



# INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare &amp; Pharmascope Foundation

Journal Home Page: <https://ijrps.com>

## Cytological and histopathological studies on the effect of honey in experimentally infected wounds in Wistar rats

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### Article History:

Received on: 11.10.2018  
Revised on: 09.03.2019  
Accepted on: 12.03.2019

### Keywords:

Wound healing activity,  
Infected wounds,  
Honey,  
Cytology,  
Histopathology,  
Wistar rats

### ABSTRACT

Honey is used as food and home remedy in of India and other countries. Scientific studies have been carried out to support these properties of honey in this investigation, incision wounds of rats that were infected with three bacterial strains viz. *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, have been treated with honey and evaluated for its effect on days 1, 3, 6, 9 and 12 post-operatively with the help of smears and biopsies collected from lesions. Cytological and histopathological investigations of infected wounds conformed significant healing property of honey in and reduction in size.



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ISSN: 0975-7538

DOI: <https://doi.org/10.26452/ijrps.v10i2.405>

Production and Hosted by

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### INTRODUCTION

Wounds pose a major threat to health and if left untreated, lead to severe complications and high cost for treatment. Healing of wounds involves a dynamic physiological process initiated and influenced by factors such as homeostasis, inflammation, proliferation and remodelling phases (Strecker *et al.*, 2007). Macrophages play a crucial role in inflammation and repair of wounds. At the site of the wound, macrophages get activated and release growth factors such as PDGF and VEGF that

initiate the formation of granulation tissue. Platelets facilitate the formation of homeostasis plug and activate fibroblasts for re-epithelisation of wounds. Within 24-48 h of injury, epidermal cells initiate margination of wounds. New granulation tissue begins to invade the wound gap after 96 h and numerous capillaries grow through new stroma with granular appearance (AL Waili 1989; Heldin *et al.*, 1996). Once the wound is filled with new granulation tissue, angiogenesis ceases, and many new blood vessels disintegrate due to apoptosis (Clark *et al.*, 1995). Many drugs and natural materials are used to heal wounds. Honey is one such product used in wound healing over the years in many countries. Its healing property has also been confirmed by researchers. In traditional systems of medicine and folklore practices, honey finds an important place in wound dressing and healing (Anonymous 1957). In this study, the influence of honey in experimentally infected incision wounds by three bacteria viz. *S. aureus*, *E. coli* and *P. aeruginosa*, has been investigated in Wistar rats based on cytological and histopathological observations of the wound tissues. Earlier studies have

indicated the efficacy of honey against anti-microbial resistant bacteria *S. aureus* and *P. aeruginosa* in wounds (Ewnetu *et al.*, 2013; Cooper *et al.*, 2002).

## MATERIAL AND METHODS

The study was conducted in the Department of Research and Development, Saveetha Institute of Medical and Technical Sciences (SIMATS), Chennai, India, after obtaining necessary approval from Institutional Animal Ethics Committee (Vide Ref. no SU/CLAR/RD/008/2016). The animals were housed in individual metabolic cages and maintained in the temperature range of 24-27°C and humidity 40-50% with the natural light/dark cycle as per Committee for the Purpose of Control and Supervision of Experiments on *Animals* (CPCSEA) guidelines for laboratory animal research. They were provided with pellet food and water *ad libitum*.

Twenty-four healthy adult male Wistar albino rats weighing 250-300 g were used in this study. Randomly selected rats were assigned to six groups (A-F) each consisting of 4 rats. Animals in groups A, C and E, were considered as test groups while groups B, D and F served as controls. Sterile pasteurised multi-floral honey was obtained from Bharat Unani Pharmacy, Hyderabad (India). It was analysed for composition and found to contain sugars (40.0–60.0%), protein (3.0%), fatty acids (1.0-32.0%), sodium (1.0%), total carbohydrate (93.0%), dietary fiber (3.0%), calcium (2.0%), vitamin C (3.0%) and iron (8.0%).

Bacterial strains that commonly cause wound infections viz. *P. aeruginosa* ATCC-27853, *E. coli* ATCC-35218 and *S. aureus* ATCC-25923 were obtained from Post Graduate Institute of Medical Education and Research, Chandigarh (India). A six-hour incubated bacterial culture suspension matching with 0.5 Mc-Farland scale standards were prepared (i.e., 10<sup>5</sup>CFU for *S. aureus* and *E. coli* and 10<sup>3</sup> CFU for *P. aeruginosa*).

