

Effect of *Punica granatum* Peels Extracts On Some Burn Infections Bacteria

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Abstract

The present study includes the isolation and identification of hundred isolates of different bacterial species were isolated from 200 patients with burn infection in Numan hospital in Baghdad city/Iraq. All isolates identified depending on morphological, cultural and biochemical tests and the results showed that the *P. aeruginosa* was the most common injuries burns with percentage 35% , followed by *K. pneumoniae*, *S. aureus* , *E. coli* (30%, 20%,15%) respectively and the susceptibility of the above isolates to nine antibiotics were tested, the results showed that the Ciproflaxacin and streptomycin were the highest effective antibiotics with a percentage 52.75%, 51.75% respectively, while all of the isolated bacteria were resistant to trimethoprim 95.25% and variable susceptibility to other antibiotics. As well as the antibacterial activity of aqueous and alcoholic extracts of *Punica granatum* peels were studied against all isolated bacteria and the results found that the MIC of aqueous and alcoholic extracts were ranged between 80-140mg/ml and 40- 80 mg/ml, While the inhibition zone were ranged between 11-18 mm and 9- 22 mm respectively . Furthermore, the results of the in vivo assay showed that the treatment of mice with the 100mg/ml of aqueous extract and Chloramphenicol have more effectiveness than other concentrations and the number of bacteria *P. aeruginosa* was reduced significantly ($P \leq 0.001$) during the treatment days .

Keywords: Burn Infections Bacteria, Medical plants, In vitro and In vivo.

Introduction

Infection is an important cause of morbidity and mortality in hospitalized burn patients (1). In spite of considerable advances in medicine and specific treatment of burn, infection continues to pose the greatest danger to burn patients and approximately 73% of all deaths within the first 5 days post burn are directly or indirectly caused by septic processes (2).The

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rate of nosocomial infections is higher in burn patients due to various factors like nature of burn injury itself, immunocompromised status of the patient (3), age of the patient, extent of injury, and depth of burn in combination with microbial factors such as type and number of organisms, enzyme and toxin production, colonization of the burn wound site, systemic dissemination of the colonizing organisms (4). In addition, cross-infection results between different burn patients due to overcrowding in burn wards (5). The burn wound represents a susceptible site for opportunistic colonization by organisms of endogenous and exogenous origin; thermal injury destroys the skin barrier that normally prevents invasion by microorganisms. This makes the burn wound the most frequent origin of sepsis in these patients (6). Currently the common pathogens isolated from burn patients are *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella spp.* and various coliform bacilli (7,8). Multidrug-resistant bacteria have frequently been reported as the cause of nosocomial outbreaks of infection in burn units or as wound colonizers in burn patients (9).

Medicinal plants are the source traditional medicine, herbal medicine and various dietary supplements. World-wide pharmaceutical scientist pushing strong demand to find out active pharmaceutical ingredients from medicinally important plant (10). *Punica granatum* L., commonly known as pomegranate, is a fruit-bearing deciduous shrub or small tree, native to Asia and belongs to the family Punicaceae (11). Different parts of the plant such as bark, leaves, immature fruits and fruit rind have medicinal significance (12). *Punica granatum* is widely employed in various countries as a source of therapeutic agent against a variety of pathogenic microbes (13).

However, to date no studies regarding the antimicrobial activity of *P. granatum* peels have been conducted in Iraq. Therefore, the goal of this study is to evaluate that.

Material and Methods

Collection of samples

Tested bacteria *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were obtained from 200 patients with burn infection and with different ages ranged between 20-50 years within four months from Jun to September, 2013 in Numan hospital in Baghdad city/Iraq. All isolates identified depending on morphological, cultural and biochemical tests as described by (14).

