

Effect of *Punica granatum* rinds ethanolic extract on healing of fungated wounds in rabbits

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Abstract

Pomegranate (*Punica granatum*) is an ancient fruit used in various systems of medicine for a variety of ailments. The study has been designed to investigate the role of ethanolic extract of rinds of *Punica granatum* as antimicrobial on 6 types of bacteria includes; *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staph aureus*, *E.coli*, *Streptococcus spp.* and *Klebsiella pneumonia* and antifungal activity on 4 types of fungi includes; *Candida albicans*, *Aspergillus niger*, *Aspergillus fumigates* and *Aspergillus valvas*. Also the study build to assessment the ability of rinds ethanolic extract of *Punica granatum* to reduced the oxidative intention as a result of fungal infection and to accelerate the wound healing in normal and wound model infected with fungi using local rabbits as experimental animals. To achieve this goal 30 adults rabbits were used and divided into three groups, one as control group and the others as treated groups. Two wounds models via incision were make and infected with fungi. The first infected group was treated with ethanolic extract of *Punica granatum* and the second group treated with 0.5 g ointment of ethanolic extract of *Punica granatum*. The results of antimicrobial assessment exhibited significant antibacterial and antifungal activity against almost all tested bacteria and fungi, and the used of rinds ethanolic extract of *Punica granatum* for each treated groups (300 mg/kg and 500 mg/kg) also showed significant ($P<0.05$) improvement in percentage of wounds contraction and healing. In addition the oxidative stress was assessed by estimating the serum levels of antioxidant enzymes: malondialdehyde (MDA), catalase (CAT), glutathione (GSH), and SOD. Also the results shows that the treatment with ethanolic extract of rind of *Punica granatum* markedly prevented oxidative stress by increasing of catalase (CAT), glutathione (GSH) and decreasing the level of MDA and SOD. In conclusion the study revealed that use of rinds ethanolic extract of *Punica granatum* as a lotion and ointment improve the wounds healing properties.

Key words: *Punica granatum* rinds, ethanolic extract, wound healing, fungated wounds, rabbits

تأثير المستخلص الايثانولي لقشور نبات الرمان *Punica granatum* على شفاء الجروح المصابة بالفطريات في الأرانب

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الخلاصة

أجريت الدراسة لمعرفة الفعالية التثبيطية للمستخلص الايثانولي لقشور نبات الرمان (*Punica granatum*) اتجاه 6 أنواع بكتيرية متمثلة *Pseudomonas pycoaneous* و *Salmonella typhi* و *Staph.aureus* و *E. coli* و *Streptococcus spp.* و *Klebsiella pneumonia* فضلا عن خمائر مثل *Candida albicans* وفطريات مثل *Aspergillus niger* و *Aspergillus fumigatus* و *Aspergillus valvas* وكذلك تهدف الدراسة إلى تقييم فعالية المستخلص الايثانولي لقشور الرمان في تقليل الإجهاد التأكسدي الناتج من الإصابة الفطرية وشفاء وإعادة بناء الجروح المستحدثة والمصابة بالفطريات في الأرانب المحلية. استخدم في هذه الدراسة 30 أرنباً بالغاً تم تقسيمها إلى ثلاث مجاميع احدها مجموعة سيطرة والأخيراتان مجاميع معالجة وتم احداث الجروح واصابتها بالفطريات وتم علاج إحدى المجموعتين

