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Wound healing: In response to natural remedies and phototherapy

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ABSTRACT

Insufficient studies had been carried on the effectiveness of (*Phoenix dactylifera*) Ajwah date in the healing of burns. The aim of the current study was to inspect the role of Ajwah date, honey and low intensity laser therapy in healing of burn as well as antimicrobial activity. Total Flavonoids contents in methanol extract of Ajwah date with an average value of 5.574 mg QE/gram. Quercetin described as the greatest flavonoids contents within the extract (2.438 mg GAE/gram fw). Moreover, quercetin extracted to examine the antimicrobial activity via TLC and recognized by IR, MS, and ¹H and ¹³C NMR. Quercetin of Ajwah date presented different responses through creating numerous inhibition zones against examined microorganisms. Chemical burn was induced to twenty local rabbits that were divided randomly into four groups of equal number received topical application of quercetin (80 %), lime honey, low intensity laser therapy (LILT) and control group. Wound surface area was measured through tracing method and 3D camera while wound swab was used to detect the microbial load. The results revealed that there were significant differences in wound surface area and microbial load for the treatment groups when compared to the control group after 7, 14, and 21 days as p<0.05, while the quercetin group was superior to honey and low intensity laser therapy respectively. This study supported that quercetin extracted from ajwah; honey and LILT are beneficial methods to accelerate the wound healing process as well as decrease the microbial load.

Keywords: Antimicrobial; Burn; Honey; Low Intensity Laser Therapy; Phoenix dactylifera; Quercetin.

INTRODUCTION

A wound is a change of the original anatomic structure and physiological capacity of tissue (Jayasutha et al., 2011). Wounds result from pathologic sequels introduced interior or exterior of the affected area. Wound healing is the means through which skin or other body tissue rebuild itself following injury (Murti and Kumar, 2012). One of the functions of the skin layers is to create a shielding barrier against the invading organisms. If this barrier is disrupted, an organized process of biochemical measures is (Bjarnsholt et al., 2008) initiated to repair the injury. This process is divided into foreseen phases: the initial phase is the blood clotting, followed by the inflammatory phase then the prolifera-

tion phase and ended with the remodeling phase. Delayed wound healing or failure to heal may be resulted from various factors like the bacterial invasion or due to the formation of biofilm (Chah et al., 2006).

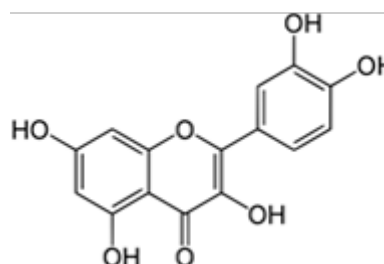


Figure 1: Structure of quercetin

Where there is a deficiency of sound proof concerning local wound treatments from systematic studies of randomized trials. Exclusion is a point of covering the wound with suitable and effective topical agent. Reasonably because of a noted ambiguity respecting what is the best in a complicated field, both complementary and alternative medicines are more noticed to be reliable than conventional therapies (Blaser et al., 2007).

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The extensive evolution of bacterial resistance to antibiotics is considered to be a challenging factor. Hence, contemporary attention is directed toward finding an alternative to antibiotics, therefore, traditional treatments, as honey, herbal extracts, essential oils, enzymes, electro-therapeutic modalities, light therapy, negative pressure, ozone therapy and hyperbaric oxygen are significant factors correlated to the process of wound healing.

Honey was proven to be an antibacterial agent working against infected wounds or those wounds of risk to be infected and has a strong potential to accelerate the wound healing process within burns, wounds, and bed sores (Visavadia *et al.*, 2008). Being a dressing covering wounds, honey affords wet healing conditions, swiftly cleans germs, purifies, and lessens inflammation and the subsequent swelling and exudate. It enhances the speed of healing via triggering the formation of new blood vessels, overgrowth of granulation tissue (Almeida-Lopes *et al.*, 2011).