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Antibiotic Sensitivity Test

The sensitivity of the isolated bacteria to different antimicrobial agents (Ampicillin (AP) 10µg/disc, Refapin (Rif) 5µg/disc, Chloramphenicol (Cm)10µg/disc , Tetracyclin (TE) 30µg/disc, Streptomycin (Sm) 10µg/disc, Ciprofloxacin (Cip)5µg/disc , Erythromycin (Er) 10µg/disc, Trimethoprim (Tm)10µg/disc, Nalidixic acid(NA) 30µg/disc were determined by using method of Kirby-Bauer as described by (15).

Source of *Punica granatum* L.

Peels of *P.granatum* were identified and purchased from alzahraa Herbarium to cure by Dr Alli Almosawee, science collage, Biology Department, University of Baghdad.

Plant Extraction

The peels of *P. granatum* were air dried and powdered, and then 25g were extracted with 500mL of water and ethanol for 72h on soxhlet apparatus separately. After cooling the contents of flasks were filtered and concentrated to obtain crud extract and stored at 4oC in dark vials until it used (16).

Phytochemical screening methods

The presence of alkaloids, flavonoids, tannins, glycosides and steroidal terpenes were determined as described by (17,18)

Bacterial susceptibility testing

Agar diffusion test

Muller Hinton Agar (MHA) (HiMedia, India) was used to determine the diameter of inhibition zone (ID) by the well diffusion methods. The plates were inoculated with the standardized suspension (comparing with McFarland tube) of the test isolates. The plates were allowed to dry in the incubator for 30minutes at 37 °C and with the aid of a sterile standard coreborer, 5 wells with 5mm in diameter were bored at equidistant. The bottoms of the wells were sealed with sterile molten nutrient agar to prevent seepage of the extract under the agar. Each of the aqueous and ethanol crude extract was dissolved in Dimethyl sulfoxide (10% DMSO), final concentration 0, 2.5, 5, 12.5, 25, 50, 100,200 mg/ml and 25µl of each extract was introduced into the appropriate well in the inoculated plate ,in addition to sterile water and DMSO were used as negative controls. The prepared plates were incubated at 37°C for 24h. The resulting zones of inhibition were measured using a ruler calibrated in millimeters. The average of the three readings was taken to be zone of inhibition of the bacterial isolates in question at that particular concentration (19,20).

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Determination of minimum inhibitory concentration (MIC)

The above mentioned extracts that showed antimicrobial activity were later tested to determine the MIC for each bacterial isolates. Four bacterial isolates *P. aeruginosa*, *K. pneumoniae*, *S. aureus* and *E.coli* were grown in nutrient broth tubes separately for 6 h. Afterwards , 100 μ L of 10^6 cells/mL of growth culture were inoculated in nutrient broth tubes containing different concentrations of the extracts (0,10, 20, 30, 40, 50, 60, 70, 80, 90,100, 110, 120, 130, 140, 150 mg/ml) for 24 h at 37 °C separately for each plant extract and each bacteria in addition to control samples, then the bacterial growth was evaluated on the basis of the turbidity of the suspension and all tubes were read by spectrophotometer (LKB-PYEUNICAM-SP 6.55, England) at 620nm. However, the MBC was determined by subculturing the test dilutions onto fresh solid medium and incubated further for 18 – 24hours (21).

Inhibitory effect of *P. granatum* peels on growth of *P. aeruginosa* (In vivo)

Thirty male Balb/c mice (26 – 28 g) aged between 12 and 16 weeks were used for all in vivo experiments. They were kept in a light, food and temperature-controlled room and all mice were acclimatized for at least 1 week prior to beginning the experiments. The dorsal back skins of the mice were shaved and ethanol (70%) was used as antiseptic for the shaved region and then burned by using inflamed knife and then contaminated with bacteria *P.aeruginosa* (1.5×10^8 bacteria /ml), which was the more common species in burn infection in present study. After two days of injury the inflammation, redness and suppuration region were observed and the experimental mice were randomly divided into the following groups (n = 30):

- Group 1: untreated mice (control).
- Group 2: burn mice treated with ointment composed of antibiotic (Cm) .
- Group 3: burn treated with ointment (concentration 50mg/ml) consisting of 0.5g of aqueous extract added to 9.5g of Vaseline.
- Group 4: burn treated with ointment (concentration 100mg/ml) consisting of 1g of aqueous extract added to 9 g of Vaseline.
- Group 5: burn treated with ointment (concentration 200mg/ml) consisting of 2g of aqueous extract added to 8 g of Vaseline.
- Group 6: burn treated synergistically with ointment Cm and 100mg/ml of aqueous crude extract of *P.granatum* .