### Wound preparation

Rats were anaesthetized by the injection of ketamine (15 mg/kg b.w.; i.p.) and xylazine (1.1mg/kg b.w.; i.p.) (Hazrati *et al.*, 2010). A wound of 500mm<sup>2</sup> was created using a sterile scalpel by removing the skin on the dorsum of the abdomen of both groups in the test as well as controls. The wound-created rats in groups A, B were inoculated with *S. aureus*, C, D was inoculated with *E. coli* and E, F was inoculated with *P. aeruginosa*. The wounds were subjected to periodical evaluation on days 1, 3, 6, 9 and 12. Each wound was examined, and photographs were taken till closure of wounds.

## Assessment of wound healing

The wounded rats in test groups (A, C and E) were daily treated with topical application of 0.1 ml /cm<sup>2</sup> of honey using plain gauze whereas those in control groups (B, D and F) did not receive any treatment. All these animals were evaluated on days 1, 3, 6, 9 and 12 of post incising the wound. After 24 hours of wound creation, the membrane formed over the wound was removed. A sterile micro slide was pressed over the wound, stained and observed with oil immersion microscope (100x) for cytological changes using hematoxylin and eosin staining. The cut biopsy specimens of the wounds at the periphery of rats in all 6 groups were preserved in 10% formalin solution. Histopathological evaluations of these biopsies were done using haematoxylin and eosin staining and observed under high power microscope (40x) on days 1, 3, 6 and 9. Clinical assessments included observations concerning the appearance, and the wound size was measured using the following formula. The percentage of wound contraction was calculated by dividing the difference in wound area of a particular day from the first day by the first-day area and then multiplying it with hundred (Sadaf *et al.*, 2006).

Wound size measurement for percentage of wound contraction was done on day 1, 3, 6, 9 and 12, while cytology and histopathology was done only on days 1, 3, 6, and 9, consider the normal process of wound healing by day 12, and taking the biopsy and a sterile micro slide was pressed over the wound was not possible on day 12.

### Statistical analysis

The data were analyzed by one-way ANOVA test All the data were presented as a mean value with its standard deviation (mean±S.D) P < 0.001 was considered statistically significant where n = 4 was considered as statistically significant.

## RESULTS

Wound healing is a natural restorative response to tissue injury. It consists of four systematic steps of hemostasis, inflammation, proliferation, and maturation in both humans and animals. The healing process was confirmed by microscopic and macroscopic studies.

### Wound Size

Compared to a normal and infected wound control group of rats, wound sizes were significantly (P<0.001) decreased in experimental group rats as observed on days 1, 3, 6, 9 and 12 after wound creation (Table 1, 2 and 3).

**Cytological Observations:** Wounds of rats in control group and experimental groups inoculated

**Table 1: Periodical evolution of wound contraction in experimental and Control groups inoculated with *S. aureus***

S. No	Parameter	Group A(T) Mean ± S.D	Group B(UT) Mean ± S.D	Statistical Analysis (p)
1	3 <sup>rd</sup> day	16.17±0.20	3.85±0.16	P< 0.001
2	6 <sup>th</sup> day	50.07±0.08	10± 0.14	P< 0.001
3	9 <sup>th</sup> day	75.1±0.14	19.5±0.16	P< 0.001
4	12 <sup>th</sup> day	90.07±0.07	29.27±0.19	P< 0.001

T= Treated with Honey; UT=Untreated

**Table 2: Periodical evolution of wound contraction in experimental and Control groups**

S. No	Parameter	Group A(T) Mean ± S.D	Group B(UT) Mean ± S.D	Statistical Analysis
1	3 <sup>rd</sup> day	18.2±0.19	3.72±0.16	P< 0.001
2	6 <sup>th</sup> day	52.15±0.16	9.57± 0.14	P< 0.001
3	9 <sup>th</sup> day	78.1±0.16	19.17±0.20	P< 0.001
4	12 <sup>th</sup> day	91.27±0.19	29.82±0.20	P< 0.001

