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Comparative Study between Effect of *Punica granatum* L. Peel Aqueous Extract and Effect of Honey on Healing of Infected Wound

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Keywords:
Pomegranate Peel Extract, Excisional Infected Wound, Wound healing, Rabbit. Honey, Agar Diffusion Method, Antimicrobial Efficacy.

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ABSTRACT

The present study reports the potential effect of *Punica granatum* L. peels aqueous extract on the in vivo wound healing. A 10 % extract based-ointment was formulated and evaluated for its wound healing in rabbit comparing with topical use of honey and penicillin-streptomycin antibiotic. The ointment was applied in vivo on the paravertebral area of induced excisional wounds once a day for 10 consecutive days. The ointment significantly enhanced the wound contraction and the period of epithelialization as assessed by daily measurement of wound diameter and the histopathological characteristics. Such investigation was encouraged by the efficiency of the pomegranate peel extract as antimicrobial. Indeed, the extract exhibited significant antibacterial activity against isolated bacteria from induced contaminated and infected wounds (*Staphylococcus aureus*). The formulated ointment might well find use as skin repair agent without hazard to health based on these results and on the fact that it has been well established that the extracts of pomegranate used in conditions similar to those applied by traditional medicine, showed no toxic effects.

دراسة مقارنة بين تأثير المستخلص المائي لقشور الرمان وتأثير العسل على شفاء الجروح الخمجة

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الكلمات المفتاحية :
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الملخص

سجلت الدراسة الحالية تأثير فعال للمستخلص المائي لقشور الرمان على شفاء الجروح. حيث تم تحضير مرهم بتركيز 10% من المستخلص المذكور وقيم تأثير هذا المرهم على شفاء الجروح في الأرانب مقارنتا مع الاستعمال الموقعي للعسل ومع المضاد الحيوي البنسلين– ستريبتومايسين واسع الطيف. استعمل هذا المرهم بوضعه على الجروح الخزعية المستحدثة في المنطقة الظهرية وذلك بالاستعمال اليومي ولمدة 10 أيام متتالية. حيث عجل وبشكل ملحوظ عملية تقلص الجروح ومرحلة اعادة تكوين الخلايا الطلائية وكان ذلك التقييم من خلال القياس اليومي لقطر الجرح والوصف النسجي المرضي.

أن فعالية مستخلص قشور الرمان كمضاد للجراثيم هي التي شجعت على أجراء هذه الدراسة. وبالفعل فأن المستخلص قد أظهر تأثير فعال ضد الجراثيم (*Staphylococcus aureus*) المعزولة من الجروح الملوثة والخمجة المستحدثة.

ان من الممكن استخدام هذا المرهم لاصلاح الجلد وبدون اي مخاطر صحية وذلك استنادا لنتائج البحوث السابقة التي اكدت على ان مستخلص الرمان قد استخدم في حالات مماثلة في الطب التقليدي وبدون اي تأثير سمي.

Introduction :

Wounds are physical injuries that result in an opening or breaking of the skin. Wound healing is a complex process that results in the contraction and closure of the wound and restoration of a functional barrier. Cutaneous wound repair is accompanied by an ordered and definable sequence of biological events starting with wounding and progressing to the repair and remodeling of damaged tissue (Bele *et al*., 2010).

Wounds in animals are a common and frequent reason for seeking veterinary attention. The way in which wounds are managed affect the rate of healing, the time to return to normal function, the final cosmetic appearance, and hence the satisfaction of customers. The management of wound is differ by the use of antiseptics and antimicrobials, adherent and non-adherent dressings, and miscellaneous topical applications (Liptak,1997).

Wound healing is a complex process involving many physiological events. Immunological resources are recruited to fight infection and debride damaged tissue. Blood supply in the healing area is reestablished (angiogenesis), Regeneration of tissue (cell proliferation, fibrolplasia) follows, replacing damaged or destroyed tissue. The area to be healed is decreased via wound contraction. % closure of the wound is achieved through epithelial cell migration .Finally, remodeling of scar tissue occurs to approximate prior appearance and function." A safe treatment should promote, or at least not impair, this process (Burks, 1998).

The presesent proplem of multiple drug resistance to pathogenic organisms has intiated a search for new antimicrobial substances from plant cource. The pomegrante tree has rich history of traditional use in medicine. Several studies have reported the efficacy of various extracts or pure compounds from the different parts of pomegranate plant against the growth of Grame posative and Grame nagative bacteria (Jayaprakasha *et al*., 2006).

Recently, the traditional use of plants for wound healing has received attention by the scientific community It attracted attention due to its apparent anti-bacterial activity (Navarro, *et al*.,1996), wound-healing properties (Chidambara *et al*., 2004) , anti-inflammatory (Rajan, *et al*., 2011) and anti-oxidative capacities (Tzulker, *et al.*, 2007). Hence, we intend to find an alternative, safe, cheap and acceptable agent to enhance the healing of contaminated and infected wound in animals. Considering above facts, the wound healing acceleration by application of topical aqueous extract of *Punica granatum* peel has been investigated on excisional wound in local bread rabbit.

Materials and Methods :

Experimental Animals:

Local bread male rabbets were used. The animals were housed in standard room temperature ($22 \pm 3C^{\circ}$). During experimental time, rabbets were given same diet.

Inducing of the Wound:

The dorsal skin of the rabbits was shaved. Animals were anesthetized with 35 mg/kg, of Ketamin and 5mg Xylazine. A full thickness of the excision wound (circular area about 120 mm±5 mm in diameter)(figure1) was created along the marking area using toothed forceps, a surgical blade and scissors (Chidambara, .,2004; Pirbalouti *et al*., 2010).