The treatment continued twice each day during 15 consecutive days and the numbers of bacteria was calculated .

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Statistical analysis: Results were expressed in Mean \pm SD and were analyzed by one way analysis of variance (ANOVA) and post hoc analysis was done using Tukeys using SPSS package version 16. Pvalue < 0.05 was considered to be significant.

Results and Discussion

In present study, 100 isolates of different bacterial species were isolated from 200 patients with burn infection and *P. aeruginosa* was the most common injuries burns with percentage 35% (Figure 1), followed by *K. pneumoniae* which achieved second place (30%), as well as recorded *S. aureus* proportion 20%, while the least isolated bacteria was *E. coli* (15%), these results were similar to that found by (22).

On the other hand, isolated bacteria showed a variation in their sensitivity and resistance to used antibiotics and the results revealed that Ciproflaxacin and streptomycin were the highest effective antibiotics against all of the isolated bacteria with a percentage (52.75%, 51.75%) respectively for both antibiotics, while all of the isolated bacteria were resistant to trimethoprim (95.25%) and the ampicillin and nalidixic acid were located in the second grade with percentage (79%, 78.75%) respectively, moreover the results of the present study showed that isolated bacteria gave variable sensitivity to other antibiotics as appeared in table (1). These finding were similar to resistant pattern of bacterial isolates in previous studies presenting public health problem (23,24). The high resistance of the bacterial isolates in this study to different antibiotics may be related to the presence and disseminations of the plasmids within heterogeneous populations of these bacteria (25), or may be due to that majority of the populace sampled purchases antibiotics in the open markets without any medical prescription, use of wrong concentration and for wrong diseases. The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants and microbes (26).

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. The main objective of the present study was to evaluate the ability of *P. granatum* peel extracts to inhibit the growth of isolated bacteria and to explore possible future use of these extracts as alternatives to common antibiotics and to determine their ability to enhance activity of antibiotics.

The MIC of both ethanolic and aqueous extracts was presented in (Figure 2). It found that the aqueous extract of the peels was effective against all

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tested bacteria with the MIC ranged between 80-140mg/ml. Moreover, the ethanol extract was the more effective against isolated bacteria with the MIC ranged between 40- 80 mg/ml, this might be results due to some components present in peel extract (Table 2) and this results were in agreement with (27).

Different concentrations of both extracts of pomegranate peels exhibited different inhibitory activity against all tested bacteria and the highest inhibition zones were between(9- 22 mm) and (11-18 mm) for both of ethanol and aqueous extracts respectively. However, the results showed that there were a significant differences at $P<0.05$ between concentrations (50,100, 200mg/ml) for aqueous extract respectively except for bacteria *K. pneumonia* which the affect were found only between (100- 200 mg/m). While for ethanol extract it was showed a stronger effect than aqueous and there were a significant differences at $P<0.05$ between concentrations (25, 50,100, 200mg/ml) for *E. coli* and *S. aureus* respectively except *P. aeruginosa* and *K. pneumonia* which the affect were found between 50,100, 200mg/ml (Figure 3). These results may be due to the possibility of containing all active compounds and various concentrations had inhibitory effects on all types of microorganisms in vitro such as flavonoids, tannins, alkaloids, saponins and glycosides (28). Antimicrobial activity may be due to numerous free hydroxyl ions that have the capability to combine with the carbohydrates and proteins in the bacterial cell wall, they may get attached to enzyme sites rendering those inactive (29), similar results were reported by (30,31).