Despite the limited impact of low intensity laser therapy (LILT) on some patients, it is extremely useful for most patients with burns, wounds, as well as bed sores. These effects primarily depend on the physical and physiological background of wounds (Samaneh *et al.*, 2015). Many animal studies implied that low-intensity laser light promotes the wound healing process through the prominent effect in enhancing the cell proliferation in the wound bed, promoting the rapid formation of collagen fibers and granulation tissue as well as proper enhancement of mRNA specific pools. ATP required by cells for energy production is synthesized within mitochondria by the effect of light therapy. Furthermore, activation of body defensive mechanisms to pathogens is promoted by activation of lymphocytes in the wound (Ghamsari and Taguchi, 1997).

Studies in the few earlier times revealed that ingredients obtained from dates seem to be a strong antioxidant, inhibit tumor cells as well as cease inflammation, give a proper alternative medicine in different diseases remedy (Jovanovic *et al.*, 1994). Dates supply the body with several nutrients including nearly carbohydrates, proteins, vitamins, and antioxidants.

This study aimed to investigate of the antibacterial activities of ajwah date palm (*Phoenix dactylifera*) extracts against some pathogenic microorganisms. Also, evaluate the effect of ajwah date extracts, honey, and low intensity laser therapy on healing of induced full thickness burns in animal model. Moreover, microbial infection and wound surface area changes were characterized during the treatment protocols.

MATERIAL AND METHODS

Chemicals

All chemicals and solvents were obtained from Sigma Aldrich Co. Ltd (St. Louis, France).

Ajwah Date extraction method

Ajwah fruit was obtained from a determined farm in the Madinah province, KSA. This farm plows solely Ajwah date palms. Of five palms, 1 kg of products was picked and incorporated in a case. By the period of the season, the products denoted in "Tamar" or matured degree, and classification was dependant on the local experience of the producer. Mature fruits of regular extent, clear of visible decay and damage from pests and fungal germs, were picked and appropriated for the whole experiments. Upon approach to the laboratory, the samples (100–150 g portions) has been inserted in polyethylene pouches, tied, and saved at 20 °C till investigated. The flesh was manually isolated from the date and dehydrated for 18 h in a drying closet (Unitemp) at 40 °C, and saved at room temperature. The dehydrated date of 100 g were smashed and extracted via 80% methanol under agitation at ordinary room temperature. After 2 days, the extract was purified by Whatman No. 2 filter paper (Whatman International Limited, Kent, England) and the filtrate was the be evaporated to dryness in a drying cabinet (Unitemp) at 40 °C, and stored in a desiccator at room temperature.

Determination of Total Flavonoids

Flavonoid component in the methanolic extract of ajwah extract was discovered through aluminum chloride calorimetric approach (Chang *et al.*, 2002).

Quercetin extraction and HPLC Analysis

The methanol extract (ME) was filtered and concentrated in vacuum to obtain a crude extract (80 g). Ethyl acetate fraction was applied to silica gel column (400 g) which was eluted with methylene chloride containing increasing amounts of methanol (up to 100%) to give 20 combined fractions according to Marzouk *et al.*, 2006 protocol. These compounds were identified by comparison of its spectral data (1H-NMR) with reported values in the literature (Marzouk *et al.*, 2006). Quercetin in the samples was identified by comparison of its retention time (tR) with the standard quercetin (Phani *et al.*, 2010).

Antimicrobial Protocol

Microbial strains

The agar diffusion test was administered according to modified Kirby-Bauer disc diffusion method (Selim *et al.*, 2013). One loopful of every test organism was suspended in 3 ml 0.9% NaCl solution distinctly. The microbial strains utilized in this trial (others than ATCC strains) were separated from humans and belonged to the microbiological laboratory samples of the Clinical Laboratory Sciences, College of Applied Medical Sciences, Aljouf University, KSA. Nutrient agar (for bacterial strains) and YEA media (for fungi) were inoculated with this suspension of the particular organism and flowed inside a sterile petri dish.