Experimental Design:

Twelve adult male rabbets were divided randomly into three groups of four animals each. Group I, represented control was treated by topical application of penicillin-streptomycin antibiotic, group II treated topically by honey, and group III was treated with ointment prepared from *Punica granatum* peel aqueous extract, each groups that treated daily for 10 days. During the wound healing period and at the presented time intervals, the wound area was traced manually and photographed. The wound area was measured using ruler daily to the 12^{th} day of the experiment was terminated and the wound area was removed from the surviving animals for histological examination.

The percentage of wound closure was calculated as follows using the initial and final measurement diameter of excised circle wound area during the experiments: % of wound closure = (wound area on day 0 × wound area on day n)/wound area on day 0 ×100 where n is a number of days (3th, 6th, 9th and 12th day) (Pirbalouti□*et al*.,2010; Kamath et al.,2006;Asif *et al*., 2007; Wall *et al*., 2002). Also analyzed the daily measurement data statistically.

Pathological Study:

Macroscopic and microscopic examinations of the wounds were performed on all rabbets at twelve days after treatment. Four rabbets used for each group.

Rabbets were anesthetized, and then the wounds were examined by naked eye and photos were taken. Longitudinal full thickness Specimens were harvested containing the contracted wounds.

The specimen was trimmed to a suitable size to, which was fixed in 10% neutral buffered formalin for 48 hours, then washed, dehydrated in a serial graduated alcohol, cleared in xylol, embedded in paraffin wax, sectioned at 5-6 microns thickness and stained with Hematoxylin-Eosin (H and E) stain, and examined under light microscope (Prophet *et al*., 1992).

Pomegranate Peel Extracts Preparation:

Dried Pomegranate peels were ground to powder. The powder was macerated with water and then poured into a tall container. Water is added until the powder is completely immersed. It is allowed to stand for 24 hours. The crude extract was filtered through muslin followed by Whatman No. 1 filter paper to remove the peel particles (Handa *et al.,* 2008). The Extract was then changed into powder by lyophilization set. This set lowers the solution temperature until it freezes and changes into a solid porous mass. The mass was separated from flask by a glassy mixer (Eslami *et al*., 2011).

Agar well diffusion method for pomegranate peel extract, honey and penicillin-streptomycin:

After isolation of bacteria (*Staphylococcus aureus*) from contaminated wound, the nutrient Agar medium was prepare and cultures of isolated and test strains of S. aureus were inoculated and evenly spread on the surface of the agar by sterile swab to get uniform lawn culture of the organism. For agar well diffusion method, a well was prepared in the plates with the help of a cup-borer (0.85cm). Into the well, 100 µl of the test compound (pomegranate peel extract, honey and penicillin-streptomycin) was introduced. The Petri dishes were incubated at 37C for 24 h, the zone of inhibition was observed and the results were recorded. Microbial growth was determined by measuring the diameter of the zone of inhibition in mm for each compound (Norrel and Messley, 1997; Raju *et al*., 2011)

Statistical Analysis:

The results were expressed as Means and Standard Errors (M±SE). The data were analysis by one way Analysis of Variance (ANOVA). Differences between the mean treatment of each group, and at each time analyzed, using least significant differences (LSD).The P value <0.05 or 5% was considered statistically significant (Snedecor and Cochran, 1973).

Results and Discussion :

Clinical Observations:

Initially the cardinal signs of inflammation (i.e. swelling, heat, pain, redness and loss) were seen locally at the site of wound at some hours post induced wounding. It was more obvious and increased after 12 hours, due to increase the bacterial colonization. These signs were gradually subsided after treatment in all groups except one case of wound necrosis in penicillin-streptomycin group).

Macroscopic Evaluation:

After topical treatment of induced wound photos were taken for all animals in each group with measurement the area of wounds from 1st to 12th day after treatment by penicillin-streptomycin, honey and pomegranate (figures**3**, **4** and **5** respectively) to show and evaluate the rate of wound sealing and contraction, which recorded in table (1).

Information in table (1) refers to the drugs were applied to the animals for 12 days. The mean closure of wound area was calculated on 3 th, 6th. 9th, 12th day post wounding. On day "0" the area of wound in mm. the area of wound in mm was 120±0.00, in all the groups.

The mean area of wound in pomegranate group (Group III) on 3rd Day was 45±0.50 mm showed significantly reduced ($p < 0.05$) wound area with respect to the Penicillin-streptomycin74±0.30 group (Group I), but there is no significantly wound reduced between the Group I and Honey group (Group II) 56±0.60.

The wound area of Group III at 6th. 9th, 12th day was 29±0.60mm, 16±0.30mm and 00.0±0.00mm respectively represent a significant differences $P<0.05$ with respect to the wound areas of Group II at the same days (6th. 9th, 12th day) which were 47±0.40, 31±0.20 and 13±0.30 respectively, also to the Group I at the 6th. 9th, 12th day with value of 70±0.60, 53±0.40 and 25±0.40 respectively.

According to the equation that use in study by Kamath *et al*.,(2006) to calculated Wound contraction as a percentage changes in the initial wound size i.e.

$$WC\ (\%) = \frac{\text{Initial wound size-specific wound size x100}}{\text{Initial wound size}}$$

The pomegranate group shows the higher percentage of wound contraction (100%) comparing with honey group (89%) and control group (79%).

Study by Aslama *et al*., (2006). Indicated the Estimated the content of hydroxyproline in the granulation tissue revealed that the animals treated with hydro alcoholic extract of *Punica granatum* peel extract had high hydroxyproline content i.e. 2009.67± 48.09 ($p<0.01$), 2103.33 ± 81.894($p<0.001$) in comparison to the control group which showed hydroxyproline contents of 700.30 ± 31.33.

