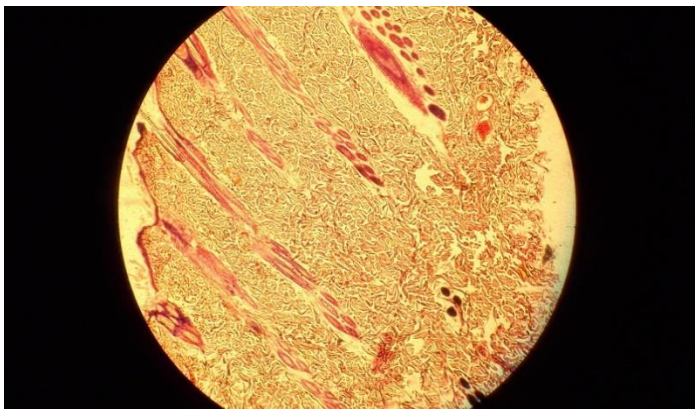

Fig. 4. Slide showing thin epidermis formation and sufficient presence of collagen fiber and some blood vessels in the dermis of healing wound tissue being treated with Manuka honey (Group A).

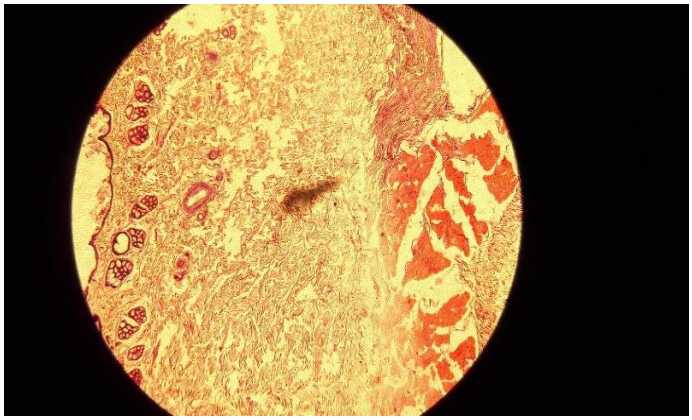

Fig. 5. Photograph of slide showing thin epidermis with some collagen fiber of blood vessels in healing wound tissue being treated with Fusidic Acid (Group B).

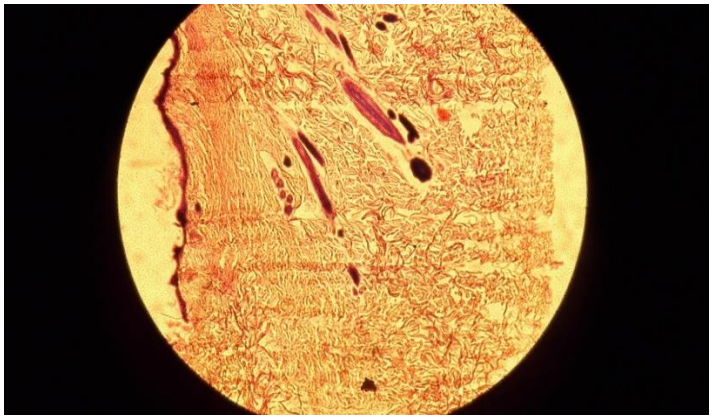

Fig. 6. Slide showing moderate to good thickness epidermis and high presence of collagen fiber in the dermis and blood vessels in the healing wound tissue being treated with combination of Manuka Honey and Fusidic Acid (Group C).

Table 1. Wound Contraction Rate (cm) SE ± 0.01 (cm).

Days of Treatment	Group A	Group B	Group C
0	0.00	0.00	0.00
3	0.09	0.05	0.08
6	0.22	0.11	0.21
9	0.37	0.16	0.37
12	0.51	0.24	0.58
15	0.66	0.36	0.74
18	0.82	0.46	0.95
21	1.00	0.57	1.00
24	1.00	0.68	1.00
27	1.00	0.77	1.00
30	1.00	0.86	1.00
33	1.00	0.97	1.00
36	1.00	1.00	1.00

Table 2. Analysis of variance for Wound contraction rate between group A vs group B vs group C.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Treatment	2	0.005	0.0025	33.784*
Error	27	0.002	0.000074	
Total	29	0.007		

* = Highly significant (P<0.01)

Table 3. Analysis of variance for Wound contraction rate between group A vs group B.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Treatment	1	0.002	0.002	36.41*
Error	18	0.001	0.000055	
Total	19	0.003		

* = Highly significant (P<0.01)