بسائل المستخلص الايثانولي والمجموعة الثانية بمرهم المستخلص الايثانولي. وأظهرت النتائج وجود تأثير تثبيطي معنوي بالنسبة للأحياء المجهرية كافة ، وان استخدام المستخلص الايثانولي لنبات *Punica granatum* لكل المجموعات المعالجة (300 ملغ/كغ و 500 ملغ/كغ) قد أظهرت وجود تحسن معنوي ($P<0.05$) في النسبة المئوية لسرعة تقلص الجروح المعالجة وشفاءها. وتم خلال فترة المعاملة قياس مستوى الإنزيمات المضادة للأكسدة MDA, TBARS, CAT, GSH, SOD وأظهرت الدراسة إمكانية المستخلص الايثانولي في تقليل الاجهاد التأكسدي من خلال زيادة CAT و GSH ونقصان MDA و SOD . وأخيراً كشفت نتائج هذه الدراسة أن استخدام المستخلص الايثانولي لنبات *Punica granatum* كمحلول او مرهم يعمل على تحسين خصائص شفاء الجروح. الكلمات المفتاحية: قشور نبات الرمان ، المستخلص الايثانولي ، الفعالية التثبيطية ، الجروح الفطرية ، الارانب.

Introduction

Many wounds that a veterinarian is asked to treat have been traumatized or have undergone some degree of tissue loss caused by microbial infection. Recently the traditional use of plants for wound healing has received attention by the scientific community (1, 2). Approximately one-third of all traditional medicines in use are for the treatment of wounds and skin disorders. In these instances, first intention wound healing is not possible. This wound can be treated, however, so they heal by second or third intention. The increased universal in medicinal plant drew the attention of the authors to investigate provoking effect of topical applications of *Punica granatum* (Romman) on second intention wound healing in rabbits. *P. granatum* is a tree plant of the family *Punicaceae*; it was locally named (Romman) and it had a known importance in Iraqi traditional medicine (3). Many studies revealed that the alcohol extract of the plant materials have antifungal activity against the fungus *Aspergillus niger* and some bacteria (4), many other fungi specially dermatophytes (5) and antiparasite (6). The extracts of traditional herbs have been shown to exert biological activity in vitro and in vivo. *Punica granatum* is employed in man medicine for the treatment of various diseases such as skin diseases, and wound healing, ulcers, fever, diarrhea, and microbial infection. In the recently years, the *Punica granatum* has been the subject of much scientific research which have showed its antimicrobial, antioxidant and anti-cancer effects (7, 8). Wound healing is a complex process characterized by homeostasis, re-epithelialization, and granulation tissue formation and remodeling of the extracellular matrix. Reports about medicinal plants

affecting various phases of wound healing process are abundant in scientific literature (9). Pomegranate is an ancient fruit used in various systems of medicine for a variety of ailments. Dried fruit peel (pericarp) is used for diarrhea and to treat respiratory infections. The fruit peel exerts diverse pharmacological functions such as antioxidant activity (10), cytotoxic activity (11), hypoglycemic activity (12), hepatoprotective activity (13) and anti-inflammatory activity (14). In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments. The synergistic action of the pomegranate constituents appears to be superior to that of single constituents. In the past decade, numerous studies on anticarcinogenic and anti-inflammatory properties of pomegranate constituents have been published, focusing on treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections and antibiotic resistance, and ultraviolet radiation-induced skin damage. Other potential applications include infant brain ischemia, male infertility, Alzheimer's disease, arthritis, and obesity. Pomegranate peel aqueous extract has shown potent dermal effect on cultures of human skin cells, stimulating dermal fibroblast proliferation and collagen synthesis while inhibiting major collagen degrading enzyme (15). Topical gel application of alcoholic extract of peel has shown beneficial healing effect in rat excision wound model (16). For that the study aim to know the antimicrobial activity of two forms of *P. granatum* peels (ointment and lotion forms), and their efficacy on wound healing.

Materials and methods

Plant materials:

The peels were brought from Basra market, then were air dried and well milled.

Preparation of plant extract:

Ten grams of dried milled peels were put in thimbles of soxhlet extractor and extracted separately by 250ml of 75% ethyl alcohol for 24h. The extracts were evaporated by rotary evaporator (Switzerland RM scientific LTD). This method was replicated three folds to obtain sufficient quantity from dried material extract (17). The dried residues were kept in tightly closed vials in deep freeze away from light until the time of use.