On the other hands, Brennan, *et. al*., (2003) a water-soluble extract made from pomegranate peel was found to inhibit elaboration of the matrix metalloproteinase-1 enzyme (MMP-1) responsible for collagen destruction in aged/photo aged skin.

A study by Hayouni EA *et al*.,(2011) reports for the *in vivo* wound healing potential of pomegranate peels. A 5% (w / w) methanolic extract-based ointment was formulated and evaluated for its wound healing in guinea pigs. The ointment significantly increased wound contraction and duration of epithelialization, as determined by the mechanical (contraction rate, tensile strength), biochemical (increasing of collagen, DNA and proteins synthesis), and histopathological characteristics.

Agar Diffusion Test :

The agar diffusion test results shows inhibition zone of 60mm,40mm, 35mm to the Punica granatum, honey, and pinecillin-streptomycin respectively for Staphylococcus aureus (fig 5, fig 6, fig 7).

In study by Bele, *et. al*., (2010) the extracts showed antibacterial activity against gram positive and gram negative microorganism such as *Escherichia coli, Klebsiella pneumoniae, Pseudomona saeruginosa, Proteus valgaris, Enterobacter aeruginosa* and gram positive microorganism *Staphylococus aureus, Bacillus subtilis.* MIC of *Punica granatum* peel ranged from 0.05mg/ml to

3.2mg/ml and was compared against marketed preparation. Formulation 5% showed maximum zone of inhibition ranging from 20.2mm to 26mm.

According to study by khan and Hanee,(2010) the hot aqueous, methanolic and ethanolic extracts of *Punica granatum* showed Zone of inhibition of at least 22mm against *P.aeruginosa* which was greater than that of Tetracycline 21 , 21mm against *E.coli* which was a little lesser than that of Standard (25mm) and 22.5mm against *E.coli* which was greater than that of standard Tetracycline (19.5mm) respectively.

Braga. *et. al*., (2005). evaluated the interaction between Punica granatum (pomegranate) methanolic extract (PGME) and antibiotics against 30 clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-sensitive Staphylococcusaureus (MSSA). Susceptibility testing of the isolates to PGME and antibiotics was performed by the broth dilution method. Synergistic activity was detected between PGME and the five tested antibiotics i.e. chloramphenicol,gentamicin, ampicillin, tetracycline, and oxacillin.

The antibacterial activity of peels of *Punica granatum* may be indicative of broad spectrum antimicrobial compounds that act against both gram +ve and gram -ve bacteria.

Microscopic Evaluation:

Penicillin-Streptomycin Group:

The lesion showed presence of cellular loose connective tissue which formed from collagen fibers extended in different orientations, in addition various fibroblasts (spindle or satellite)(fig.10), with infiltration of large number of inflammatory cells, presence of blood capillaries and the wound still exhibited incomplete re-epithelialization (fig.8). Hyalinized and thickened collagen bundles with multidirectional growth represent abnormal collagen fibers (fig.9).

Honey Group:

The main feature at this period reflected the presence of dense irregular collagen fibers with scattered fibroblasts cells and few inflammatory cells (fig.13). These are complete re-epithelialization (fig.11, fig.12).

Pomegranate Group:

The pathognomonic feature at this group is the complete replacement of injured dermis with mature collagen fibers (regular orientation) and the presence of extracellular matrix between collagen fibers with few blood capillaries (fig. 14). Section revealed band of collagen fibers arranged in a wave-like fashion with few scattered fibroblasts (fig. 15) which considered as an indicative for organized healing.

In the present study and in the penicillin-streptomycin group, the wound gap filled with highly cellular loss connective tissue and the wound still exhibited obvious inflammation and incomplete re-epithelialization.

Some sections in this group revealed abnormal hyalinized and thickened collagen bundles as well as their multidirectional growth.

Despite the fact that collagen synthesis continues at a higher rate, no further increase in scar mass occurs. At this point new collagen is created and old collagen is broken down in a balance fashion with the aid of an enzyme collagenase (Nwomeh, *et .al.*, 1999).

Histological picture showed the presence of huge number of neutrophils and this finding agree with a study of (Al-salamah, *et. al.*, 2006) and disagree with Anderson and Miller (1984), who said that most wound heal without infection because of the microbiocidal capacity of the macrophage population.

The collagen deposition at the 12^{th} day still remained in irregular direction in honey group and this give an indication of immature connective tissue formation, this result was inaccordence with (Rahban and Garner, 2003), who stated that collagen turnover allows the randomly deposited scar

tissue to be arranged, in both linear and lateral orientation, while in pomegranate group, collagen fibers impacted and assumed linear direction and appears as a wave-like fashion with complete re-epithelialization, this means there is significant wound healing . This process continues until the remodeling phase end at months post injury.

The microscopic findings in the present study resembled the normal repair of incised wound which is divided into three stages that mentioned by several authors (Falanga, 1993; Nedelec, *et. al.*, 2000; Hart, 2002). The first stage, is a period of cellular activity include autolysis, phogocytosis and accelerated production of mucopolysaccharid, the second stage, fibroblastic stage, characterized by formation of granulation tissue, collagen production, and stage three differentiations which include maturation of collagen fibers and scar tissue formation.

In conclusion, the results obtained from this study reflected accelerated healing of the induced excision wound by applying ointment of *Punica granatum* peel extract which was highlighted by the full thickness coverage of the wound area by an organized epidermis and re-epithelialization. The enhanced capacity of wound healing could be explained on the basis of its tannin and astringent effects that are documented in the literature.

Table (1). Effect of topical application of penicillin, honey and pomegranate extract on excision wound contraction.

