

Study the Inhibitory Effect of Aqueous Extract of *Punica granatum* L. on Resistant *Staphylococcus aureus* Isolate from Mastitic milk

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Abstract

Were collected 154 milk samples from 120 animals included (25 sheep ,25 goats,70 cows), subjected these samples to California Mastitis Test, (60) sample showed a positive result. These samples were grown at different common, selective culture media, and depended on the study of the cultural characteristic and microscopic examination and biochemical tests, 32 isolates diagnosed as *Staphylococcus aureus* , 11 isolates diagnosed as *Staphylococci* coagulase negative and 17 isolate as gram negative bacteria, the diagnosis was confirmed by using the API STAPH system .The *staphylococcus aureus* isolates were tested for their sensitivity to commonly used antibiotics .the isolates are more susceptible to Chloramphenicol ,Streptomycin and Ciprofloxacin, and the least sensitivity was observed to Ampicillin, Gentamycin and Cefixime. then selected one isolate from *S. aureus* (isolate exhibit resistant to large number of antibiotic), for determination antibacterial activity of *Punica granatum* in different concentration 25mg/mL, 50mgL/ mL, 75mgLmL, 100mg/mL150, mgL/mL ,the mean of inhibitory zone diameter ware 20.66, 23, 24.66, 27, 29.66 respectively The present study provides evidence antibacterial effect of aqueous extract of Punica granatum on resistant staphylococcus aureus isolated from mastitic milk.

Key words: mastitic milk, *S. aureus*, antibiotic susceptibility, aqueous extract of *Punica granatum*

دراسة التأثير التشبيطي للمستخلص المائي لقشور الرمان على المكورات العنقودية الذهبية المقاومة للمضادات
الحياتية المعزولة من حالات التهاب الضرع

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الخلاصة:

تم جمع 154 عينة حليب من 120 حيوان شملت (25 أغنام, 25 ماعز, 70 أبقار), أخضعت هذه العينات لأختبار الكالفورنيا, (60) عينة أظهرت نتيجة موجبة نميت هذه العينات على مختلف الأوساط الزرعية الشائعة والانتخابية وبالأعتماد على دراسة الصفات الزرعية والفحص المجهرى والأختبارات الكيمو حيوية, 32 عينة شخصت على أنها المكورات العنقودية الذهبية و 11 عينة شخصت على أنها المكورات العنقودية السالبة للأنزيم الكوكليز و 17 عينة شخصت على أنها بكتريا سالبة لصبغة كرام وتم تأكيد التشخيص باستخدام نظام ال API STAPH, أجري أختبار الحساسية للمضادات الحيوية شائعة الاستخدام على عزلات المكورات العنقودية الذهبية, وكانت العزلات أكثر حساسية أتجاه الكلورم فينكول والستربتومايسين والسايبروفلوكساسين وكانت أقل حساسية أتجاه الأمبسلين والجنتاميسين والسيفكسيم, بعد ذلك أختبرت عينة واحدة من المكورات العنقودية الذهبية (العزلة التي أظهرت مقاومة أتجاه أكثر عدد من المضادات الحيوية) لتحديد مدى فعالية المستخلص المائي لقشور الرمان كمضاد بكتيري وبالتراكيز 25 مغ/مل, 50 مغ/مل, 75 مغ/مل, 100 مغ/مل, 150 مغ/مل, كانت معدلات أقطار التنشيط هي 20,66, 23, 24,66, 27, 29,66 على التوالي. هذه الدراسة اظهرت مدى فعالية المستخلص المائي للقشور الرمان كمضاد بكتيري على المكورات العنقودية الذهبية المقاومة للمضادات الحيوية والمعزولة من حالات التهاب الضرع.

Introduction:

Mastitis is a general term which refers to inflammation of the mammary glands, regardless of the cause (1). characterized by physical, chemical, and usually bacteriological changes in the milk and by pathological changes in the udder (2). Mastitis is the total or partial inflammation of the mammary gland provoked by one or more pathogenic microorganisms, which can appear either in clinical or subclinical forms (3). It is a disease that can cause devastating effects to a farmer because of serious economic losses (4).

Mastitis is one of the major diseases in the veterinary field. The worldwide losses due to the disease are approximately \$35 billion annually. In addition to the huge direct and indirect economic losses, the

presence of certain pathogens in the milk threatens public health (5).

Over 50% of dairy cows are infected with pathogens which cause mastitis, with 20-30% of the individual quarters being infected (6). Prevalence of mastitis in dairy cattle has been reported as 31% in Finland and 26% in Germany (7). Overall prevalence of sheep mastitis including both clinical and subclinical cases has been reported as 39%, 35%, 14% and 30% in Iran, Spain, Australia, and Jordan, respectively (8). prevalence of goat mastitis including both clinical and subclinical cases has been reported as 36%, 36%, 33% and 18% in the United States, Britain, France, and Spain, respectively (9).

