



EVALUATION OF HEALING EFFECT POTENCY OF RAW HONEY ON EXCISION WOUNDS IN ALBINO RAT MODEL

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Abstract

Cutaneous wound healing is a highly complex process. Hence, the utilization of natural products such as raw honey, which has antimicrobial, anti-inflammatory and antioxidant activities to accelerate healing process, was an important target. The aim of this work was to assess the possible effectiveness of raw honey on wound healing in albino rats model. A total of 60 healthy, adult albino rats were used in this study. Utilizing aseptic surgical technique, a 1.5 cm incision was made on the skin of the dorsal area of each rat. Experimental rats were randomly divided into four equal groups (negative control, positive control, raw honey and standard drug formulation (tetracycline ointment 1%). Then, each group was subdivided into three subgroups that received treatment for 4, 7 and 14 days. The wounds were submitted to topical applications (twice every day) with a fixed amount of honey and standard tetracycline, the negative control group did not receive any treatment, while the positive control group treated with normal saline solution (0.9 % Na Cl). Experimental animals were sacrificed on 4th, 7th and 14th days and entire wound tissue were excised as well as surrounding normal skin. Wound sizes were measured to assess wound contraction % as a parameter of healing. Histological study was performed using H&E staining technique. Neutrophil, macrophage, fibroblasts and new blood vessels were counted in the wound bed to evaluate the advancement of healing from the inflammatory to the repair phase. All measured parameters were followed by statistical analysis. Our data suggest that raw honey had a greater anti-inflammatory effect and shorter healing time in incisional surgical wounds in albino rats, in comparison to standard tetracycline ointment. The results of present study indicated that raw honey can be used safely and effectively in management of cutaneous tissue wounds. This might be an effective strategy to manage delayed healing of wounds.

Keywords : Open cutaneous wound, wound healing, honey, experimental rats.

Introduction

Cutaneous wound repair is a highly complex process. It consists of several overlapping phases affecting each other, including haemostasis, inflammatory response, proliferation, remodeling and scar formation (Dzobo *et al.*, 2016). Wound management is a major clinical challenge and places a large financial burden, especially in developing countries (Guest *et al.*, 2017). It is known that the current therapy modalities using chemo drugs dissimulate multidrug resistance besides to other side effects (Castro *et al.*, 2006). In the recent years, the importance of utilizing alternative treatments and natural products in wound management has rapidly increased (Pereira and Bartolo, 2016). Among natural products, honey has clinched the attention of researchers as an alternative and complementary remedy. Honey is a natural sweetener that is extensively available around the world. In general, raw honey consists of approximately 75–85% sugars, the majority of these are simple sugars fructose and glucose (80–90%), respectively. Water is the second main constituent in honey (15–20%). Honey contains also trace amounts of ash, proteins, amino acids, vitamins, enzymes and other vital constituents (Roshan *et al.*, 2017). Other medicinal compounds, including flavonoids, phenolic acid, organic acids as well as essential minerals elements are present in honey, which are based mainly on its geographical location and botanical origin (Ciulu *et al.*, 2011). Historically, honey has been well used for its therapeutic properties in numerous civilizations since ancient times (Dias, 2016). Many studies suggested that the biological activity of raw honey on wound repair and tissue regeneration may be attributed to its antimicrobial, anti-inflammatory, antioxidant and immunomodulatory properties (Oryan *et al.*, 2016). Thus, target of this study was to assess and compare the efficiency

of topical application of raw honey in the treatment of excision wounds versus a standard drug formulation.

Materials and Methods

Honey sample

Approximately one kg of natural, freshly honey was obtained from the mountainous region Isfahan /Iran during late spring season of 2018. Honey sample was passed from 0.5 mm Whatman filter at 25 °C temperature “high temperatures are not utilized in any way because high heat causes the loss of useful honey constituents”. The honey used was sterilized by gamma-irradiation (25 kGy) and kept at laboratory temperature (20°C) in the dark until the analysis was performed (Javadi *et al.*, 2018).

Physicochemical analysis of honey

Honey sample were investigated for physicochemical properties including moisture, pH, electrical conductivity, acidity, fructose, glucose, proline and hydroxymethyl furfural (HMF). All of these analyses were done following AOAC Method (2005), at Ministry of Science and Technology/Materials Research Unit, Baghdad, Iraq.