The in vivo assay revealed that the treatment of mice with the different concentrations of aqueous extract of *P. granatum* peel and Cm antibiotic during 15 days and it was found to have marked effects on the number of *P. aeruginosa* especially the group six which represent 100mg/ml of aqueous extract and Cm antibiotic which showed more effectiveness than other groups and the number of bacteria was reduced significantly ($P\leq 0.001$) from 120 to 5×10^6 cell/ml during the treatment days and this results due to *P. granatum* extract which have antibacterial activities and exhibited synergistic effects when used with antibiotic Cm as well as have wound healing properties. In addition, according to the results presented in table (3) it was noted that the group four (100 mg/ml aqueous extract) significantly ($P\leq 0.001$) was more effective than other groups and generally the skin of the mice was healed after sex days of treatments. Therefore, the current data clearly demonstrated the importance of plant extracts in the control of burn infection resistant bacteria which are becoming a threat to

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human health and the aqueous extracts of pomegranate peels are reported to have therapeutic properties.

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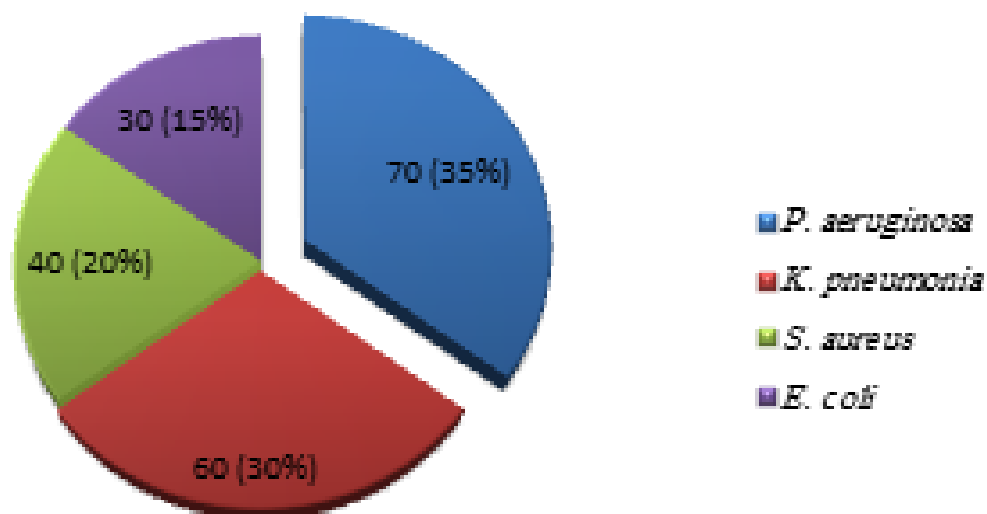


Figure 1: Number and percentage of bacteria detected in burns samples.

Table 1 : Antimicrobial resistance percentage of isolated bacteria from burned infections.

Isolated Bacteria	No. of isolates	Tm		AP		NA		Cip		CM		Sm		Rif		Er		TC	
		S%	R%	S%	R%	S%	R%	S%	R%	S%	R%	S%	R%	S%	R%	S%	R%	S%	R%
<i>P. aeruginosa</i>	35	0	100	28	72	28	72	10	90	81	19	81	19	28	72	28	72	10	90
<i>K. pneumonia</i>	30	0	100	18	82	9	81	82	18	18	82	9	91	18	82	27	73	0	100
<i>S. aureus</i>	20	19	81	19	81	10	90	100	0	28	72	82	18	28	72	35	65	82	18
<i>E. coli</i>	15	0	100	19	81	28	72	19	81	28	72	35	65	28	72	10	90	28	72
Total (%)		4.7	95.25	21	79	18.75	78.75	52.75	47.25	38.75	61.25	51.75	48.25	25.5	74.5	25	75	30	70