Several pathogens can cause mastitis but *Staphylococcus spp.* are the most frequently diagnosed causal

microorganisms. Commonly isolated pathogens include *Staphylococcus aureus* (10). *Staphylococcus aureus* is one of the main pathogens isolated from contagious bovine mastitis cases in many countries (11). *Staphylococcus aureus* is recognized as the most common causal agent of goat mastitis (12). *S. aureus* has been reported to be the most common causal organism in both meat (13) and dairy ewes (14). This organism is able to produce a host of structural changes in udder and keeps on developing resistance against the most commonly used antibiotics.

Synthetic drugs are not only expensive and inadequate but also often had issues with adulterations and side effects. The widespread use of antibiotics on dairy farms, could lead to the selection and to the emergence of antibiotic resistant bacterial strains (15).

Plants have been known to be a reservoir of secondary metabolites which are being exploited as source of bioactive substance for various pharmacological purposes.

SO here we are trying to investigate an alternative approach to the treatment of mastitis cases, caused staphylococcus aureus infections resistant to common use antibiotics. This alternative approach has been evaluated by using in vitro aqueous extracts of *Punica granatum L.*

Material and method:

Sample collection:

154 milk sample were collected from different animals (25 from sheep, 25 from goats & 70 from cows) from some Salah Al-deen areas (ballad ,yathreb ,Al-salam, Al-ishaqi and samarra city) in the period from 22/12/2013 to 15/3/2014.

Were collected aseptically from lactating animals suspected for mastitis. After proper sanitization of teat orifice with 70% ethyl alcohol, 10-20ml of milk samples from the teats were collected aseptically after discarding first few streams. The ice box containing milk samples in tubes was taking to the microbiology lab of the Veterinary College of Baghdad University to isolate the strains. where the milk samples were kept at 4-80C in refrigerator for further laboratory investigation.

Testing of milk samples for mastitis:

All the milk samples were subjected to the California Mastitis Test (CMT) according to (16).

Isolation and identification of *Staphylococcus aureus*:

A total of sixty milk samples positive for mastitis were subjected to isolation and identification of bacteria on the basis of morphological, cultural and biochemical characteristics (17)

The positive samples collected from the field were subjected to cultural examination using blood agar, nutrient agar and Staph 110 agar,

The inoculated plates were incubated aerobically at 37°C for 24-48 hours. Colony morphology, gram staining and catalase test were used to identify bacterial genus and were followed by further culturing in selective and differentiated media (mannitol salt agar and Baird-Parker agar (ISO) bas) and also culturing in other media, then flow by biochemical tests (Coagulase, phosphates, oxidase and urease activity) final confirmative diagnosis by using API-Staph system.

Antibiotic Susceptibility test:

For antibiotic susceptibility test, disk diffusion method proposed by (18), was followed. Isolates were incubated in nutrient broth at 37°C for 12 h. plates were made with Muller Hinton agar and allowed to solidify. By spread plate technique the cultures were inoculated in the plates using sterile swab. The antibiotics disk of Ciprofloxacin ,Nitrofuranton , Methicilin , Ampicillin , Tetracyclin , Streptomycin , Penicillin , Gentamycin , Cefixime , Chloramphenicol and Vancomycin were placed in the plates. Agar plates with antibiotic disk were then incubated for 24 h .the diameters of inhibition zone were measured using a ruler ,the results were expressed as sensitive(S)intermediate (I)and resistant(R(as pre the recommended standers.

Preparation of *Punica granatum* aqueous extract:

The *Punica granatum* L. Fruits were collected from a local market, after cleaned the rind, were obtain and

put in dark room until complete drayed ,then grounded into fine powder using a electric blender. Then aqueous extract prepared according (19).

Determination Antibacterial Activity of *Punica granatum*:

After choosing *S. aureus* isolate that exhibit resistant to large number of antibiotic(isolate which are exhibit resistant to Ciprofloxacin, Nitrofuranton, Methicillin , Ampicillin , Tetracyclin , PenicillinG , Gentamycin , Cefixime , and Vancomycin) in antibiotic sensitivity test. Antibacterial activity was determination by agar diffusion method (20).three replicates were carried out for each concentration extract, the diameter of inhibition zone was measured and the mean values were recorded.

Result and discussion:

From 154 milk samples, 60 were found positive for mastitis. The overall occurrence of mastitis was 38.96%. These findings agree well with those of (21), who recorded 32.91% mastitis cases in cattle, also agree with (22) and (23), who recorded 23.59 to 34.5% and 32% mastitis cases respectively.