Experimental animals

In this study, a total of 60 healthy adult, male albino rats age 3-4 months and weight 250-300 25± gram were procured from Biotechnology Research Center of AL-Nahrain University. The rats were housed in individual cages with adequate size under strict hygienic conditions of temperature (24°C) and brightness (light/dark cycle of 12 h) and proper fed by standard diet and clean water ad libitum. The cages were cleaned every day to avoid the infection to the wound. This study has been done according to the ethics

committee for animal research of the animal house of Biotechnology Center of AL-Nahrain University-Baghdad following international ethics and regulations for animal research in laboratory applications (Gluck *et al.*, 2002).

Wound creation

All the surgical procedure was performed under anesthesia with an intraperitoneal injection of ketamine (200 mg/kg) and xylazine (10 mg/kg) (Eyarefe and Amid, 2010). The wide dorsum area of each rat was prepared for aseptic surgery by shaving and sterilization with chlorhexidine and alcohol (Alam *et al.*, 2012). The linear full thickness skin wounds at distance 1.5 cm were created on the dorsum of each rat between the 6th and 8th thoracic vertebrae symmetrically.

Experimental groups and treatments

Experimental rats were divided randomly into 4 equal groups, with 15 rats in each group. Each group was subsequently divided into 3 subgroups, each of 5 rats, corresponding to the 4th, 7th and 14th days:

Group one (negative control group): Normal rats without any surgical procedure.

Group two (positive control group): Excision wound-induced rats were treated with normal saline solution (0.9 % NaCl).

Group three: Excision wound-induced rats topically treated with raw honey.

Group four: Excision wound-induced rats topically treated with standard drug ointment (tetracycline 1%).

The therapy schedule was twice every day by topical administration of experimental substances as above. Experimental rats were treated in accordance with Guide for the Care and Use of Laboratory animals by National Research Council, (2010).

Wounds contraction evaluation

The degree of wounds closure was determined after every two days of wound formation on (3, 5, 7, 9, 11 and 13 days), respectively. The wound size was measured based on the initial area (measured on day zero), using the following formula:

Percentage of wound contraction= [(original wound area - unhealed area)/original wound area] × 100% (Subalakshmi *et al.*, 2014).

Histopathological examinations

For histological studies, during the 4, 7 and 14 days experimental animals were sacrificed and skin specimens and adjacent skin were excised from the wound edge of each rats. The tissue sections were kept in histological cassettes and fixed with buffered formaldehyde solution at 3.7% for 24 hours. Afterward, they were processed according to the standard light microscope tissue protocols. The wound sections were embedded in paraffin blocks and sliced into 5 μ m-thick sections. The slides were observed under a light microscope after staining with haematoxylin and eosin (H&E). Afterward, sections the were photo'd via

(CX31-Olympus, Japan) at 400x objective magnification (Masson-Meyers *et al.*, 2013).

Morphometric study

The number of neutrophil, macrophage, fibroblasts and blood vessels were calculated in 10 non overlapping high power fields in a magnification of x40 and the measurement were done using software 3 tools.

Statistics

The Statistical Analysis System-SAS (2012) program was utilized to expose the influence of difference factors (days and groups) in experiment parameters. Least significant difference (LSD) test (Analysis of Variation-ANOVA) was utilized to determine statistically significant variances between the mean values in this investigate.

Results

Physicochemical parameters of honey

The quality standard for honey include: Moisture, pH, electrical conductivity, acidity, glucose, fructose, proline, hydroxymethyl furfural (HMF). In general, findings as shown in (Table 1) were within the standard limit reported by Codex Alimentarium (Codex Alimentations, 2001).

Table 1: Physicochemical parameters of honey

No.	Physicochemical parameter	Results
1	Moisture %	18.8
2	pH	3.69
3	Electrical conductivity ms/cm	0.13
4	Acidity meq/kg	27.5
5	Sucrose	7.8
6	Fructose %	38.86
7	Glucose%	34.91
8	proline mg/kg	26.62
9	HMF mg/kg	33.00

Neutrophils number

Through all the experiment periods, a large number of neutrophils were observed in the wound tissue in all experimental treatments groups as compared with the normal rats in negative control groups. Moreover, there was a significant decrease in the number of neutrophils per mm² in all experimental groups was observed on the 14th day in comparison with the previous periods (4th and 7th days). There was also significantly decreases difference (P<0.01) in the number of neutrophils per mm² in the in honey-treated group in comparison with positive group control and tetracycline-treated group on the 4th day. Whereas on the 7th day, there was no significant difference between honey-treated group and tetracycline-treated group. On the 14th day, the honey group tended to have less neutrophils than that of positive control group and tetracycline-treated group where significantly reached near to normal rats in negative control group with (3.80 ± 0.45) as shown in (Table 2).