Days	Penicillin-streptomycin group	Honey group	Pomegranate group
0 day	120±0.00	120±0.00	120±0.00
3rd day	74±0.30	56±0.60*	45±0.50
6th day	70±0.60	47±0.40*	29±0.60**
9th day	53±0.40	31±0.20*	16±0.30**
12th day	25±0.40	13±0.30*	00.0±0.00**

Values are expressed as mean ± SE; N = 4 animals in each group; Test used: ANOVA, * **refer to a significant differences P<0.05**, between the control and honey group, ** **refer to a significant differences P<0.05**, between the honey group and pomegranate group.

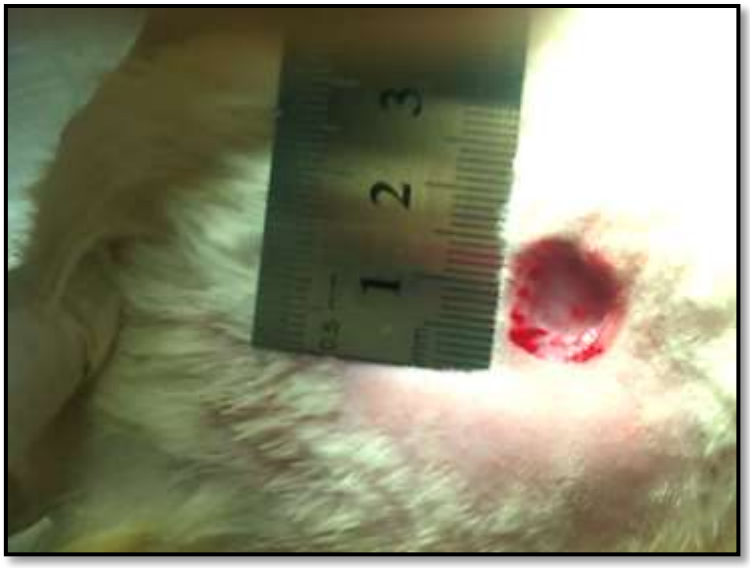

Fig.1- The induced excisional skin wound.

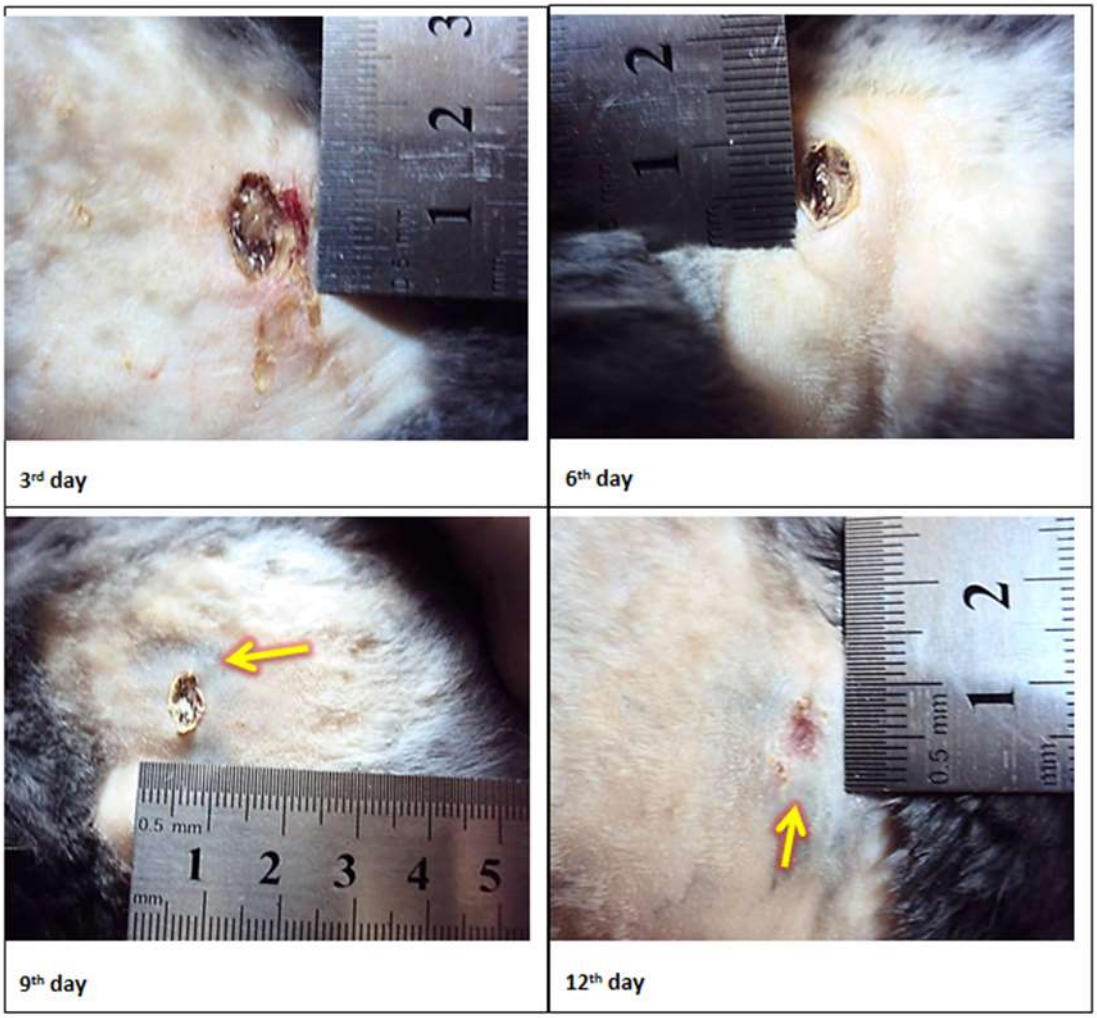

Fig.2–Macroscopic feature of Penicillin-Streptomycin Group, 3rd, 6th, 9th and 12th day post treatment reveal's, the measurement of wound area and show incomplete wound closure with blow area of necrosis surrounded the wound (arrows).