Disc-diffusion assay

The extract was suspended in dimethylsulfoxide (DMSO) to a definitive concentration of 30 mg/ml and sterilized through filtration via 0.45 µm Millipore filters. Antimicrobial analyses were then brought with disc diffusion process (Selim, 2011) utilizing 100 µl of a suspension comprising 108 cfu/ml of bacteria and 106 cfu/ml of yeast spread on nutrient agar (NA) and Sabouraud dextrose agar (SDA), respectively. The discs (6 mm in diameter) were soaked with 5 mg/disc and located on the inoculated agar. Negative controls were fixed using the identical solvent used to melt the extract. Amoxicillin (30 µg/disc), gentamycin (30 µg/disc) and streptomycin (30 µg/disc) were employed as positive evidence standards to ascertain the sensitivity of one strain/isolate in each microbial species tested. The inoculated plates were incubated at 37 °C for 24 h for clinical bacterial strains and 48 h for yeast isolates. Antimicrobial activity was assessed by estimating the zone of inhibition against the test organisms.

Micro-well dilution assay of MIC and MBC

The minimal inhibitory concentration (MIC) rates were investigated for the bacterial strains, remaining sensible to the extract in the disc diffusion assay. The inocula of the bacterial strains were made from 12 h. Broth cultures and suspensions were standardized to 0.5 McFarland regular turbidity. The extract was primarily dissolved in 10% DMSO and then diluted to the highest concentration (5 mg/ml) to be tested, and then serial two-fold dilutions were prepared in a concentration range from 0.1 to 5 mg/ml in 10 ml sterile test tubes containing nutrient broth. MIC values of the extract against microbial strains isolates were determined based on a micro-well dilution method as described by Selim, 2011.

Wound healing activity Protocol

Experimental animals

The current study was carried on 20 local rabbits (10 male and 10 female) of age 16 weeks and weights varied between (1200- 1400 gms) obtained from the college of pharmacy, Aljouf University, Saudi Arabia. All rabbits were given one week for adaptation.

Burn induction

Each rabbit back skin was shaved mechanically and given one-day rest. On the second day, each rabbit was anesthetized with ketamine at a dose of 30 mg/kg before induction of the burn. three drops of concentrated HCl (38%) were topically dropped on the shaved skin. the rabbits were housed separately under sterile conditions with persistent environmental settings that provided good air circulation, food supplements, and lighting. rabbits had free access to water and food (standard food as a pellet) in all groups and cages were cleaned periodically. anesthesia was induced by keta-

mine and xylazine (bioethics permission according to Aljouf University Ethical Committee, Saudi Arabia).

Experiment design

This is a double blind randomized comparative clinical trial conducted on animal model. After induction of burn, rabbits were divided in a random way to four groups of equal number each group contains five rabbits; group I; Quercetin group that received quercetin extracted from an ajwah date (80%) through a daily application on the wound. Group II that received a daily application of honey (local lime honey with pH 3.2) with a volume of 3 ml that dogged by a disinfected syringe then emptied to the burn area and set aside by non-adherent sterile gauze. Group III; Laser group that received low intensity laser therapy in which disinfected plastic wrap was supported to the burn area and its neighboring parts to avoid contamination from the laser device. The plastic wrap should cover the burn area by about 3 cm and fixed on the rabbit through the use of adhesive strap. Infrared LILT cluster device was applied to the burn area every other day for 4.5 minutes, 4J/cm², and power intensity was 14 x 50 mw while the wavelength was 785nm (LDU 8015 PN, SN: LDPN91100, Germany). Firm grasping of the rabbit is important to ensure complete and adequate application of LILT for gaining better benefit. The control group (group IV) received daily local wound care by normal saline. The treatment was started in each group after 24 hours of the burn induction and debridement of the burn bed to remove the dead tissue. The same procedure was applied for three successive weeks.