Table 2 : Comparing the neutrophils count in the wound bed of groups on the 4 , 7 and 14 days of experiment.

Groups	Treatment days Mean \pm SE			LSD value
	4 Day	7 Day	14 Day	
Negative control	1.80 \pm 0.59 C a	1.80 \pm 0.21 C a	1.40 \pm 0.23 D a	1.180 NS
Positive control	35.60 \pm 2.77 A a	28.20 \pm 0.36 A a	17.40 \pm 3.14 A b	7.517 **
Honey	18.80 \pm 1.33 B a	12.40 \pm 0.95 B b	3.80 \pm 0.45 CD c	3.101 **
Tetracycline ointment	32.80 \pm 1.34 A a	15.80 \pm 1.48 B b	9.60 \pm 0.74 B c	3.831 **
LSD value	5.278 **	3.760 **	4.199 **	---

Means having with the different big letters in same column and small letters in same row differed significantly, ** (P<0.01).

Macrophage number

like neutrophils, a significant increase in the number of macrophages was revealed in the wound tissue of positive control and other treated groups as compared with the negative control groups through all the study periods. On 4 and 7 days during the inflammatory and early proliferative

periods, the number of macrophages per mm² in the in the honey treated group was significantly larger compared to those in other experimental groups (P<0.01); however, on the day 14, honey-treated group indicated to have fewer macrophage than the other experimental groups (P<0.01) (Table 3).

Table 3 : Comparing the macrophages count in the wound bed of groups on the 4 , 7 and 14 days of experiment.

Groups	Treatment days Mean \pm SE			LSD value
	4 Day	7 Day	14 Day	
Negative control	2.33 \pm 0.41 D a	1.83 \pm 0.30 C a	1.67 \pm 0.31 C a	1.076 NS
Positive control	7.83 \pm 0.32 C a	7.67 \pm 0.72 B a	7.16 \pm 0.78 A a	1.931 NS
Honey	12.67 \pm 1.12 A a	10.67 \pm 1.09 A a	3.79 \pm 0.48 C b	2.836 **
Tetracycline ointment	9.16 \pm 0.41 BC a	8.50 \pm 0.43 B a	5.33 \pm 0.85 AB b	1.787 **
LSD value	2.246 **	2.201 **	49.087 **	---

Means having with the different big letters in same column and small letters in same row differed significantly, ** (P<0.01).

Fibroblasts number

During all the experimental periods, the number of fibroblasts as shown in (Table.4) exhibited a significant increase in all experimental groups compared to negative control (normal rats). The number of fibroblasts per mm² in honey-treated group significantly peaked on the 7th day and

after that decreased severely on the 14th day. While, tetracycline-treated groups and positive control groups revealed significant gradual increase from the 4th day to 14th day. There was a significant difference between honey-treated group when compared with tetracycline and positive control groups.

Table 4 : Comparing the fibroblast count in the wound bed of groups on the 4, 7 and 14 days of experiment.

Groups	Treatment days Mean \pm SE			LSD value
	4 Day	7 Day	14 Day	
Negative control	5.40 \pm 0.88 D a	6.60 \pm 0.61 D a	5.60 \pm 0.75 D a	2.308 NS
Positive control	64.80 \pm 1.72 C b	81.80 \pm 9.48 C ab	84.40 \pm 1.93 C a	17.515 *
Honey	87.60 \pm 1.71 B c	147.80 \pm 2.21 B a	122.40 \pm 6.78 B b	13.067 **
Tetracycline ointment	70.20 \pm 1.43 C c	86.80 \pm 1.36 C b	91.60 \pm 1.48 C a	4.373 **
LSD value	6.149 **	12.035 **	9.675 **	---

Means having with the different big letters in same column and small letters in same row differed significantly, * (P<0.05), ** (P<0.01).