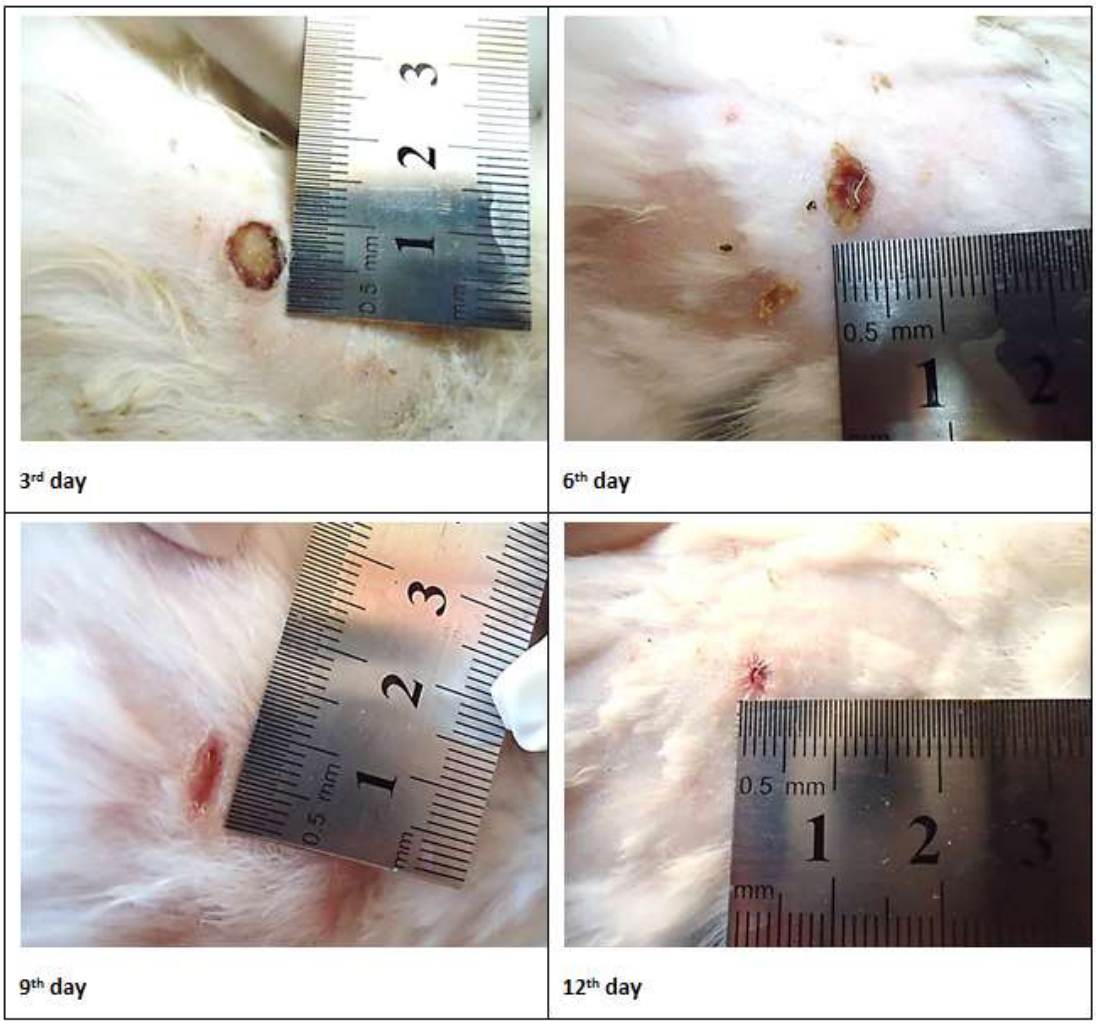

Fig.3–Macroscopic feature of honey Group, 3rd, 6th, 9th and 12th day post treatment reveal's, the measurement of wound area and show incomplete wound closure at 12th day with significant wound contraction.