Microorganisms test:

Six types of pathogenic bacteria and four species of fungi were isolated and identified previously. To study the antimicrobial activity of alcoholic extract of *P.granatum*, Muller-Hinton agar medium was used for bacterial growth, plates were incubates at 37°C for 24-48hrs. For fungal growth; potato dextrose agar (PDA) medium was used of *Candida albicans* and sabroud dextrose agar (SDA) was used for *Aspergillus* species. Plates were incubated at 25°C for 3-5 days. In both cases the method of well contain extract in two concentrations (300 and 500 mg/ml) were used and the inhibition zones were measured by scale and compared with the control (18). They were directly transferred to nutrient broth bacteriology media using the usual microbiology methods.

Preparation of animals:

The work was undertaken on apparently healthy 30 rabbits (aged 6-7 months, and weighing 1.37-1.4 Kg) randomly divided into three groups ten animals of each. They were kept in cages, fed with commercial food, and water given ad libitum. Each group was treated with *Punica granatum*. The first group has clean wound (wound induced under clean and aseptic condition), treated with ethanolic extract of *Punica granatum* and consider as control group. Second group has infected wound with fungi, where treated with 0.5 g ointment of ethanolic extract of *Punica granatum* for 14th days. Third group

infected wound with fungi treated with 0.5 g lotion of ethanolic extract of *Punica granatum* for 14th days and as following; A-0-day was wounding, B-7th-day post wounding, C-14th-day post wounding. To induce full thickness skin incision, animals were anesthetized with I/M injection of xylazine 5mg/kg, and ketamine 40 mg/kg. The dorsal aspect of each animal was prepared for aseptic surgery by clipping, shaving and apply antiseptic. Two full thickness skins circular excisional wounds 1.5 cm in diameter were done with scalpel, one on the right side for treatment, and the other on the left side which left without treatment as a control in the same animal. The ointment was prepared by mixing the ethanolic extract of *Punica granatum* with Vaseline base at the ratio of 1:3 respectively. The lotion was prepared by mixing the ethanolic extract of *Punica granatum* with distilled water in the ratio of 1:3 respectively.

Macroscopic evaluation:

The wounds were clinically examined daily for color exudation, granulation tissue formation, vascularization and general appearance. Two swabs were collected from the depth of the wound of each animal under complete aseptic condition for microbial examination.

Contracting ability and the closure time:

The contracting ability can be defined as the ability of the wound to become narrower than the beginning volume; throughout the observation of the periphery around the wound. The closure time is the time where the infected-wound looks like normal appearance. The growing hair time is the time where the hairs can be seen on the epidermis. In order to determine the course of healing and scar size (percentage of wound contraction) the wound size were measured at the 0, 7th and 14th days of wounding and the percentage of wound contraction were estimated by the following equation.

$$\text{Percentage of wound Contraction} = \frac{\text{Initial day wound size} - \text{Specific day wound Size}}{\text{Initial day wound size}} \times 100$$

Biochemical Analysis

Blood samples were taken in order to measure the malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GSH-Px) levels, and catalase (CAT) enzyme activities. The values of MDA reactive material were expressed in terms of the malondialdehyde. Values were expressed as MDA equivalents in $\mu\text{mol/L}$ (19). GSH-Px activity was determined using cumene hydroperoxide and reduced GSH as co-substrates and the loss of GSH following enzymatic reaction was measured spectrophotometrically with Ellman's reagent at 37°C and 412 nm according to (20), of where the color developed was read at 412 nm. Values were expressed as GSH equivalents in $\mu\text{mol/L}$. Enzymatic determination of CAT in blood was performed according to the method of (21) in which the decomposition of H_2O_2 was followed spectrophotometrically at 240 nm. The difference in absorbance/unit time was a measure of CAT according method to (22). **Statistical analysis:** Data were expressed as Mean \pm SD and were analyzed by one way analysis of variance (ANOVA)(23).