T= Treated with Honey; UT=Untreated

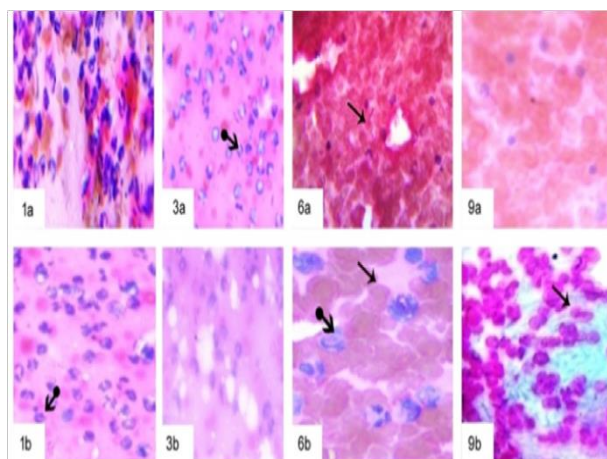
**Table 3: Periodical evolution of wound contraction in experimental and Control groups**

S.No	Parameter	Group A(T) Mean ± S.D	Group B(UT) Mean ± S.D	Statistical Analysis
1	3 <sup>rd</sup> day	15.15±0.14	4.17±0.14	P< 0.001
2	6 <sup>th</sup> day	49.85±0.16	10± 0.14	P< 0.001
3	9 <sup>th</sup> day	72.15±0.16	20.07±0.08	P< 0.001
4	12 <sup>th</sup> day	88.85±0.16	30.15±0.16	P< 0.001

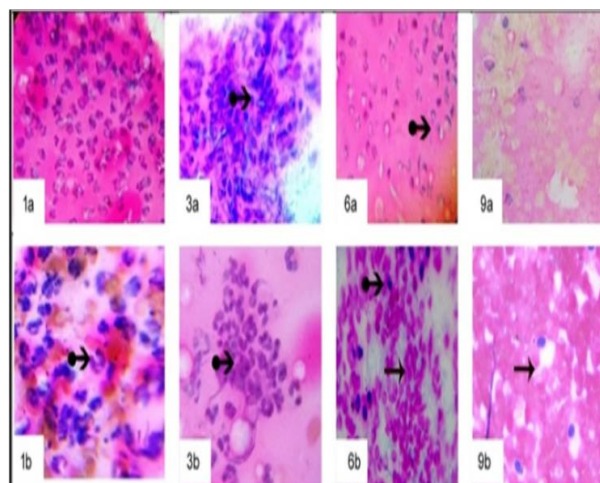
T= Treated with Honey; UT=Untreated; Statistical Analysis is done with One Way ANOVA Test P < 0.001 was considered statistically significant where n = 4

with bacterial strains showed an increased number of macrophages, neutrophils, decreased number of fibroblasts, collagen fibres, blood vessels and RBC on day 1.

However, those in experimental groups showed a significant decrease in the number of macrophages and neutrophils (Figure1).



**Figure 1: Inoculation of *S. aureus* strains (1a) is 1<sup>st</sup> day control, (1b) is 1<sup>st</sup> day test; (3a) is 3<sup>rd</sup> day control, (3b) is 3<sup>rd</sup> day test; (6a) is 6<sup>th</sup> day control, (6b) is 6<sup>th</sup> day test; (9a) is 9<sup>th</sup> day control, (9b) is 9<sup>th</sup> day test; ●→ Indicates neutrophils; → Indicates RBC**



**Figure 2: Inoculation of *E. coli* strains (1a) is 1<sup>st</sup> day control, (1b) is 1<sup>st</sup> day test; (3a) is 3<sup>rd</sup> day control, (3b) is 3<sup>rd</sup> day test; (6a) is 6<sup>th</sup> day control, (6b) is 6<sup>th</sup> day test; (9a) is 9<sup>th</sup> day control, (9b) is 9<sup>th</sup> day test; ; ●→ Indicates neutrophils; → Indicates RBC**

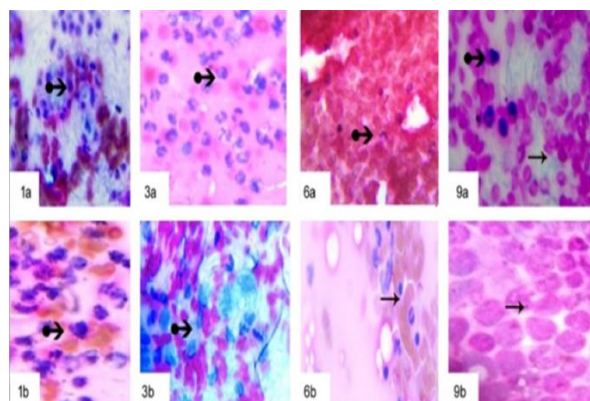
Histopathological Observations: Wounds of rats in control, as well as experimental groups, showed ulceration of stratified squamous epithelium and collection of inflammatory cells (pmns) in the subepithelial zone on day 1. However, wounds of experimental group rats that received honey followed by inoculation of *S. aureus*, *E. coli* and *P. aeruginosa* strains, showed partial ulceration of stratified