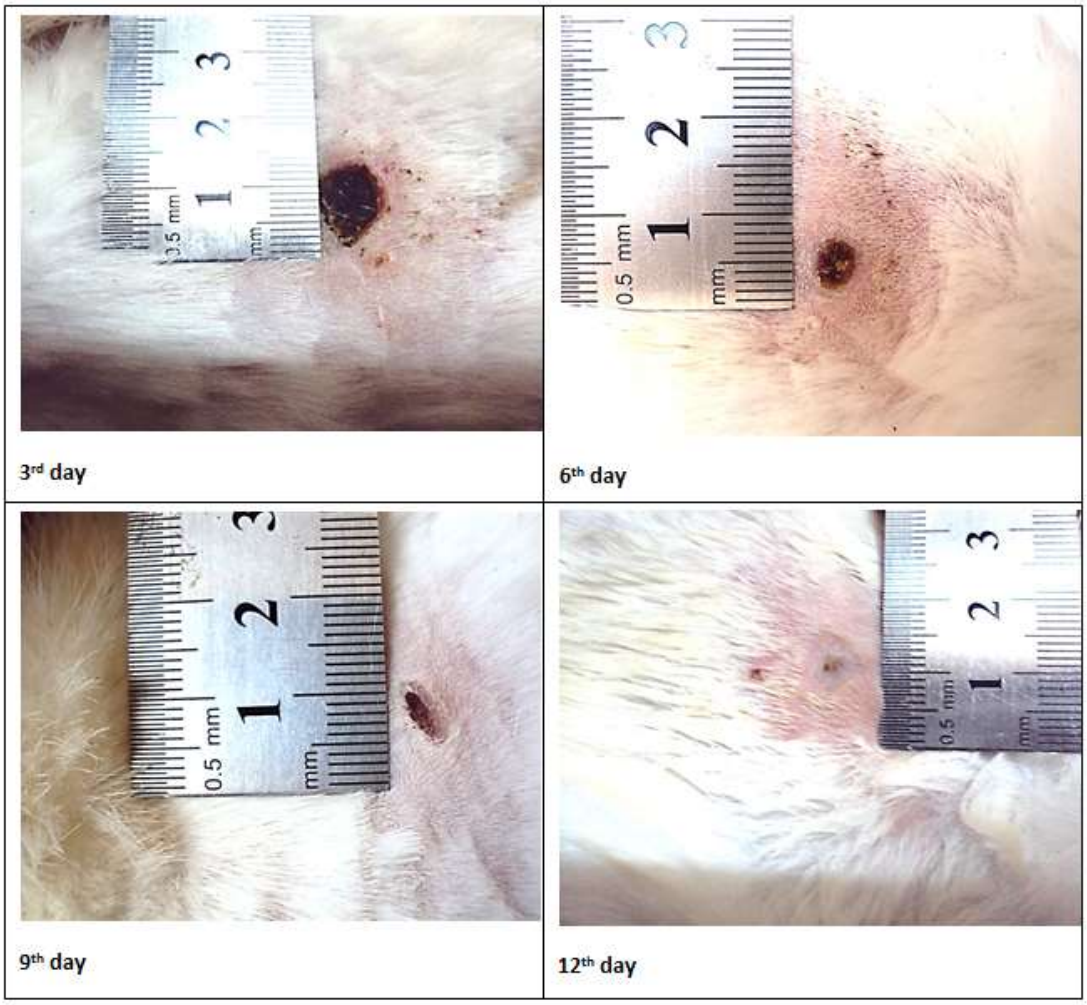

Fig.4 –Macroscopic feature of pomegranate Group, 3rd, 6th, 9th and 12th day post treatment reveal's, the measurement of wound area and show complete wound closure at 12th day.

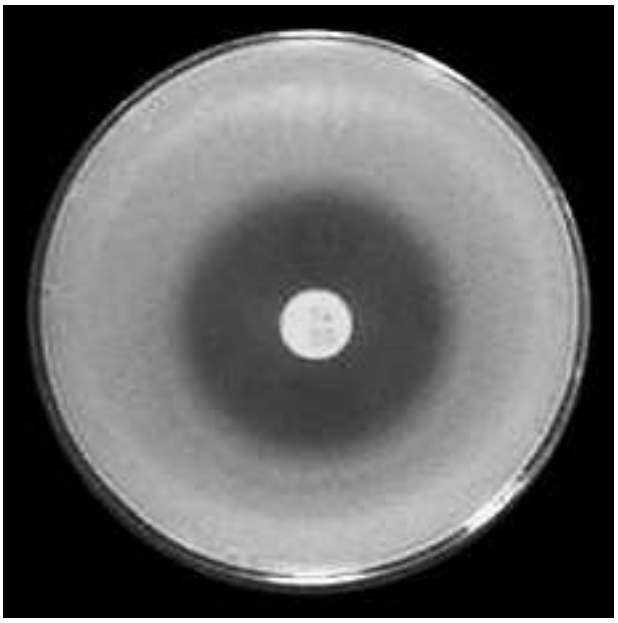

Fig.5– *Staphylococcus aureus* zone of inhibition to penicillin-streptomycin (35 mm)

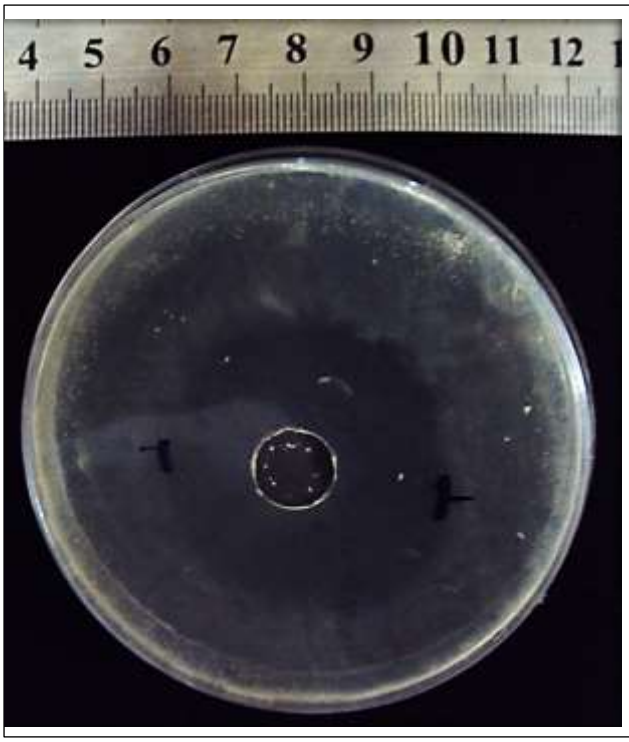

Fig.6– *Staphylococcus aureus* zone of inhibition to honey (40 mm).