Results

One week post wounding the animals of infected groups and control showed bright red healthy granulation tissue starting from the wound edges toward the center. There was very scanty granulation tissue of uneven surface and some wound showed purulent discharge. The microbial examination revealed that infected wound groups offered the least incidence of bacteria followed by control. The highest incidence of fungal infection such as *Aspergillus fumigatus*, *Aspergillus niger* and *Candida albicans* was seen in the infected groups. However, the wounds of infected groups were seen expanded and covered by white greenish or white yellowish layer of infection. By time the wounds were severely inflamed and became deeper and wider than the first week post wounding. The averages width was $4.23\text{-}8.48\text{ cm}^2$. Two weeks post wounding all animals of groups treated and control showed contracted wound with accelerated healing.

At the end of the third week some of the wounds became rough and covered by thick hard material (Table 1) (Fig.1), without any repairing of skin architecture or hair growing. Thus the closure time was not easy to determine in some wounds. However, the closure time was between 14 to 21 days.

Table 1: Effect of topical ethanolic extract of *Punica granatum* rind on wound contraction (%) in infected wound with fungi models in rabbits (Values are expressed as Mean \pm SD).

Groups	Weight of animals kg	Treatment	% of wound healing		
			0 day	7 day	14 day
1	1.38 \pm 0.52	Control	40 \pm 1.57	95 \pm 6.43	100 \pm 0.00
2	1.40 \pm 0.29	Infected wound treated with lotion	44 \pm 2.78	87 \pm 3.96	94 \pm 3.12
3	1.37 \pm 0.71	Infected wound treated with Ointment	46 \pm 5.73	89 \pm 4.87	98 \pm 1.26

Table 2: Mean of diameter of the inhibition zones induced by alcoholic extract of *Punica granatum* on microorganisms used in this study.

Microorganisms	Concentration of alcoholic extraction (mg/ml)	
	300	500
Bacteria	DIZ(mm)	DIZ(mm)
<i>Pseudomonas aeruginosa</i>	8.6 \pm 0.14	9 \pm 0.11
<i>Salmonella typhi</i>	12 \pm 0.37	15.5 \pm 1.32
<i>Staphylococcus aureus</i>	10 \pm 0.26	12 \pm 0.15
<i>Escherichia coli</i>	9.5 \pm 0.31	12.8 \pm 1.80
<i>Streptococcus spp.</i>	10.9 \pm 0.63	13 \pm 2.74
<i>Klebsiella pneumoniae</i>	9.5 \pm 0.47	11 \pm 0.12
Fungi		
<i>Candida albicans</i>	8.6 \pm 0.71	10.7 \pm 0.31
<i>Aspergillus niger</i>	9 \pm 0.13	11 \pm 0.22
<i>Aspergillus fumigatus</i>	8 \pm 0.19	10.5 \pm 0.29
<i>Aspergillus valvas</i>	9.4 \pm 0.21	11.9 \pm 0.15

DIZ = Diameter of inhibition zone measured in millimeter.

The results of antimicrobial activity test, of ethanolic rind extract, at different concentrations screened by the mean value of zones of inhibition were assessed in millimeter diameter. Ethanolic rind extract show activity against microbial such as: *Pseudomonas pycoaneous*, *Salmonella typhi*, *Staph. aureus*, *E. coli*, *Streptococcus spp.*,

Klebsiella pneumonia, *Candida albicans*, *Aspergillus fumigatus*, *Aspergillus niger* and *Aspergillus vlavus*. The ethanolic extract of rind of *Punica granatum* (L.) in two concentrations (300, and 500 mg/ml) show highest diameter of inhibition zone against *Salmonella typhi* (15.5±1.32 mm) (10.9±0.63mm), *Streptococcus spp.* (13±2.74mm) (10.9±0.63), *Escherichia coli* (12.8±1.80mm) (9.5±0.31mm) *Staphylococcus aureus* (12±0.15mm) (10±0.26mm), *Klebsiella pneumonia* (11±0.12mm) (9.5±0.47), *Pseudomonas aeruginosa* (9±0.11mm) (8.6±0.14mm). The ethanolic extract of rind of *Punica granatum* (L.) showed highest diameter of inhibition zone against *Aspergillus valvas*