Blood vessels number

There was a significant increase in the number of the new blood vessels in all experimental groups in comparison with normal control as shown in (Table 5). In honey-treated groups, the number of new blood vessels per mm² in the wound bed increased rapidly from the 4th day to the 7th day

(each P<0.05) and it peaked on the 14th day. While those of the tetracycline-treated group and positive control group until 14th day statistically remained almost the same as on the 4th day. Moreover, there was a significant difference between honey-treated group compared with other experimental groups (always P<0.01).

Table 5 : Comparing the new blood vessels count in the wound bed of groups on the 4, 7 and 14 days of experiment.

Groups	Treatment days Mean ± SE			LSD value
	4 Day	7 Day	14 Day	
Negative control	2.40 ± 0.52 D a	2.60 ± 0.41 D a	3.00 ± 0.46 C a	1.404 NS
Positive control	8.80 ± 1.03 C a	10.80 ± 0.74 C a	12.40 ± 1.61 B a	3.618 NS
Honey	16.20 ± 1.29 A b	18.60 ± 0.52 A a	20.20 ± 1.21 A a	3.252 *
Tetracycline ointment	12.80 ± 1.03 B a	14.20 ± 0.81 B a	14.40 ± 1.61 B a	3.664 NS
LSD value	2.658 **	2.228 **	3.379 **	---

Means having with the different big letters in same column and small letters in same row differed significantly, * (P<0.05), ** (P<0.01).

Measurement of wounds contraction

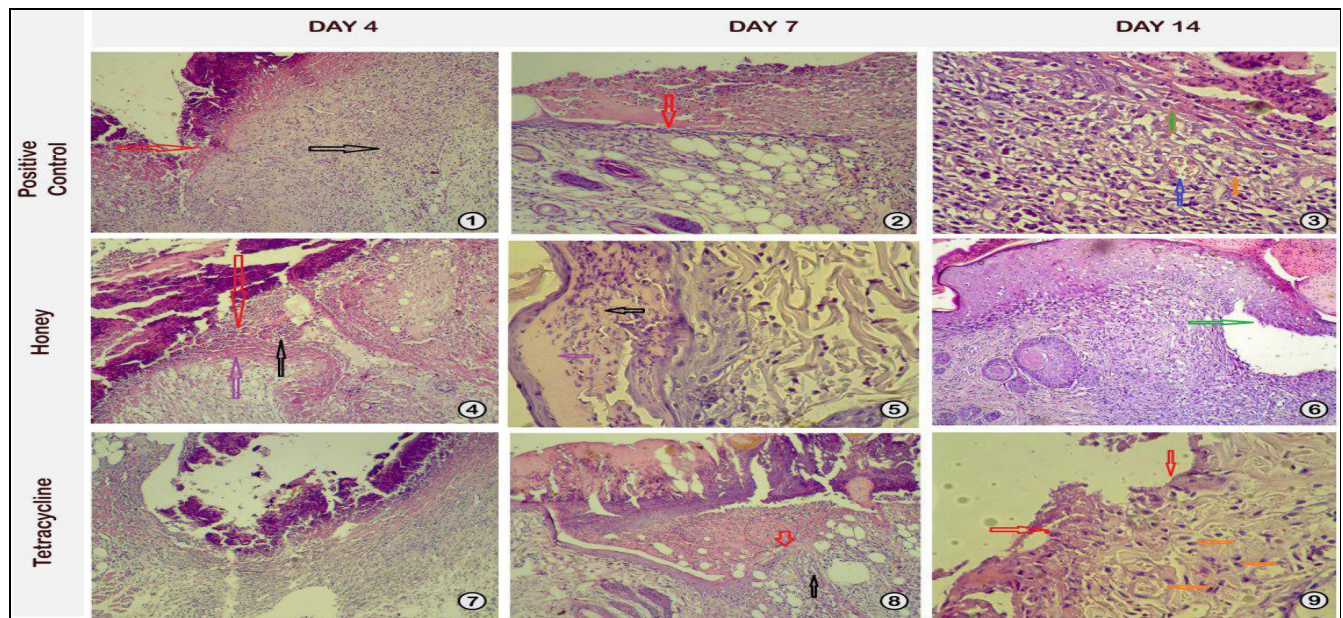
The ratios of tissue repair process of experimentally induced cutaneous wounds in rats was measured as shown in (Table 6). Wound contraction was gradual in all experimental

groups from days 0-13. The rate of epithelial closure was significantly increased in the honey-treated wounds as compared with tetracycline-treated group and positive control group.