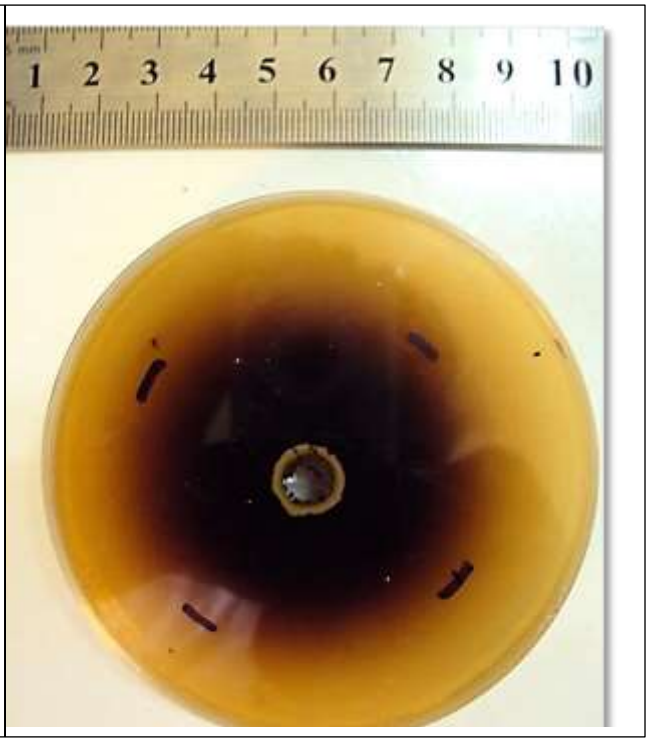

Fig.7– *Staphylococcus aureus* zone of inhibition to pomegranate (55 mm).

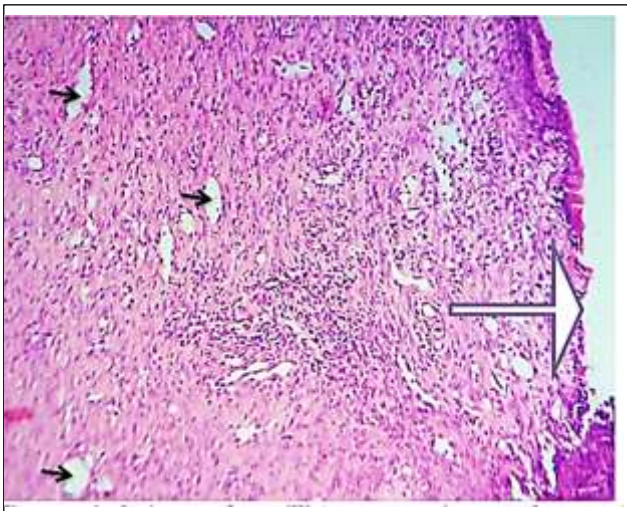

Fig. 8 - Microscopical picture of pencillin-streptomycin control group, 12days post wounding, show's complete infilling of excised wound with proliferation of cellular loose connective tissue, infiltrated by large number of inflammatory cells and blood capillaries (black arrows) wound still exhibited incomplete re-epithelialization

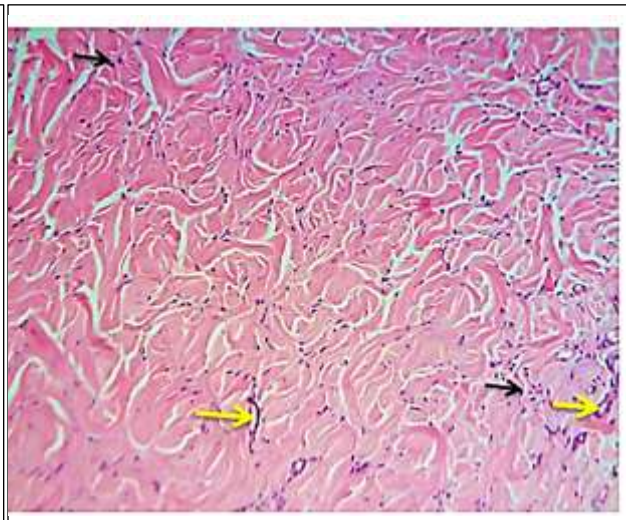

Fig. 9- Microscopical picture of pencillin-streptomycin control group, 12days post wounding, show's infilling of excised wound with hyalinized and thickened collagen bundles as well as their multidirectional growth (immature collagen fibers) infiltrate with inflammatory cells (black arrows) and

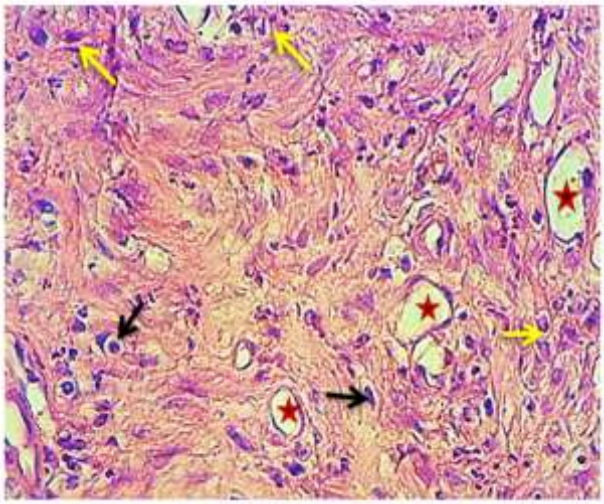

Fig. 10 - Microscopical picture of penicillin-streptomycin control group, 12days post wounding, show's infilling of excised wound with proliferation of cellular loose connective tissue infiltrated by large number of inflammatory cells (white arrow) and blood capillaries (stares) with fibroblasts (black arrows) (H&E 40X).