(11.9±0.15mm) (9.4±0.21mm), *Aspergillus niger* (11±0.22mm) (9±0.13mm), *Candida albicans* (10.7±0.31mm) (8.6±0.71mm), *Aspergillus fumigates* (10.5±0.29mm) (8±0.19mm) (Table 2), (Fig. 2, 3). The oxidative stress was assessed by estimating serum malondialdehyde (MDA), thiobarbit - uric acid reactive substances (TBARS), catalase (CAT), glutathione (GSH). Rabbits exposed to infected wound with fungi produced marked increase in oxidative stress, which was assessed in terms of TBARS and SOD along with decrease in the level of catalase and glutathione. The ethanolic rind extract was strongly inhibiting the growth of bacteria and fungi and accelerate the healing of the infected wound with fungi (Table 3).

Table 3: Effect of ethanolic extract of *punica granatum* rind on activates of antioxidant enzymes in rabbits induced infected wound with fungi. (Values expressed as Mean ±SD).

Treatment Parameters	Control treated with ethanolic extract of <i>P. granatum</i>			Infected wound + treated with ethanolic extract of <i>P. granatum</i> (lotion)			Infected wound + treated with ethanolic extract of <i>P. granatum</i> (Ointment)		
	0day	7days	14days	0day	7days	14days	0day	7days	14days
MDA µmol/L	0.59 ±0.02	0.56 ±0.07	0.53 ±0.04	4.56 ±0.54a	2.86 ±0.21b	1.17 ±0.03c	4.56 ±0.54a	2.142 ±0.10b	1.08 ±0.01c
GSH µmol/L	8.34 ±0.32	9.51 ±0.62	9.95 ±0.52	3.82 ±0.54	6.73 ±0.21b	8.98 ±0.22a	3.82 ±0.54	6.73 ±0.21b	8.98 ±0.22a
SOD U/ml	8.25 ±0.32	8.93 ±0.20	9.02 ±0.11	2.96 ±0.62	7.28 ±0.42b	9.23 ±0.45a	2.67 ±0.83	7.67 ±0.46b	8.98 ±0.22a
CAT U/L	7.38 ±0.42	7.46 ±0.32	8.01 ±0.56	4.04 ±0.39	6.13 ±0.81b	7.13 ±0.68a	4.82 ±0.67	6.58 ±0.29b	7.23 ±0.08a