Table 6 : The percentage of wound closure obtained with different treatments in all study groups .

Groups	Mean ± SE						LSD value
	3 Day %	5 Day %	7 Day %	9 Day %	11 Day %	13 Day %	
Positive control	20.16 ± 1.05 C d	27.44 ± 2.46 D c	41.04 ± 4.22 D b	45.55 ± 3.52 D b	40.94 ± 4.12 C b	53.27 ± 2.01 D a	6.04 **
Honey	37.77 ± 2.22 A e	58.02 ± 2.86 A d	70.52 ± 3.31 A c	75.22 ± 4.43 A c	89.38 ± 0.58 A b	98.67 ± 0.69 A a	8.56 **
Tetracycline ointment	24.61 ± 1.55 C d	40.16 ± 3.59 C c	52.16 ± 2.16 C b	56.83 ± 1.65 C b	71.01 ± 5.85 B a	76.88 ± 5.12 C a	8.25 **
LSD value	4.85 **	9.55 **	9.17 **	10.69 **	9.87 **	7.76 **	---

Means having with the different big letters in same column and small letters in same row differed significantly, ** (P<0.01).



Figs. (1-9): Photomicrographs of sections of skin in wound rat model stained with H & E, 40x magnification showing: (1) surface epidermal discontinuity, massive inflammatory cellular infiltration, filling of wound bed with more cellular granulation tissue (2) surface epidermal discontinuity with heavy inflammatory cells infiltration and abundant formation of granulation tissue (3) heavy inflammatory cells infiltration, granulation tissue and start re-epithelization as a cell lining (4) less infiltration of inflammatory cells, with beginning formation of granulation tissue and the wound look like a clean wound (5) filling of wound bed with thick mature collagen fibers covered with a newly formed epidermis under the crust. Note the presence of less inflammatory cellular infiltration (6) filling of wound bed with mature collagen fibers in some parts and others with fine immature collagen fibers covered with a newly formed epidermis. Note the appearance of hair follicles in wound bed. A small area with incomplete filling with granulation tissue is observed (7) surface epidermis with inflammatory cells infiltration and beginning formation of granulation tissue (8) surface epidermal discontinuity with inflammatory cells infiltration and beginning formation of granulation tissue (9) surface epidermis with heavy inflammatory cells infiltration with granulation tissue but still there was no re-epithelization.

Discussion

Wound healing is a highly complex process characterized by four distinct overlapping phases: haemostasis, inflammation, proliferation and tissue maturation or remodeling (Dzobo *et al.*, 2016). Immediately after an epithelial injury, blood clotting occurs, this clot is constituted of thrombins, platelets, and fibronectin, and acts as a temporary matrix for other cells (comprising neutrophils, monocytes, and endothelial cells). This temporary matrix aids the migration and maintenance of these cells at the site of the injury (Park and Barbul, 2004).

During the inflammatory response, a large number of neutrophils are typically found within 24-36 hours at the injury site, followed by monocytes that rapidly differentiate into macrophages (Powell and Huttenlocher, 2016). Neutrophils release a high levels of reactive oxygen species (ROS) and several pro-inflammatory cytokines to sterilize the injury (Frykberg and Banks, 2015). Upon completion of this process, they are phagocytized via the newly arrived macrophages (Gantwerker and Hom, 2012). Whereas excessive neutrophil activity can cause delayed repair and even harming surrounding healthy tissues (Wilgus *et al.*, 2013).

The histological examination in this study showed that treatment with raw honey nearly normalized the count of neutrophils as compared with those in positive control group and tetracycline-treated group (Tables 2). Honey has moisture absorption features that can reduce edema of the injury. This results in faster healing and early proliferation phase of inflammation response (Yaghoobi and Kazerouni, 2013). The mechanism by which honey affected the neutrophil count may be attributed to its anti-inflammatory activity which included: Inhibition of the classical complement pathway, inhibition of ROS formation, inhibition of leukocyte infiltration, inhibition of cyclooxygenase-2 (COX-2) and inducible the expression nitric oxide synthase (Majtan *et al.*, 2013). The present findings were similar to the previous review by Suguna *et al.* (1993) and Takzaree *et al.* (2017) who revealed the same findings.

