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Antimicrobial activity of ethanolic and aqueous extracts of pomegranate peel against Extended Spectrum Beta-Lactamase producing bacteria

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Abstract:

Background: Pomegranate *(Punica granatum*) is an ancient, mystical, unique fruit with potential antimicrobial activity.

Objective: This study was performed to explore the antimicrobial activity of ethanolic and aqueous extracts of pomegranate peel against extended spectrum beta-lactamase producing bacteria *(Klebsiella pneumonia* , *Escherichia coli* and *Aeromonas hydrophila*).

Material and Methods: 100 swabs were collected from the skin of burned patients. The bacterial strains were identificated according to their cultural , morphological , microscopically and biochemical characteristics, then subjected to ESBL-producing screening by double-disc synergy test. The ethanolic and aqueous extracts of pomegranate peel were prepared by using a Soxhalet apparatus and their antimicrobial activities were studied and compared with a commercial antibiotics against tested bacteria by using agar well diffusion method.

Results: Out of total 100 swabs, only three isolates produced ESBLenzyme, namely (*Klebsiella pneumoniae, Escherichia coli* and *Aeromonas hydrophila*). Both extracts showed an effective antibacterial activity against all these bacteria. The ethanolic extract was found to be more effective than aqueous one against all the tested microorganisms.

Conclusion: The pomegranate peel extracts have a strong antimicrobial activity against extended spectrum beta-lactamase producing bacteria. Therefore it is an important source of new antimicrobial compounds to treat infections caused by multidrug-resistant bacterial.

Keywords: *Pomegranate, antimicrobial activity, antibacterial activity, Klebsiella pneumonia, Escherichia coli, and Aeromonas hydrophila.*

فعالية المستخلصات الايثانولية والمائية لقشور الرمان ضد البكتريا المنتجة لأنزيم البيتا لاكتميز الواسع الطيف

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الخلاصـــــة:

الهدف: اجريت هذه الدراسة لتقييم فعالية المستخلصات الايثانولية والمائية لقشور الرمان ضد البكتريا المنتجة لأنزيم البيتا لاكتميز الواسع الطيف.

طريقة العمل: تم جمع 100 مسحة من جلد الاشخاص المصابين بالحروق, ثم اجريت عدة اختبارات للبكتيريا المعزولة لتحديد نوعها و قابليتها لإنتاج انزيم البيتا لاكتميز الواسع الطيف. تم تحضير المستخلصات الايثانولية والمائية لقشور الرمان باستخدام جهاز السوكسليت, ثم استخدمت طريقة الاكار لتحديد تأثير المستخلصات على هذه البكتيريا.

النتائج: من مجموع مائة مسحة , تم الحصول على ثلاث عزلات بكتيرية منتجة لانزيم البيتا لاكتميز الواسع الطيف وهي *Klebsiella pneumonia* , *E. coli*،*Aeromonas hydrophila*

أظهر المستخلص الايثانولي والمائي نشاطا فعالا عاليا ضد هذه البكتريا, حيث وجد أن المستخلص الإيثانولي أكثر فعالية من المستخلص المائي.

الاستنتاج: اظهرت مستخلصات قشور الرمان نشاطا فعالا عاليا ضد البكتريا المنتجة لانزيم البيتا لاكتميز الواسع الطيف, والذي يمكن ان يستخدم كعقار ضد البكتيريا المقاومة للعديد من الادوية وعلاج الالتهابات الناجمة عنها.

Introduction:

Antimicrobial resistance is a major cause of morbidity and mortality in the world, which can result from the widespread use of commercially available antimicrobial agents that lead to the emergence of microbial resistant pathogens and thus pose a serious threat to global health (Khan and Hanee 2011). This resistance increases among Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* due to their ability for production of beta-lactamases enzymes .These enzymes are capable for hydrolyzing penicillins, broad-spectrum cephalosporins such as cefotaxime, ceftazidime and monobactams such as aztreonam (Chaudhary and Aggarwal 2004; Bush and Fisher 2011). Infections caused by extended spectrum beta-lactamases (ESBLs) producing bacteria either hospitals or community-acquired infections are serious and more difficult to treat because of antibiotic resistance, therefore it is necessary to search for new antimicrobial agents of plant origin (Paterson and Bonomo 2005;Zaouia *et al*., 2010; Sadeghian *et al*., 2011).