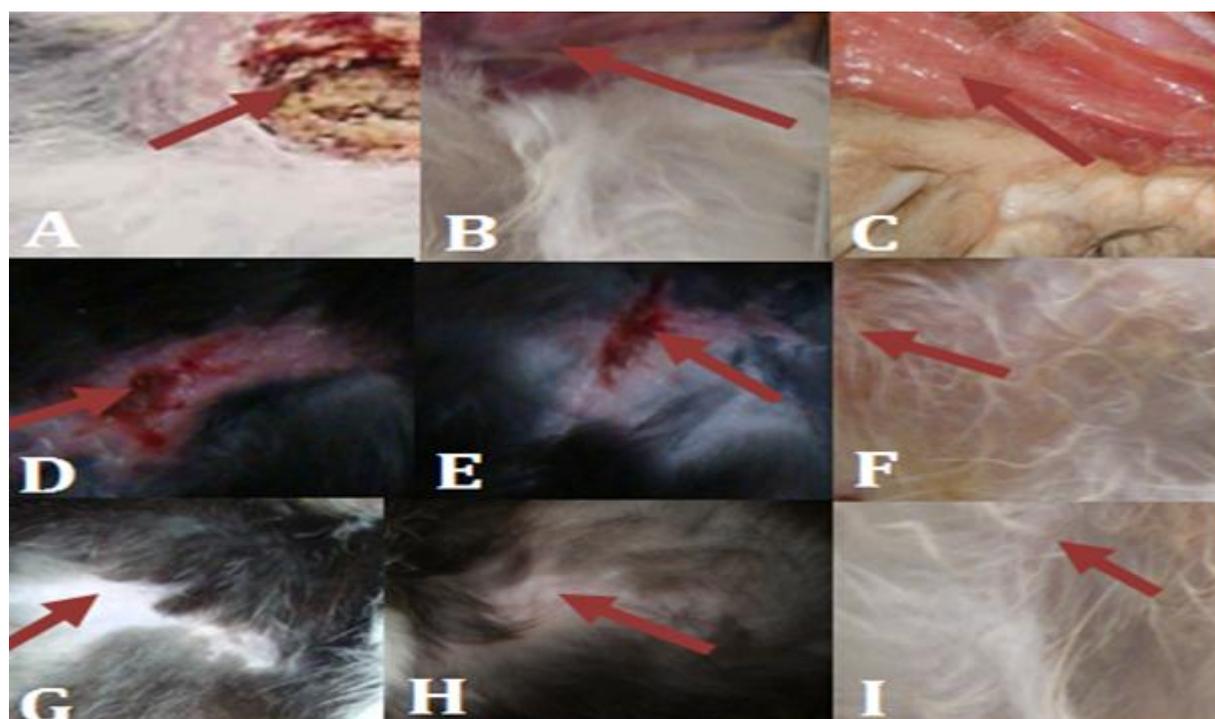


Fig. (1): Showing the induced infected wound with fungi in rabbits, before and after topical application of treatments with ointment and lotion of *Punica granatum*. A and B: Induced wound infected with fungi, C: Induced wound (control), D: Infected wound + Ointment of *Punica granatum*, E: Infected wound + Lotion of *Punica granatum*, F: Treated wound of control with ethanolic extract of *Punica granatum* after 7 days, G: Healing of infected wound after treated with ointment of *Punica granatum*, H: Healing of infected wound after treated with lotion of *Punica granatum*, I: Healing wound of control with treated ethanolic extract of *Punica granatum* after 14 days.