Table 4. Analysis of variance for Wound contraction rate between group A vs group C.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Treatment	1	0.001	0.001	18.94*
Error	18	0.001	0.000055	
Total	19	0.002		

* = Significant (P<0.05)

Table 5. Analysis of variance for Wound contraction rate between group B vs group C.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Treatment	1	0.005	0.005	97.18*
Error	18	0.001	0.000055	
Total	19	0.006		

* = Highly significant (P<0.01)

Table 6. Analysis of Variance for wound healing time.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-value
Treatment	2	1367.150	683.575	1123.6849*
Error	27	16.425	0.608	
Total	29	1383.575		

* = Highly significant (P<0.01)

4. Discussion

Perhaps for as long as the occurrence of wound has been reported, both the human and the animals had been searched for the latest ways to aid and stimulate the healing process. Modern research necessitates the searching of ways that stimulate the wound healing process and much of the information has been yielded concerning the common wound infections and infectious agents, their methods of control, nature of wound and better methods of wound care and management than previously existing.

Wound healing process usually demands sustenance at three different levels: initially at enhancing the general support and resistance, then at accelerating the repair and regenerative phenomenon and last but not the least, the therapeutic and nutritional processes **(Gutteridgde, 1995; Simon et al., 2006; Nisbet et al., 2010).** Congregation of these needs was well facilitated by *Nigella sativa* honey. Histopathological examination revealed the commencement of proliferative phase on the third day of treatment group that was delineated by the formation of granulation tissue, depicting that *N. sativa* stimulated the synthesis of human interleukins and caused the alertness of macrophages. Macrophages are capable of phagocytosing bacteria and serve as a second line of host defense **(Hag et al., 1995).** A number of chemotactic and many growth factors **(Corton et al., 2003)** were also released such as transforming growth factor (TGF), interleukin-1 (IL-1), fibroblasts growth factor (FGF) and epidermal growth factor (EGF) which were responsible to circumscribe the proliferative phase. Formation of collagen fibers was triggered by certain growth factors and growth hormones. This was also helpful in kindling the proliferation of fibroblasts leading to the development of granulation tissue, hastening through the re-epithelialization and collegenization. However it was reported to delay granulation tissue formation and scar development. Besides that the *N. sativa* oil contains some fatty acids which help in the construction of collagen contents sustaining the resilience of skin.

The significantly improved contraction rate in wounds treated with combination of manuka honey and antibiotic was in accordance with the results of several in-vitro studies on the synergistic activity of manuka honey with different antibiotics. Manuka honey enhanced the susceptibility of MRSA to oxacillin by down regulating the *mecR1* gene (known to enhance methicillin resistance in MRSA **(Jenkins and Cooper, 2012a).** Methylglyoxal (MGO) present in manuka honey showed additive affect with Rifampicin and completely inhibited the growth of MRSA **(Mandal and Shyampada, 2011; Muller et al., 2013).** A combination of Imipenum with manuka honey and Tetracycline with manuka honey showed synergistic affects in clearing MRSA growths in-vitro **(Jenkins and Cooper, 2012b).**

Manuka honey when used alone, and in combination with fusidic acid, in both circumstances possess anti-inflammatory property. Therefore, in this way they reduce the chances of inflammatory edema, improve sloughing off of devitalized tissue, attract macrophages for clearing the wound, act as energy source for the local wound tissue and mask the wound acting as a protective barrier against possible contaminations. A hyperosmolar and acidic nature of manuka honey confers to its antibacterial properties **(Gupta et al, 1992; Sudhakar et al., 2003).**

Histopathological evaluation in current study revealed that the group C of wounds (treated with combination of manuka honey and fusidic acid mixed in equal proportions) showed best development of epidermis among the three groups and also greatest thickness of the skin layer from animals of other groups A and B (treated with manuka honey alone and Fusidic acid alone respectively). However, the presence of collagen fibers was less significantly different in group C from that of group A, but still highly significant from group B. These results were in accordance with the findings of **Gupta et al. (1992)** and **Sudhakar et al. (2003).**

5. Conclusion

From the current study, it was concluded that manuka honey in combination with fusidic acid not only improves the wound healing by promoting the wound contraction rate, but also reduced the antibiotic resistance of MRSA. Therefore, it is recommended to use manuka honey in mixture of equal proportions with fusidic acid for the treatment of fresh cut infected wounds in animals and in humans is very efficient and can be used with utmost confidence without any fear of side effects.

6. Authors Contributions

All authors participated equally to the design of the research, methodology, and writing of the manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.

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