Measurement Protocols

All measurement protocols were conducted at 24 hours post burn, and at the end of each successive week for a period of 3 weeks. The measurement of wound surface area was conducted by tracing method according to (Bohannon and Pfaller, 1983). A sterilized transparency film was placed on the wound. The wound perimeter was traced by using fine tipped transparency marker. Each wound area was traced three times to establish measurement reliability. After tracing, the transparency film face which faces the wound was cleaned by a piece of cotton and alcohol. The carbon paper was placed over the metric graph paper 1 mm². The traced transparency film was placed over a carbon paper with a white paper in between and transcribed the tracing on metric graph paper. The number of squared millimeters on the metric graph paper was counted to determine the wound area. This area was converted to cm². The mean of the three trials was calculated and considered as a wound surface area.

Microbiological load measured by collecting specimens from induced burns in rabbits by swabs. A swab of each pus sample was suspended in 3 mL of water peptone. Drops of the prepared suspensions were spread on surface of plates containing *Pseudomonas* selective

Table 1: Showed the mean \pm standard deviation, p- values of wound surface area for all groups of the study at 0, 7, 14, and 21 days within and between groups

Group	Group I (Quercetin)	Group II (Honey)	Group III (LILT)	Group IV (Control)	P- value
0 days	14.44 \pm 0.21	14.14 \pm 0.27	14.00 \pm 0.40	14.19 \pm 0.28	0.123
7 days	11.24 \pm 0.50*	12.90 \pm 0.23*	12.48 \pm 0.53*	14.10 \pm 0.20	0.001
14 days	8.24 \pm 0.77*	10.26 \pm 0.3*	8.94 \pm 1.91*	14.00 \pm 0.16	
21 days	5.50 \pm 0.49*	7.98 \pm 0.41*	9.50 \pm 0.34*	13.90 \pm 0.19	

Where: P: Probability Value and *significant

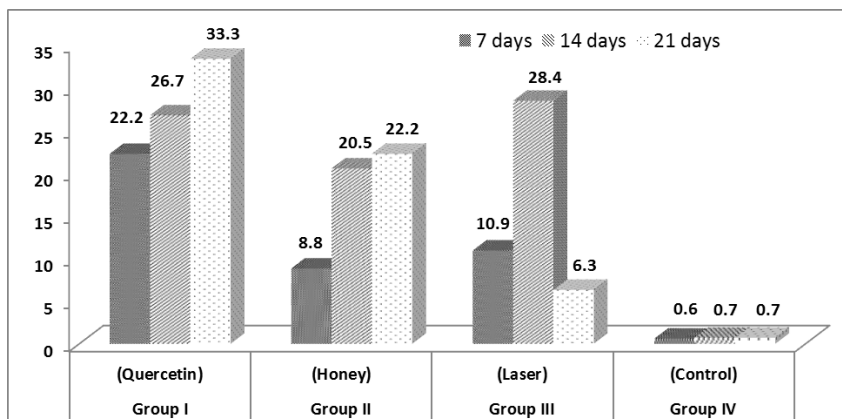
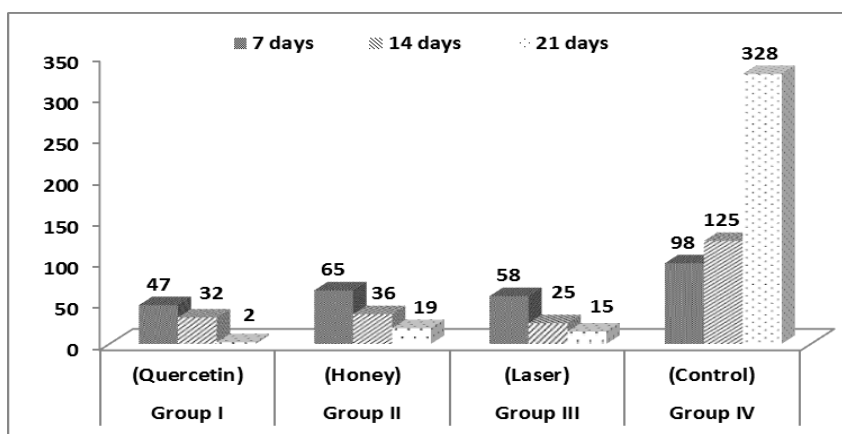
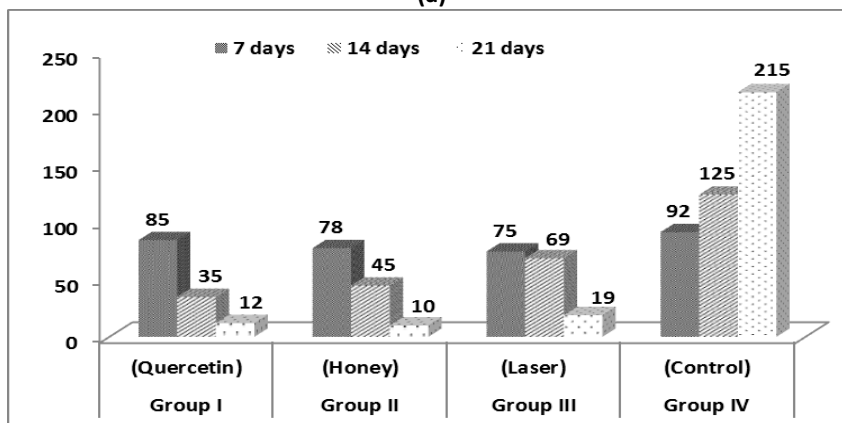