The pomegranate is an ancient, mystical, unique fruit has strong antioxidant and anti-inflammatory properties, used widely for the treatment of many diseases (Yehia *et al*.,2011). The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, and protection from ultraviolet (UV) radiation" (Adhami and Mukhtar 2007; Neelam and Singh 2012) . The most therapeutically beneficial pomegranate constituents are ellagic acid, ellagitannins (including

punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones (Jurenka 2008).

Several studies used pomegranate extracts against a range of both Gram positive and negative bacteria, these studies showed that pomegranates extracts have antimicrobial activity through their ability to inhibit the growth of bacteria as well as inhibit the production of enterotoxin. (Meléndez and Capriles 2006; Panchal *et al*.,2013). The current study was carried out to evaluate the antimicrobial activity of ethanolic and aqueous extracts of pomegranate peel against extended spectrum β-lactamase producing bacteria.

Materials and methods:

Plant collection and extraction:

Pomegranate fruits were purchased and collected from the local market of Al-Nasiriya city. The peels were removed manually and rinsed well with sterile distilled water and dried under shade, then grounded into fine powder using an electric blender. Then, ethanolic extract was prepared by mixing 100 g of Pomegranate peel powder with 500 mL of ethanol (70%) in a Soxhlet apparatus for 6 hours. The extraction procedure was same as used for aqueous extract. Both extracts were filtered by filter paper and dried using rotary evaporator at 50°C. The extracts were stored at 4°C in pre sterilized air tight flasks until use to prepare 100 mg/mL concentrations from each extract (Pai *et al*., 2011).

Microorganisms and Culture:

One hundred swabs were collected from the skin of burned patients, from both sex with age 3-50 year in burn unit in Al-Hussein hospital at Thi-Qar province in Iraq for the period from 1st of January 2016 to the end of May 2016. All swabs were cultured on Blood agar, MacConkey agar and Nutrient agar and incubated at 37°C for (24 - 48) hours. The bacterial strains were isolated and identificated according to their cultural , morphological , microscopically and biochemical characteristics (Finegold and Martin 1982 ; Koneman *et al*., 1992; Steven, *et al*., 2001 ;Retty *et al*., 2007) . For final identification of isolates had been used kits API 20E kit (BioMeriux).

Detection of Extended-spectrum β-lactamase (ESBL) -producing bacteria by double-disc synergy test (DDST):

A disc of ceftriaxone (30 µg) and ceftazidime (30 µg) were placed at a distance of 16-20 mm from the Augmentin disc(20µg amoxicillin plus10µg clavulanic acid) center to center on a Mueller-Hinton Agar (MHA) plate swabbed with the test isolate. After incubation the plate at 37°C for 24 hours, the organisms were considered to be producing ESBLs when the zone of inhibition around any of the expanded-spectrum cephalosporin discs showed a clear-cut increase towards the Augmentin disc. (Jarlier *et al.*, 1988 ; Jabeen *et al.*, 2003).

Estimation of antibacterial activity :

The antibacterial activity of the extracts against three burn isolates was evaluated by using the agar well diffusion methods. 0.1 mL of diluted inoculum of each tested bacteria was aseptically spread onto the surface of Mueller Hinton agar and then left to dry for 30 min. Wells of 8 mm in diameter were made into agar plates containing the bacterial by using a sterilized stainless steel borer and filled with 50 µl of each extracts. Parallel with fruit extracts, the antibacterial activity was also analyzed with twelve commercially available standard antibiotic discs (Ciprofloxacin, Norfloxacin, Imipenem, Gentamicin , Netilmicin, Amikacin, Ticarcillin , Aztreonam , Cefotaxime , Ceftriaxone , Ceftazidime , and Amoxicillin-clavulanate) for comparative study. The prepared plates were left at room temperature for 30 minutes allowing the diffusion of the extracts into the agar, then incubated at 37 °C for 24 hours. The antibacterial activity was evaluated by measuring the diameter of inhibition zone produced by the extracts against tested bacteria (Perez *et al*., 1990 ; Ahmad and Beg 2001).

Phytochemical analysis of pomegranate peel extracts:

Phytochemical analysis was carried out on the ethanolic and aqueous extracts of pomegranate peel for the presence of bioactive components such as Carbohydrates, Glycosides, Tannins, Saponins, Flavonoids, Phenolic compounds, Alkaloids, Triterpenoids and Steroids according to the methods described by Raman (2006), Kumar *et al*. (2009) , Banu and Cathrine (2015).

Results:

In this study from 100 swabs from the skin of burned patients, only three isolates produced ESBL, namely (*Klebsiella pneumoniae, Escherichia coli* and *Aeromonas hydrophila*) .