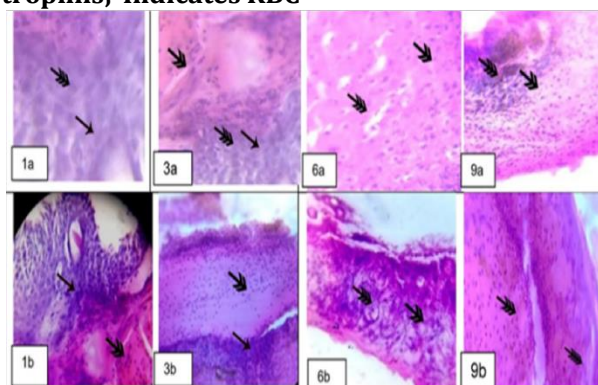
squamous epithelium, subcorneal exudate, haemorrhage, congested vessels and acute inflammatory cell collections on day 3, decrease in inflammatory cell collections, congested vessels and proliferation of fibrovascular stroma on 3<sup>rd</sup>, 6<sup>th</sup> and 9<sup>th</sup> days compared to control groups respectively (Figure 2).

**DISCUSSION**

The wound healing property of honey is widely known. Studies have demonstrated that it has antibacterial activity also (Zumla *et al.*, 1989; Maeda *et al.*, 2008). A small number of clinical studies show that the application of honey to severely infected cutaneous wounds is capable of clearing infection from the wound and improving tissue healing (Nasrin *et al.*, 2017). This is significant, as microbial resistance to conventional therapeutic agents is constantly on the rise and no new antimicrobial agents are being discovered. Our study reiterates the effectiveness of honey on wound healing in bacterially contaminated wounds.

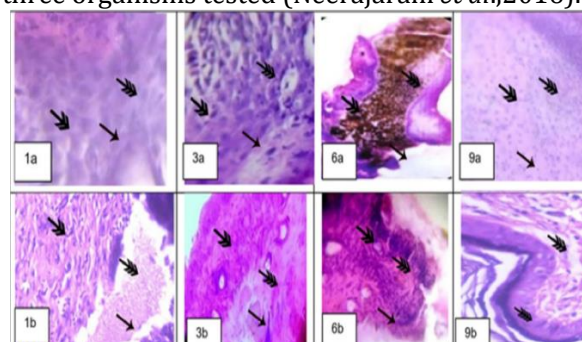


**Figure 3: Inoculation of *P. aeruginosa* strains (1a) is 1<sup>st</sup> day control, (1b) is 1<sup>st</sup> day test; (3a) is 3<sup>rd</sup> day control, (3b) is 3<sup>rd</sup> day test; (6a) is 6<sup>th</sup> day control, (6b) is 6<sup>th</sup> day test; (9a) is 9<sup>th</sup> day control, (9b) is 9<sup>th</sup> day test; ; → Indicates neutrophils; ↗ Indicates RBC**

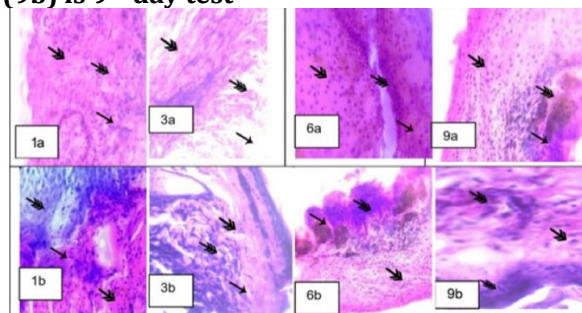


**Figure 4: Inoculation of *S. aureus* strains (1a) is 1<sup>st</sup> day control, (1b) is 1<sup>st</sup> day test; (3a) is 3<sup>rd</sup> day control, (3b) is 3<sup>rd</sup> day test; (6a) is 6<sup>th</sup> day control, (6b) is 6<sup>th</sup> day test; (9a) is 9<sup>th</sup> day control, (9b) is 9<sup>th</sup> day test**