A previous study have revealed that macrophages play crucial functions in all phases of wound healing, including host defense, promotion and resolution of inflammation, removal of apoptotic cells, and support of cell proliferation and tissue restoration (Wynn *et al.*, 2013). Macrophages are derived from two different sources: (a) a tissue-resident macrophage established before birth (b) circulating monocytes that are recruited to the injury site and rapidly transformation into macrophages (Vannella and Wynn, 2017).

In this study, results showed that the number of macrophage in the honey-treated group was significantly higher ($P<0.01$) compared to other experimental groups on the 4th and 7th days. On the contrary, on the 14th day, macrophage number in the honey-treated group was significantly decreased ($P<0.01$) compared to other groups (Table 3). These result is supported by a previous studies done by Nakajima *et al.* (2013) and Takzaree *et al.* (2016).

In spite of extensive investigations, the specific molecular mechanisms by which honey regulates the macrophages count remain poorly understood. Thus, it might

be to its immunomodulatory effect (Majtan, 2014). Another research of Ahmed *et al.* (2009) supported the hypothesis of anti-inflammatory effect of honey through inhibition of activated macrophages.

It is known that fibroblasts are the most common cells of connective tissue, which are crucial in supporting wound healing, participating in essential processes (breaking down the fibrin clot, synthesizes the extra cellular matrix and collagen structures) (Darby and Hewitson, 2007).

Our findings indicated a significant increase in the counting of fibroblasts in the honey-treated group as compared to other experimental groups (Table 4). These results were consistent with previous findings that reported an increase in the proliferation of fibroblasts in the honey-treated group compared with the control group (Nisbet *et al.*, 2010; Takzaree *et al.*, 2017).

Indeed, the acidity of honey mainly releases oxygen from hemoglobin and leads to increased activity of fibroblasts (Burlando and Cornara, 2013). Hydrogen peroxide (H_2O_2) in honey also promotes growth of fibroblasts and epithelial cells involved in the migration and proliferation stages (Molan, 2001). Likewise, trace elements in honey (mainly zinc, copper and manganese) also participate to cell growth and proliferation (Coger *et al.*, 2019).

During wound healing, angiogenic vessel sprouts invade the wound clot rich in fibrin/fibronectin and organize into a microvascular network throughout the granulation tissue within a few days (Tonnesen *et al.*, 2000).

In this review, the rats that treated with raw honey showed significantly increase ($P<0.01$) in the counting of new blood vessels compared to those in other experimental groups (Table 5). These results may be attributed to hydrogen peroxide (H_2O_2) which is released from honey (with its insulin-like features) where could stimulate the development of new blood vessels in the injury site (Yaghoobi and Kazerouni, 2013). Additionally, the pH of the honey alone might be adequate to stimulate capillary sprouts creation, as it has been recorded that an acidic circumstances may provoke angiogenesis (Rossiter *et al.*, 2010). The angiogenic effectiveness of raw honey was also evidenced in several studies such as Nisbet *et al.* (2010) and Nakajima *et al.* (2013).

It is known that that the contraction of the wound has a crucial role in wound healing processes through reduces the wound size and shortens the healing period (Nedelec *et al.*, 2000).

The results of this work showed a significant increase ($P<0.01$) in the percentage of wound contraction in honey-treated groups compared to other experimental groups through all the experiment periods (Table 6). This is similar to the results reported by several researchers on the effectiveness of topical application of honey in wound management (Iftikhar *et al.*, 2010; Gill *et al.*, 2019). Acceleration of wound contraction rate might be due the characteristics of honey that provides energy required for contractile action of myofibroblasts (Yusof *et al.*, 2007). Increased number of fibroblasts with subsequent deposition of collagen also shared in improvement of wound healing with decreased wound size (Aljady *et al.*, 2000). Honey also contains high levels of proline and methionine which have a

crucial role in collagen formation and deposition (da Silva *et al.*, 2006) and has hydroscopic effect that assists in repair processes (Giusto *et al.*, 2017).

Conclusion

It can be concluded, there is biological plausibility of topical administration of raw honey in accelerates the inflammatory reaction and wound healing rate of excisional wounds of albino rats model, but further exploratory studies are needed to support large clinical trials.

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