Figure 2: Percentage of wound surface area improvement for all groups



(a)



(b)

Figure 3: Frequency of microorganisms (a) Staphylococcus Counts and (b) Pseudomonas counts colonizing and infecting burn wounds

Table 2: Antimicrobial activity of Quercetin extracted from Ajwah date

Microorganisms	DD ^a	MIC	MBC
Gram Positive Bacteria			
<i>Streptococcus sp</i>	16	250	250
<i>Staphylococcus aureus</i>	31	125	125
Gram Negative Bacteria			
<i>Escherichia coli</i>	22	250	250
<i>Klebsiella pneumoniae</i>	10	250	250
<i>Pseudomonas aeruginosa</i>	26	125	250
Yeast			
<i>Candida albicans</i>	10	500	500

MIC (minimum inhibition concentration) and MBC (minimum bactericidal concentration) as $\mu\text{g/ml}$ of quercetin; (-) no antimicrobial activity. Values are average of triplicate. ^a Inhibition zone in diameter (mm) around the discs impregnated with quercetin ($100 \mu\text{g/disc}$).

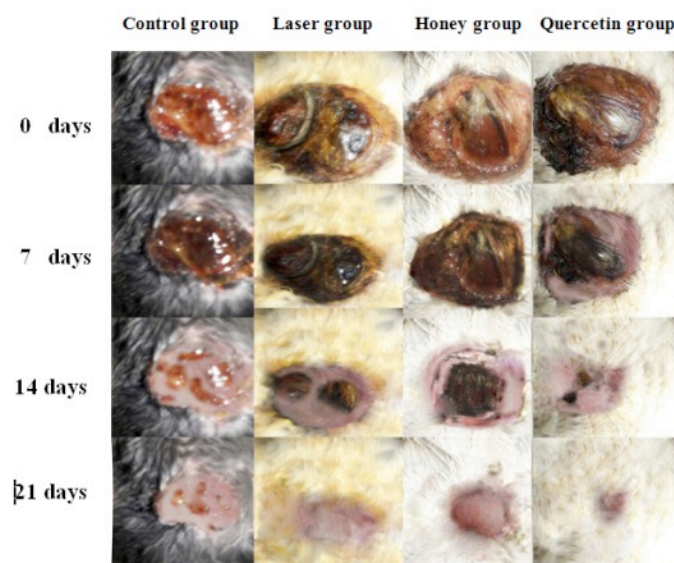


Figure 4: Illustrations of taken images by digital camera showing the decline of wound surface area during the course of the treatment protocol in the four groups of the study

medium and *Staphylococcus* 110 medium. All plates were incubated face down and the bacteria were allowed to grow at 37°C for 24-48 hours prior to enumeration and further identification.