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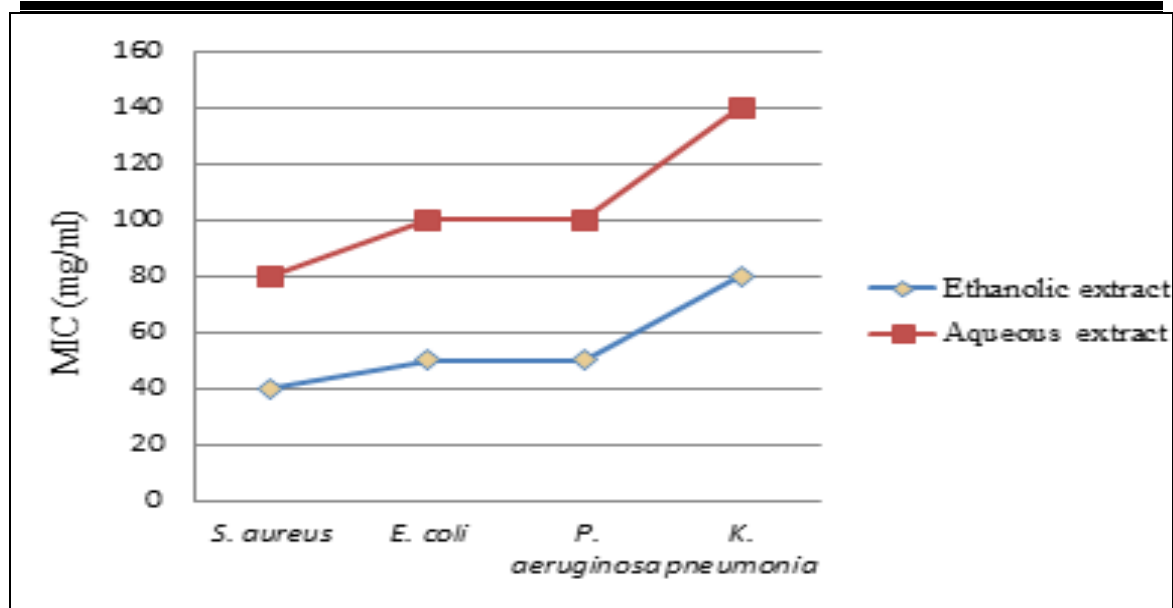


Figure 2: Minimum inhibitory concentration (MIC) of ethanolic and aqueous extract of *Punica granatum* peel on bacterial burn isolates.

Table 2: Phytochemicals of *Punica granatum* peel.

Chemical constituents	Ethanolic extract	Aqueous extract
Tannins	+	+
Flavonoids	+	+
Alkaloids	+	+
unsaturated sterols and triterpenes	-	-
carbohydrates and glycosides	+	-