Both ethanolic and aqueous extracts (100mg / mL) of pomegranate exhibited potent antibacterial activity toward all extended-spectrum β-lactamase producing tested bacteria, but ethanolic extracts showed higher antibacterial activity in comparison with aqueous extracts (Table 1).

Ethanolic extracts showed zone of inhibition 14 mm against *Klebsiella pneumonia* , which was greater than that of CIP 11 mm, NOR 11mm, GM 12 mm, and comparable to that of TI 14 mm, AT 14mm, while it was lesser than that of other antibiotic discs. Whereas the aqueous extracts showed zone of inhibition 10 mm against *Klebsiella pneumonia*, which was comparable to that of CIP 11 mm, NOR 11mm, and lesser than that of other antibiotic discs.

Regarding *Escherichia coli*, the zone of inhibition produced by ethanolic extracts was 30 mm which was greater than that of all tested antibiotic discs, while the zone of inhibition produced by aqueous extracts was 17mm which was greater than that of CIP 10 mm, NOR 11mm, GM 11 mm, TI 12 mm, AT 15mm,CAZ 15mm, and comparable to that of AUG 17 mm, CTX 18mm, and CTR 18 mm, but it was lesser than that of other antibiotic discs.

For *Aeromonas hydrophila*, Ethanolic extracts demonstrated zone of inhibition 23 mm, which was greater than that of TI 13 mm, AT 15mm, GM 17 mm, AUG 18 mm, CIP 20 mm, NOR 20 mm, CAZ 21mm, and comparable to that of NET 22 mm, AK 22mm, while it was lesser than that of other antibiotic discs. Whereas the aqueous extracts showed zone of inhibition 19 mm, which was greater than that of TI 13 mm, AT 15mm, GM 17 mm, and comparable to that of AUG 18 mm, but it was lesser than that of other antibiotic discs.

Phytochemical analysis:

The phytochemical analysis of ethanolic and aqueous extracts of pomegranate peel showed the presence of Carbohydrates, Glycosides, Tannins, Saponins, Flavonoids, Phenolic compounds, Alkaloids, Triterpenoids and Steroids (Table 2) .

Table (1): Antibacterial activity of ethanolic and aqueous extracts of pomegranate peel and commercially available standard antibiotics.

Diameter of inhibition zone (mm)			
Standard Antibiotics and extracts	***K. pneumoniae***	***E. coli***	***A. hydrophila***
CIP 10 µg	11	10	20
NOR 10 µg	11	11	20
IPM 10 µg	25	26	25
GM 10 µg	12	11	17
NET 30 µg	21	22	22
AK 30 µg	20	21	22
TI 75 µg	14	12	13
AT 30 µg	14	15	15
CTX 30 µg	19	18	26
CTR 30 µg	17	18	28
CAZ 30 µg	24	15	21
AUG 10 µg	18	17	18
AEP 100 mg/mL	10	17	19
EEP 100 mg/mL	14	30	23

CIP: Ciprofloxacin, NOR: Norfloxacin , IPM: Imipenem, GM : Gentamicin , NET: Netilmicin, AK: Amikacin, TI: Ticarcillin , AT: Aztreonam , CTX: Cefotaxime , CTR: Ceftriaxone , CAZ: Ceftazidime ,AUG: Amoxicillin-clavulanate , AEP: aqueous extracts of pomegranate, EEP: ethanolic extracts of pomegranate.

Table(2):Phytochemical analysis of the ethanolic and aqueous extracts of pomegranate peel.

Chemical Constituents	**Chemical Test**	**Ethanolic extracts**	**Aqueous extracts**
Carbohydrate	Molish's test	+	+
Glycosides	Keller Killiani Test	+	+
Tannins	Gelatin Test	+	+

Saponins	Foam Test	+	+
Flavonoids	Alkaline reagent Test	+	+
Phenolics`	Ferric chloride Test	+	+
Alkaloids	Hager's Test	+	+
Triterpenoids	Liebermann Burchard Test	+	+
Steroids	Liebermann Burchard Test	+	+

+ : indicates presence of phytochemicals.
- : indicates absence of phytochemicals.

Discussion:

Observed during the past few years, outbreaks of infection caused by the bacteria that produce beta-lactamases due to their resistance to antibiotics that ultimately led to the threat to global public health. Therefore plants have been evaluated as sources of antimicrobial agents to avoid resistance and undesirable side effects of commercially available antimicrobials (Paterson and Bonomo 2005; Prajapati 2012) .

In the present study both ethanolic and aqueous extracts of pomegranate peel exhibited antimicrobial activity against all extended-spectrum β-lactamase producing tested bacteria.