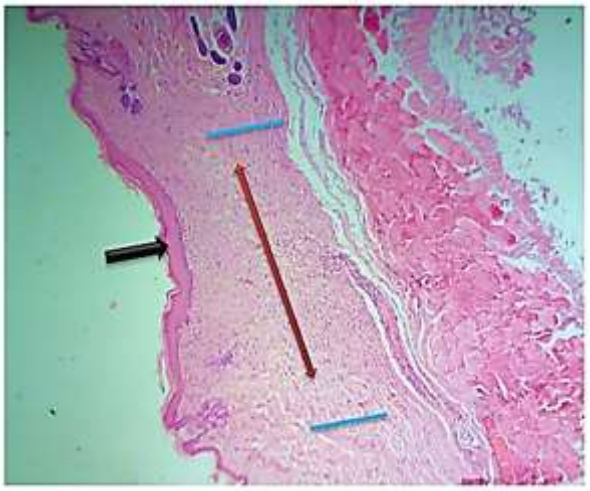

Fig.11 - Microscopical picture of honey group, 12days post wounding, show's normal skin (lines), infilling of excised wound with denser collagen deposition in dermis (dabble head arrow) infiltrated by large number of fibroblasts and inflammatory cells with complete re-epithelialization (thick arrow) (H&E 4X).

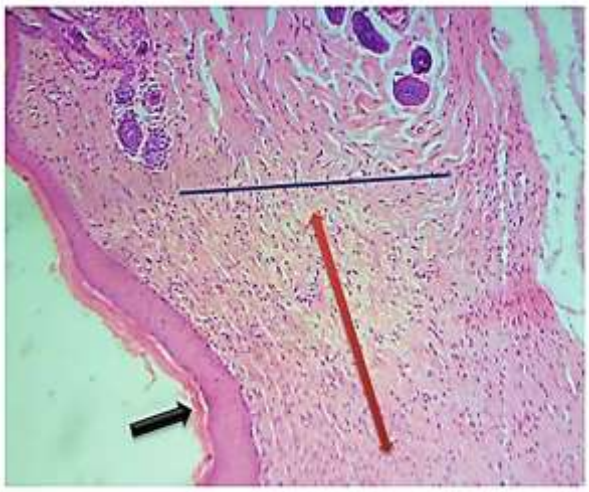

Fig.12 - Microscopical picture of honey group, 12days post wounding, show's normal skin (line), infilling of excised wound with denser collagen deposition in dermis (dabble head arrows), infiltrated by large number of fibroblasts and inflammatory cells and blood capillaries with complete re-epithelialization (thick arrow) (H&E 10X).

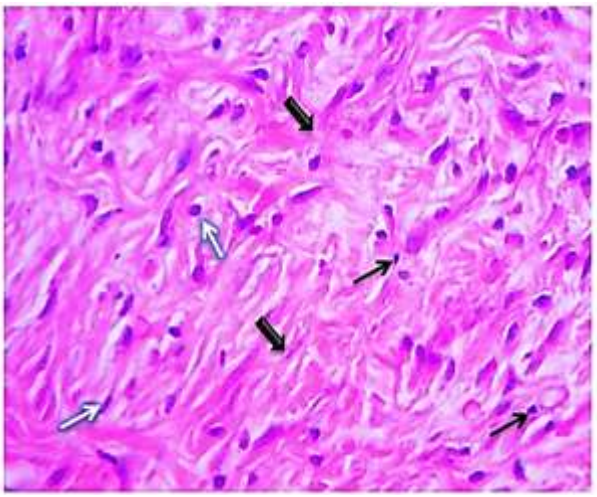

Fig.13 - Microscopical picture of honey group, 12days post wounding, show's infilling of excised wound with dense irregular collagen fibers (thick arrows) with scattered fibroblasts cells (white arrow) and few inflammatory cells (thin black arrows) (H&E 40X).

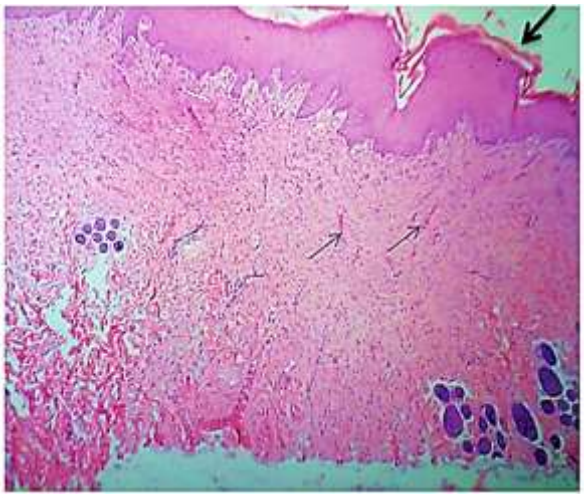

Fig. 14- Microscopical picture of pomegranate treated group, 12days post wounding, show's complete infilling of excised wound with denser collagen deposition in dermis with small blood capillaries (thin arrows), and re-epithelialization was complete (thick arrow) (H&E 10X).

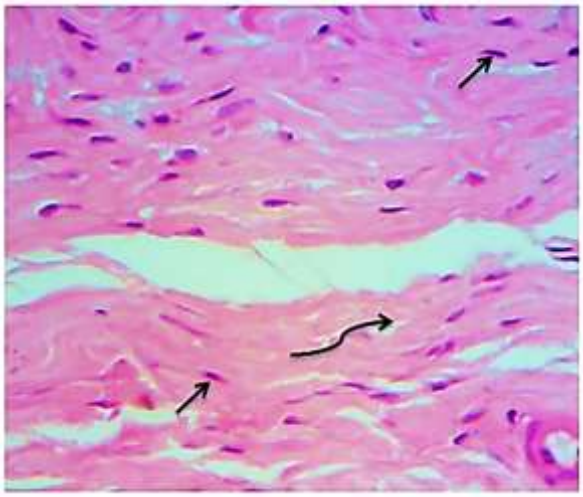

Fig. 15- Microscopical picture of pomegranate treated group, 12days post wounding, show's wave-like fashion collagen fibers (carved arrow) with scattered fibroblasts (short arrows) (H&E stain 40X).

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