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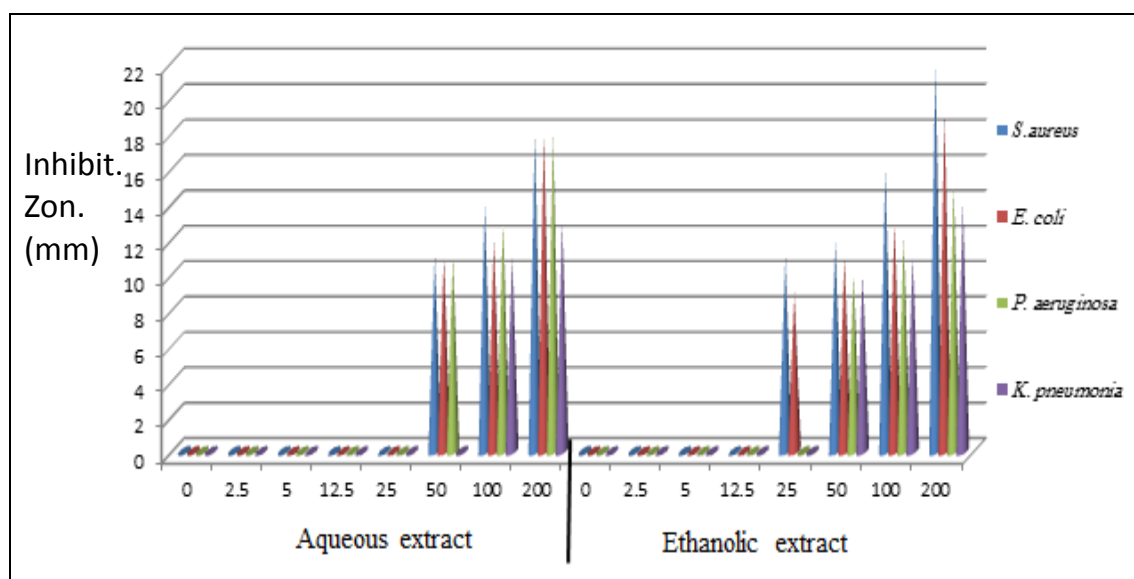


Figure 3: Diameter of inhibition zones (mm) of ethanolic and aqueous extracts of *P. aeruginosa* peel against isolated bacteria.

Table 3: Numbers of *P. aeruginosa* between the mice groups.

Duration of treatment per day	Group(1) Control	Group(2) CM	Group(3) Aqueous extract 50mg/ml	Group(4) Aqueous extract 100mg/ml	Group(5) Aqueous extract 200mg/ml	Group(6) CM + Aqueous extract 100mg/ml
3	0	150	180	171	162	120**
6	0	120	85	75*	88	5*
9	0	29	19	12	37	5*
12	0	0	3	1	6	0
15	0	0	0	0	0	0

* Significant at $P \leq 0.001$

تأثير مستخلصات قشور الرمان على بعض الأصابات البكتيرية للحروق

الخلاصه:

شملت الدراسة الحالية عزل وتشخيص مائة عزلة لأنواع بكتيرية مختلفة من مئتي مريض يعانون من اصابات الحروق والراقدين لدى مستشفى النعمان في مدينة بغداد -العراق وإستنادا الى الصفات المزرعية، الشكلية و التفاعلات الكيميائية الحياتية اظهرت النتائج ان نسبة البكتريا الزائفه الزنجارية اكثر انتشارا وبنسبة 35% ويتبعها بكتريا الكبسيلا , مسبقيات العنقوديه وبكتريا القولون وبنسبه 30%, 20%, 15% وعلى التوالي. وتم اختبار حساسية العزلات لتسعة من المضادات الحياتية وتبين ان المضاد سبروفلاكساسين وستربتومايسين هم الاكثر تأثيرا وبنسبة 52.75%, 51.75% وعلى التوالي بينما العزلات كافه كانت مقاومة للمضاد ترايمثبريم وبنسبة 95.25% وكذلك أظهرت حساسية متباينة للمضادات الأخرى .

تمت دراسة تأثير الفعالية التثبيطية لكل من المستخلص المائي والكحولي لقشور الرمان ضد العزلات كافه وتم معرفة كل من التراكيز المثبطة الدنيا للمستخلص المائي والكحولي وتراوحت مابين 80-140 و 40-80 مايكروغرام/مل وعلى التوالي بينما سجلت مناطق التثبيط مابين 11-18 و 9-22 ملم, وبالإضافة الى نتائج اختبار in vivo اظهرت معالجة الفئران بتركيز 100 مايكروغرام/مل للمستخلص الكحولي مع المضاد الحيوي كلورامفينيكول هي الاكثر فعالة اذا ماقورنت بالتراكيز الأخرى وان عدد الزائفه الزنجارية قد انخفضت وبنسبة معنوية خلال ايام المعالجة ($p \leq 0.001$).