Pai *et al*.,(2011) reported that ethanolic extracts of pomegranate peel showed greater antibacterial activity in comparison with that of aqueous extracts when tested against various enteric pathogens (like *Vibrio cholerae*, species of *Shigella*, *Salmonella*, Enteropathogenic *Escherichia. coli*, Enterotoxigenic *Escherichia. coli* ,Enteroaggregative *Escherichia. coli*, and *Aeromonas hydrophila*). Also data reported by Unnisa *et al*. (2012) revealed that ethanolic and aqueous extracts of pomegranate produced highly antibacterial activity against all four wound isolates (*S. aureus*, *E. coli*, *P. aeruginosa* and *K. pneumonia*), also demonstrated that ethanolic extracts of pomegranate possess highly antibacterial activity than aqueous extracts which agreed with our results.

Other study demonstrated that alcoholic extracts of pomegranate fruit peels showed potent antibacterial activity against *S. aureus*, *Listeria monocytogenes*, *E. coli* and *Yersinia enterocolitica* (Al-Zoreky 2009).The same activity has been reported for extracts of pomegranate against *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Bacillus subtilis* and *Salmonella typhi* (Prashanth *et al*., 2001). Melendez and Capriles (2006)

have also reported that extracts from pomegranate fruits possess antibacterial activity against many tested bacteria (*E. coli, Enterobacter cloacae, P. fluorescens, Proteus vulgaris, Alcaligenes faecalis, Serratia marcescens, E. aerogenes, S. aureus, Arthrobacterglobiformis, M.luteus, B. cereus, B. subtilis, B. coagulans ,Micrococcus roseus, M. phlei, M. rodochrus, M. smegmatis*).

Several studies showed that the alcoholic extracts of pomegranate produced highest antibacterial activity than that of aqueous extracts when evaluated against both Gram-positive and Gram-negative bacteria (Negi and Jayaprakasha 2006; Dahham *et al*., 2010).

These results are compatible with the results obtained in this study, which confirms the effectiveness of pomegranate against bacteria, also confirms that the alcoholic extracts have highest antimicrobial activity as compared to aqueous extracts. So, it is reasonable to assume that the principal chemical constituents with antimicrobial activity were concentrated in the alcoholic fraction. This was in agreement with Ahmad *et al*. (1998) who found alcohol as a better solvent for extraction of antimicrobial active substances compared to water and hexane.

The antibacterial activity of pomegranate peels may be indicative of presence of some metabolic toxins or broad spectrum antibiotic compounds like tannins, flavonoids and other phenolic compounds (Voravuthikunchai *et al*.,2004; Li *et al*.,2006).Tannins are considered toxic to microorganisms (Viuda-Martos 2010), in which their hydrophilic part interacts with the polar region of the membrane, whereas the hydrophobic part is immersed in the non-polar inner region of the bacterial membrane, which can cause instability of the membrane, thus affecting the transport of substrates into the cell, therefore causing leakage of cell membrane of the microorganism and aiding cell lysis which ultimately leads to cell death (Cristani *et al*., 2007; Olapour *et al*., 2009; Endo *et al*., 2010). In addition, tannins create stable complexes, mainly with proteins and, to a lesser extent, with carbohydrates or physiological metal ions (such as Fe and Cu) (Chung *et al*., 1998).

The complexation of tannins with enzymes changes their structural conformation, thereby inhibiting enzymatic activity, also tannins decrease metal ion availability to bacteria when forming stable complexes with these metal ions. Subsequently, metal depletion may adversely affect the activity of metallo enzymes in microbial cells (Goel *et al*., 2005).

Naz *et al*. (2007) showed the mechanism that responsible for phenolic toxicity to microorganisms was related to their reactions with sulfhydryl groups or through more non-specific interactions with proteins leading to loss of function.

On the other hand, phenols may also render substrates unavailable to microorganisms or interfere with bacterial protein secretions (Machado *et al*. 2003). This confirms by Devatkal *et al*. (2013), where they revealed that pomegranate peels extracts significantly reduced the growth of bacterial by reduction of protein content in the bacterial cells .

In conclusion, the results obtained in this study demonstrated that the ethanolic and aqueous extracts of pomegranate peel extracts had broad spectrum antimicrobial activity against extended spectrum beta-lactamase producing bacteria. The ethanolic extract was found to be more effective than aqueous one against all the tested microorganisms. Therefore pomegranate peel is an important source of new antimicrobial compounds to treat infections caused by multidrug-resistant bacterial.

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