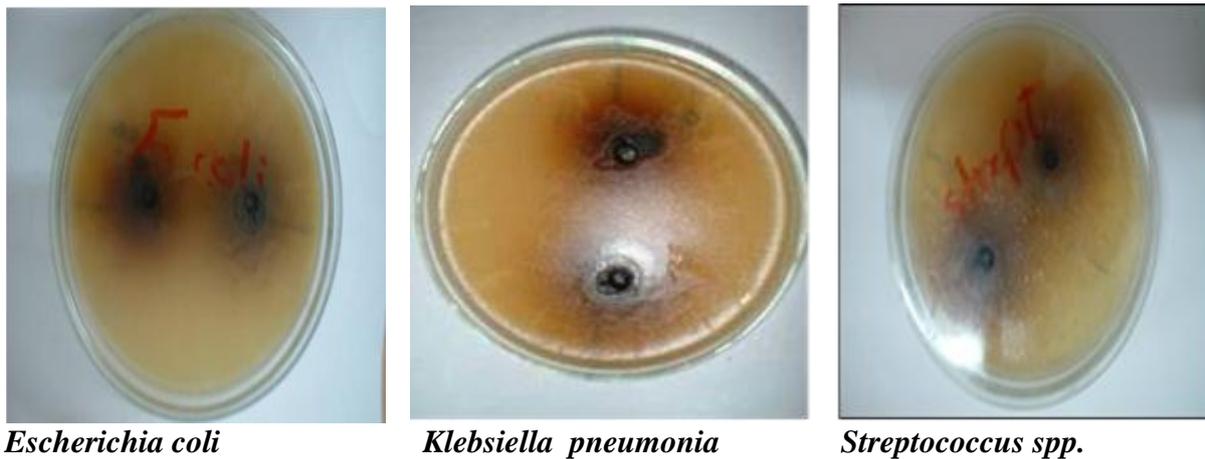
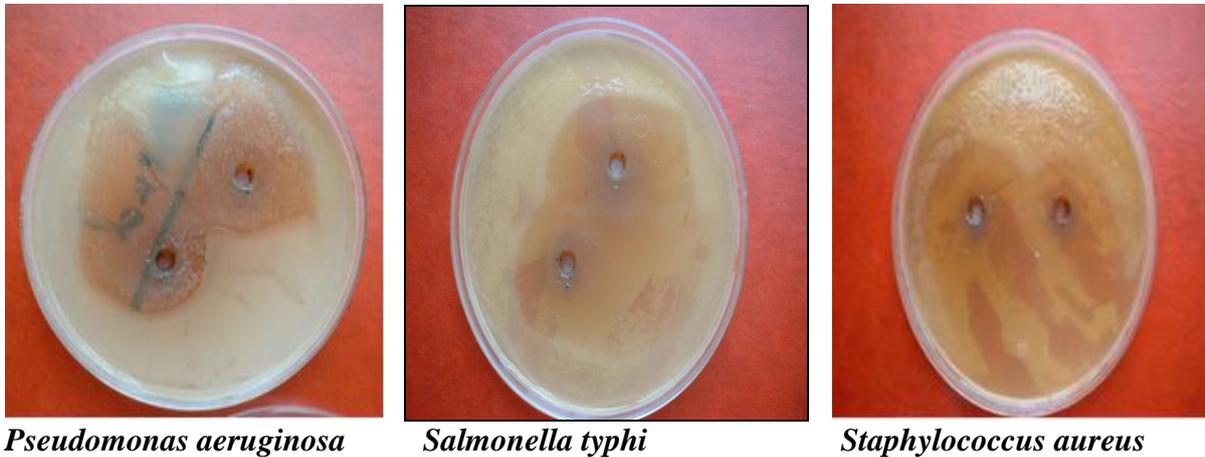


Fig. (2): Inhibition zones induced by alcoholic extract of *Punica granatum* rinds on some bacterial growth.

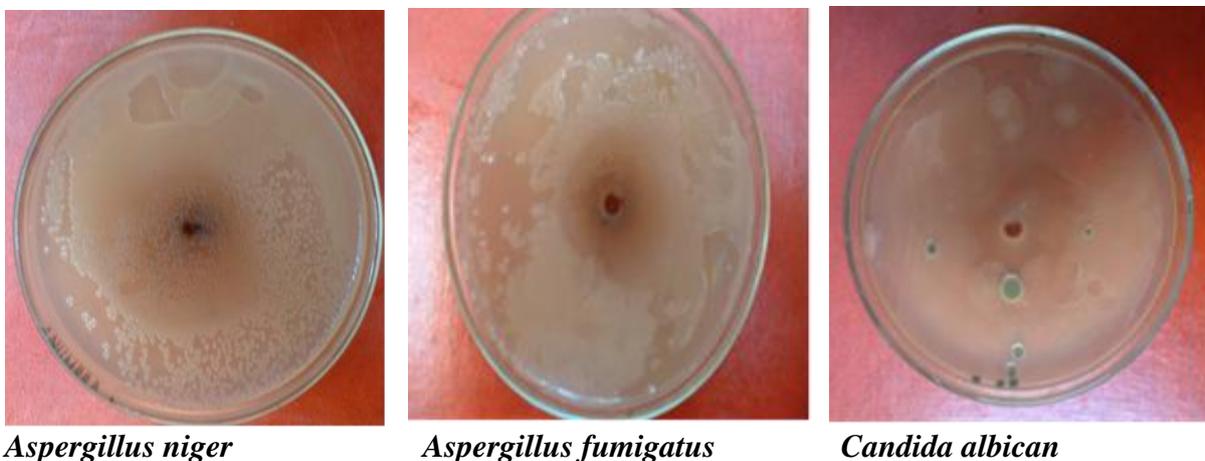


Fig. (2): Inhibition zones induced by alcoholic extract of *Punica granatum* rinds on some fungal growth.