Photographic techniques were used to observe the progression of the healing process at 0, 7, 14 and 21 days by using a digital camera with a distance of 1.5 ft between the burned area and the camera exactly at 90° above the center of the burn.

Data Analysis

Data was analyzed by SPSS v.20 using two-way ANOVA and Tukey's tests and the results were shown as mean \pm standard deviation (Mean \pm SEM). P values less than 0.05 were considered as significant.

RESULTS

Flavonoid component in the methanol extract of Ajwah date includes an average value of 5.574 mg QE/gram. Quercetin expressed as the greatest flavonoids content in the extract (2.438 mg GAE/gram fw). To examine the pureness of the active fraction, it was investigated by

TLC and HPLC techniques. Preparatory classification of the extract was based on a comparison of its Rf value and retention time among those of authentic compounds. Subsequently, the structure assignment of the fraction was based on its spectral data IR, MS, and ^1H and ^{13}C NMR, and it was distinguished as a known compound, quercetin (Fig. 1). The faithful and reproducible outcomes accomplished utilizing the regular disk diffusion technique revealed that quercetin extracted from ajwah date pits displayed powerful antimicrobial potential (Table 1). Different degrees of microorganisms sensitivity was perceived, intimating a differential fundamental tolerance of microorganisms and/or the distinct characteristics and aggregation of the flavonoid compounds present in the ajwah date. The powerful antimicrobial activity of quercetin was designated against *Staphylococcus aureus*, and the lowest activity was observed against *Candida albicans* with inhibition zone diameter 31mm and 10mm respectively. Furthermore, the extracts showed both bacteriostatic and bactericidal activities with MIC and MBC values found equal and ranged from 125 to 500

µg/mL (Table 1). The obtained results suggest that quercetin of Ajwah date was more efficient to inhibit Gram-positive than Gram-negative bacteria.

The results of the current study revealed that there was no significant difference in wound surface area (WSA) between all groups at the baseline ($p = 0.123$) which reflects the homogeneity of the groups. ANOVA revealed a significant group X time interaction effect ($p = 0.001$) (Table 2). The quercetin group showed a significant decrease in wound surface area at 7, 14 and 21 days compared with baseline and to the other groups at the same periods of time by 22.2%, 26.7% and 33.3%, respectively. While the honey group showed a significant decrease in the WSA compared to the base line at the same periods of time by 8.8%, 20.5% and 22.2%. LASER group also showed a significant decrease in the WSA when compared to the baseline at 7, 14 and 21 days by 10.9%, 28.4% and 6.3% respectively (Figure 2). A quantitative microbiology culture was used to provide the CFU per cm^2 of burn surface area. The surface swab taken from the induced burns showed decrease in bacterial counts of *Staphylococcus* and *Pseudomonas* in treated burns in comparison to control group (Figure 3). Photographic assessment of burn areas showed observable reduction of wound surface area in favor of the quercetin extracted from ajwah more than honey, LILT and control group respectively (Figure 4).

DISCUSSION

Wound healing process is complemented through a tidy and definable series of natural proceedings that are beginning by means of wound closing and moving ahead to the healing or restoration of the impaired tissue. Wound repair for a full-thickness burn runs through many phases comprising of; granulation tissue formation, epithelial tissue production, wound approximation and finally scarring. Earlier Studies revealed that wound restorative process could be hastened and intensified by the aid of natural or simulated biochemical composites. Plant ingredients could apparently increase the rate of wound restorative process (Middleton and Kandaswami, 1994).