In this study, out of the total 60 mastitis positive milk samples, 32(53.33%) isolates, were diagnostic *Staphylococcus aureus* ,this agree with (24) which find (49/110)44.45% give *S. aureus* growth from subclinical mastitis, 11 isolates diagnostic as coagulase negative *staphylococci* and 17 isolate as gram negative bacteria.

The high prevalence of *S. aureus* can most likely be attributed to the wide distribution of the organism inside mammary glands and on the skin of teats and udders (25).

The of antibiotic susceptibility test are summarized in Table 1. the highest sensitivity was to Chloramphenicol and Streptomycin(93.75%), followed by the sensitivity to Ciprofloxacin (87.5%).The highest resistance to the antimicrobials used was to Ampicillin (90.625%), Gentamycin(90.625%) and Cefixime (96.875%). Resistance to the beta-lactam antibiotics is most often due to a plasmid-encoded penicillinase(beta-lactamase) (26). Because of prolonged treatments with same antibiotics frequently is noticed the emergence of resistant variants of bacterial strains (27). Antibacterial activity assay of aqueous extract of *Punica granatum L.* were done on one isolate of

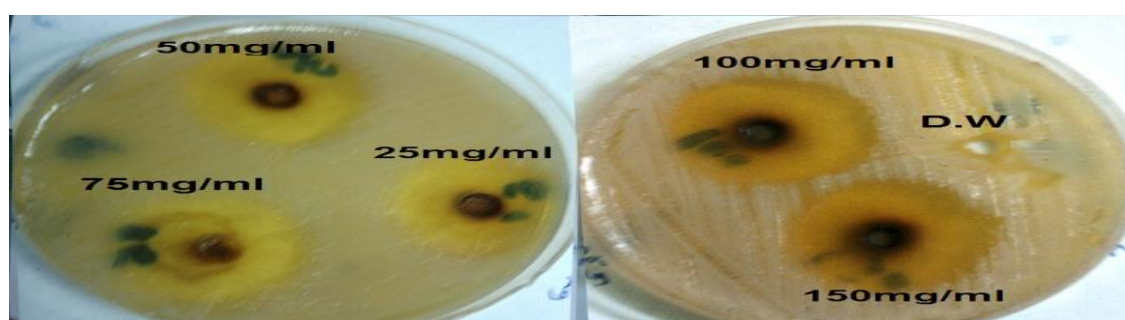
Staphylococcus aureus, isolate that resist large number of antibiotic in Susceptibility test . Antibacterial activity of aqueous extracts of *P. granatum L.* at concentration (25mg/mL,50mg/mL,75mg/mL,100mg/mL and150mg/ mL) were evaluated by measuring the diameters of zones of growth inhibition on bacterial strain and the results are presented as shown in Table 2 and Figures 1. Several metabolites including tannins, flavonoids and other active substances have been previously associated with antimicrobial activity and extensively used in the replacement of the chemically produced antimicrobial agents (28). The antibacterial activity of the pomegranate peel extracts might be due to the presence of various phytochemicals such as phenolic punicalagins, gallic acids, catechin, quercetin and retin(29)

Table 1. Antibiotic susceptibility test

Antibiotic	sensitive		Intermediate		resistance	
	Number from 32	Ration%	Number from 32	Ration%	Number from 32	Ration%
Ciprofloxacin 10mcg(cip)	28	87.5%	2	6.25%	2	6.25%
Nitrofuranton 100mcg(F)	7	43.75%	11	34.37%	14	43.75%
Methicillin 10 mcg(ME)	1	3.12%	6	18.75%	25	78.12%
Ampicillin 25mcg(AM)	3	9.37%	-	-	29	90.62%
Tetracycline 10 mcg(TE)	18	56.25%	8	25%	6	18.75%
Streptomycin 25mcg(S)	30	93.75%	1	3.12%	1	3.12%
Penicillin G 10U(P)	15	46.87%	-	-	17	53.12%
Gentamycin 10mcg (CN)	2	6.25%	1	3.12%	29	90.62%
Cefixime 5mcg(CFM)	1	3.125%	-	-	31	96.87%
Chloramphenicol30mcg(C)	30	93.75%	-	-	2	6.25%
Vancomycin 10mcg(v)	4	12.5%	3	9.37%	25	78.37%

Table 2. Antibacterial activity of aqueous extracts of *P. granatum*

Concentration mg/ml	Size of zone inhibition for three replications (mm)			Means
25	20	22	20	20.66
50	22	23	24	23
75	24	25	25	24.66
100	27	26	28	27
150	30	29	30	29.66

Figures 1. Antibacterial activity of aqueous extracts of *P. granatum***Reference:**

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