Bacterial susceptibility to honey varies from species to species. Abd-El Aal *et al.*, (2007) showed that honey had a more pronounced inhibitory effect (85.7%) on Gram-negative bacteria (*Pseudomonas aeruginosa*, *Enterobacter* spp. and *Klebsiella* spp.) and 100% inhibition in the case of Gram-positive methicillin-resistant *Staphylococcus aureus* in comparison to commonly used antimicrobial agents (Abd-El Aal *et al.*, 2007) Mohapatra *et al.* (2011) also reported that honey was effective against both Gram-positive (*S. aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Enterococcus faecalis* and *Micrococcus luteus*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *Salmonella typhi*) (Mohapatra *et al.* 2011). Recent reviews on the successful usage of honey as a dressing on infected wounds support the use of honey in infected wounds, and some suggest the prophylactic use of honey on the wounds of patients susceptible to MRSA and other antibiotic-resistant bacteria (Molan 2011). This is similar to the results obtained in our previous study which showed that *P. aeruginosa*, *E. coli* and *S. aureus* were most sensitive to undiluted honey samples tested with an average zone of inhibition of 39.96, 30.1 and 28.2 mm respectively. It was observed that zone of inhibition was indirectly proportional with the dilution of honey as the dilution was less, the zone of inhibition was more in all three organisms tested (Neerajarani *et al.*, 2016).



**Figure 5: Inoculation of *E. coli* strains (1a) is 1<sup>st</sup> day control, (1b) is 1<sup>st</sup> day test; (3a) is 3<sup>rd</sup> day control, (3b) is 3<sup>rd</sup> day test; (6a) is 6<sup>th</sup> day control, (6b) is 6<sup>th</sup> day test; (9a) is 9<sup>th</sup> day control, (9b) is 9<sup>th</sup> day test**



**Figure 6: Inoculation of *P. aeruginosa* strains (1a) is 1<sup>st</sup> day control, (1b) is 1<sup>st</sup> day test; (3a) is 3<sup>rd</sup> day control, (3b) is 3<sup>rd</sup> day test; (6a) is 6<sup>th</sup> day control, (6b) is 6<sup>th</sup> day test; (9a) is 9<sup>th</sup> day control, (9b) is 9<sup>th</sup> day test**

Studies reported that honey not only limits the growth of wound pathogens but also promote wound healing (Molan *et al.*, 1999; Vallianou *et al.*, 2014). Honey provides a moist environment and improves wound healing by abating oedema, inflammation, and exudation. It also stimulates the growth of epithelial cells and fibroblasts (Yaghoobi *et al.*, 2013). Cytopathological results of the present study showed a considerable decrease in the number of macrophages, neutrophils and increase in the number of fibroblasts, collagen fibres, blood vessels, and RBC's. The results of the histological study showed partial ulceration of stratified squamous epithelium, a decrease in inflammatory cell collections, congested vessels and proliferation of fibrovascular stroma. The antibacterial effect of honey is not only due to its osmolarity, but also due to its important factors that are present in the composition of honey (Carnawath *et al.*, 2014).

Until now, there has been no report of bacterial resistance to honey. This is likely due to the complex composition of honey, which causes the individual components to act either individually or in synergy to prevent resistance (Cooper 2002). Studies have also been conducted on the histopathology of tissues of rats. It has been shown that the application of honey can increase the thickness of granulation tissue and the area of the re-epithelisation also increases collagen metabolism during wound healing (Suguna *et al.*, 1992).

Clinical observation of wound healing properties of honey has shown that it derbies wounds rapidly, replacing sloughs with granulation tissue, promotes rapid epithelisation and absorption of oedema (Efem 1988). Noori., (2011) investigated the antimicrobial activity of natural honey and its effects on the pathogenic bacterial infections of surgical wounds and conjunctiva (Noori *et al.*, 2011). A prospective randomised clinical and histological study of superficial burn wound healing with honey and silver sulfadiazine revealed (Subrahmanyam 1998).

In the present study, partial ulceration of stratified squamous epithelium, a decrease in inflammatory cell collection, congested blood vessels and proliferation of fibroblasts, collagen fibres, blood vessels and RBC's were observed in honey treated animals when compared to control group. To our knowledge, this seems to be the first study on cytopathology of wound tissues to explore possible advantages of honey in wound healing at the cellular level.

## CONCLUSION

Our study further confirmed the role of honey in aiding infected wounds when applied topically. Its antibacterial and antioxidant properties might be

the main factors in accelerating the wound healing process supported by cytological and histopathological observations.

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