In this study, a burn was induced chemically by concentrated HCL (38%) for the estimation of wound healing activity of quercetin extracted from Ajwah date, Lime honey and Low intensity Laser Therapy. The outcomes of the contemporary study presented proofs for the efficacy of local application of quercetin to intensify the wound healing process and to reduce the healing time in rabbits. Essentially our study issues revealed that; a notable persistent decline in wound surface area throughout the treatment stages after 7, 14 and 21 days for the four preliminary groups with advantage to the quercetin group followed by the honey group then the LILT group as p -value < 0.05 . Whichever determine that topical *Phoenix dactylifera* has an effect on wound healing in rabbits. These outcomes may be

interpreted as follow; dates have been proved to be one of the well-liked fruits filled with a remarkable inventory of crucial nutrients, vitamins, and mineral deposits to be essential for typical development, and well-being. They have health beneficial flavonoid polyphenolic antioxidants identified as tannins that were recognized to own anti-infective, anti-inflammatory, and anti-hemorrhagic (avoid uncomplicated blood loss tendencies) capacities (Al-Farsi et al., 2005). They have a generous amount of vitamin-A (contains 149 IU per 100 g), which is appreciated to have antioxidant strengths and furthermore, it is necessary for supporting normal mucus membranes and skin as well (Martin, 1996).

Quercetin denotes such flavonoid that limits oxidant injury and cell destruction through eliminating oxygen radicals, guarding against the oxidative degradation of lipids (Lim et al., 2003). Furthermore, it eliminates the radical reactions, activates metal ions transport, to form inactive complexes that cannot lead to the conversion of superoxide radicals and hydrogen peroxide into hydroxyl radicals. Investigations of the local employment of composites possessing free radical elimination characteristics at patients have been proven to promote healing of wounds and defend them from oxidative injuries (Phan et al., 2003).

Wound plague is a major circumstance that impedes or restrains wound healing. Wound healing requires a good healthy situation so that the standard physiological process will result in a regular healing process besides insignificant scar development. One of the extremely well-known procedures to keep the process of healing ongoing is to disinfect spoiled tissue from unspecified microbial infection (Nunan et al., 2014). Honey has various effects, as it resists bacteria, resists free radicals, ceases inflammation, prevents tumors, and different metabolic effects. Concerning antibacterial action, interference with bacterial growth has been shown using fertilized honey dishes or combining honey within agar plates (Oladejo et al., 2003). How much of that impediment is due to honey's antimicrobial characteristics or through its acidity and hyperosmolar nature is not well settled. In this concern, the hyperosmolar sugar paste also has antibacterial activity and is better than antiseptics (Iftikhar et al., 2010).

Several studies investigate that honey produces useful outcomes on wound healing in addition to its antibacterial capacities. The role of honey in the enhancement of wound healing is relevant to its physical characteristics as it is hygroscopic, has low PH and due to the presence of various chemical compounds (Mphande et al., 2007). The chief effects of LILT on photoreceptors are not yet definitely settled, although there are amazing suggestions. Following the penetration of light to the tissue, cytochrome c oxidase becomes excited and creates electron shift in the respiratory chain. A different opinion prevails that a portion of the excited ener-

gy is turned into heat, creating a limited and temporary heat in photoreceptors (Byrnes *et al.*, 2004).

Another opinion assumed that facilitating the stream of particles in the respiratory chain through light leads to an improvement in the generation of superoxide anions. A fourth hypothesis implied that porphyrins and flavoproteins consume photons and produce reactive classes of oxygen (Reddy, 2003). On the other hand some investigators assume that LILT results in physiological consequences such as changes in cell membrane permeability beside modifications in cellular calcium, enhancement of cellular metabolism (Medrado *et al.*, 2003), new synthesis of both DNA and RNA, fibroblast reproduction, improvement of T lymphocytes activity, activation of macrophages and mast cells, expansion of secretion of endorphins and decrease the production of bradykinin (Beckmann *et al.*, 2014).

CONCLUSION

Topical application of quercetin and Honey as well as low intensity laser therapy (LILT) have auxiliary influence in hastening of wound healing process in rabbits with favor of the effect of quercetin, honey and LILT respectively, so all of these modalities can be added to the treatment protocols for open wounds and burn.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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