Discussion

Medicinal plants are potential sources of novel antimicrobial compounds against pathogenic microorganisms, which are responsible for various human and animals' infections. The use of medicinal plants still plays a vital role to develop the basic health needs in developing countries. Nearly 80% of the world population depends on the traditional medicine for primary health care, most of which involves the use of natural products (24). The antibacterial effects of ethanolic extract of *Punica granatum* (pomegranate) peel is mostly used in vitro studies. Nevertheless, few studies have showed the antifungal effect of peels pomegranate. This study showed the antifungal activity of ethanolic extract of *Punica granatum*. There are many reports of antimicrobial activity of Pomegranate. Showing that pomegranate juice is inhibitory to *Staphylococcus* and *Klebsiella pneumoniae*. Similar results are recorded in present study which shows highest antibacterial activity after rind extract. (25) reported that the antibacterial action of pomegranate juice varied with variety and depended on the contents of phenolic compounds, pigments and citric acid. Pomegranate fruit peel compound punicalagin have antimicrobial activity against *S. aureus* and *P. aeruginosa*. Fungistatic activity of pomegranate peel varied with test organisms. *In vitro* studies have revealed that the ethanolic extract of pomegranate inhibited the growth of bacteria and fungi. *Punica granatum* show an acceleration of healing in infected wounds with fungi which attributed to the activity of the *Punica granatum* in inhibiting the growth of fungi and this result approved by the studies of its antifungal activity *in vitro*. These results agreement with (24) where studied antifungal effect of pomegranate peels extracts and also the pomegranate peel extract has been found to stimulate type 1 procollagen synthesis and inhibit matrix metalloproteinase (which breaks down skin protein) produced by dermal fibroblasts. The ointment significantly enhanced the wound contraction and the period of epithelialization

as assessed by the mechanical (contraction rate, tensile strength), the biochemical (increasing of collagen, DNA and proteins synthesis). Plant extracts and their oils were used to improve wound healing in burn-wound model, such as *Celosia argentea* extract which accelerated the wound closure and promoted cell motility and proliferation of primary dermal fibroblasts and keratocytes (26). Regarding the use of medicinal in treatment of open inflected wounds (24), mentioned that their use in developing countries depend on the traditional ethno-medical beliefs. In the mean time (27), stated that plant to be used for wound dressing should have anti-inflammatory, astringent, analysis antiseptic and vulnerary action. On the other hand, the microbial studies proved that the antimicrobial effect in vitro varied form one treatments to another. The antimicrobial potency of the used treatments are graded from best to worst in this order, *Punic granatum* and 2% Tincture of iodine. variety of degenerative process and disease. These include acute and chronic inflammatory conditions such as wound healing (28). Pomegranates are a source of polyphenols and other antioxidants (31) pomegranate peel extract has markedly higher antioxidant capacity than the pulp extract in scavenging superoxide anion, hydroxyl and peroxy radicals. The contents of total phenolic, flavonoids and proanthocyanidens are known to be higher in peel extract than pulp extract (29). The free radical scavenging activity of plant phenolic and flavonoids help in wound healing (30). This could be the reason for the prowound healing activity of *Punica granatum* peel extract. The exact phytochemical component of the extract responsible for this effect however was not investigated. The various bioactive pomegranate fractions singly or in combination are to be identified to find its therapeutic potential use in the management of wound healing. In conclusion, the obtained results proved that use of extract of *Punic granatum* gave encouraging results in the treatment of infected wounds. This plant

had antiseptic vulnerary and desiccating effects besides but remains stain of plant in the tissue. They are non-expensive, available

anywhere, easily prepared and has no harmful side-effect.

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