Antibacterial activity of *Punica granatum* L and *Oregno.vulgare* L extranet against ESBL type of bacterial

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Abstract

Antimicrobial activity of the crude ethanol extracts of *punica granatum* L and *Oregano. vulgare* L against multidrug resistant UTi isolates were investigated. Two strains of *Escherichia coli* and one *Acinetobacter* that showed resistance against maximum number of tested antibiotic were selecte for an antibacterial plant extract assy. Double –disk synergy (DDS) test was use for detection of Extended Spectrum Bets-Lactamese (ESBLs) in the three studied UTi isolates. Antibacterial activities of the two plants extract were measure by well diffusion, CUF/ml, and turbidity (O.D595) methods before and after treatment. The results showed that *punica granatum*L peels extract have a significant antimicrobial activity against the three tested UTi isolates. Diameters of inhibition zone were 25mm, 23mm, cuf/ml 6.6x 10^{-1} 2.0 x 10^{-1}, O.D595 0.2 and 0.219 for *Escherichia coli* and *Acinetobacter* sequentially compare with control. Antibacterial activity of ethanol extract was depended on plants extract concentration and bacterial type. This study showed that plant extracts have negative impact on the level of protein in treated bacterial isolates comparing to the control.

Keywords: *punica granatum* L, *Oregano. vulgare* L, plants extract, *Escherichia coli* ,*Acinetobacter*, beta-lactamase

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INTRODUCTION

Antibiotic resistant strains of clinically important pathogens have been increased as result of overuse or misuse of antibiotics which have led to the eminence of new bacterial strains that are multidrug resistant WHO (1, 2). The worldwide emergence of B-Lactamase produces has become a major then aphetic problem (3, 4, 5). Resistant bacterial infections are associated with increased morbidity, mortality, and healthcare cost. There has been recent dramatic increase in the prevalence of resistant negative bacteria such as ESBL_producing enterobateriaceae, Carbapenem_ enterobateriaceae, MDR_pseudomonas aeruginosa, MDR_acinentobacter baumannii (6). Therefore, the need to search for substances from other source with proven antimicrobial activity such as plants increased medicinal (7, 8, 9, 10). Plants would be the best source for obtaining variety of drugs (11) and became it have been used for treatment of different diseases (12). Plant extracts are highly effective because they contain many active substances that have an effective inhibitory in microorganisms such as phenol compound (13). The aim of the present study was to investigate the antibacterial activity of ethanolic extracts of *punica granatum L* and Oregano. VulgreL against extended spectrum beta-lactamases (ESBLs) isolated from UTI patients

Materials and methods

The plant and extract preparation

Punica granatum L and *Oregno.vulgare* L. extraction peals were dried, a weight 40g of air dried peals were carried out by using soxhlet extractor. Powdered dried leave (40g) was extracted with (300ml)of methanol using Rotary Vacuum evaporator for 30 minutes at 60°C.

Bacterial sample

Gram-negative bacterium *E.coil* and *Acinetobacter* is isolated from UTI patient and defined by Misurata center laboratory they Maintained on Mulls Hinton Ayer medium for 24 hours old culture were prepared for three each time.

Antimicrobial susceptibility testing

UTI isolates were tested for antimicrobial susceptibility was determine by disk diffusion tests according to the method Clinical and Laboratory Standards Institute (CLSI)(12). Ceftriaxone,(30g); Amoxicillin-clavulanic acid, (30g); Nalidixic acid, (30 g); Imipenem, (10g); Ofloxacon, (5g); Trimethoprim-sulfamethoxazole, (25g); Amikacin, (30 g); Nitrofurantoin,(300 g); Cefotaxime,(30 g); Gentamicin, (30g); Doxycycline (30g) and a ptrnam at a distance of 19_15 mm center to center on a Muller Hinton agar plate on incubated at 37°C.

Screening for ESBL producing isolates

All isolates were tested for ESBL production by their susceptibility to the third generation cephalosporins, Ceftriaxone (30g),Augmentin(30g) and Cefotaxime,(30g) by using Kirby Bauer disk diffusion method (following the guidelines for CLSI, 2008).The isolates that showed inhibition zone 22 mm for Ceftazidime; 25 mm for ceftriaxone were considered to be probable producers of ESBL. The isolates that showed

resistance to at least one of the two antibiotics (ceftazidime, , ceftriaxone were tested for ESBL production by the double-disk synergy test (DDST method) (14).

Agar diffusion assay

Serial dilution of stock 3 mg/mL were made to 10^{-1}, 10^{-2}, 10^{-3}mg/ml 100 of bacteria suspension in liquid broth (0.5Macfarland stamen) was uniformly inculcated on medium plate. Asterilecork borer of 7 mm diameter made wells on the medium. 0.1 ml of plant extract with different dilution was dropp into each well with appropriate labeled (Shahidi 2004). The plates were incubating at 37°C for 24 hours. Antimicrobial activity was determined by measuring diameter of inhibition zone produced after incubation H2O was use as control.

Minimum Inhibitory Concentration (MIC) assay

Difference Concentra of plant extract stock were prepared 10^{-1}.,10^{-2},10^{-3}mg/ml assayed against the tested bacteria. The MIC was defined as the lowest concentration able to inhibit any visible bacterial growth.

Turbidity test method

0.1 ml of each plant extract concentrations were transferee to nutritious broth 0.9 ml andincubate with 0.1 ml of bacterialthenincubate at 37°C for 42 hrs. The turbidity degree was measure at wavelength 595mn spectrophotometer

Protein extraction

Bacteria culture growing at 37°C overnight had (culture harvesting). 10 min centrifuge then transfer the pellet to microcentrifuge tube then wash the pellet by phosphate buffer for 3 time at 4000 rpm for 5 min. Sanitation 30 sac at centrifugation. Add chloroform then but in ice few minutes then centrifuge for 5 min. Discard top transparent containing RNA remove RNA. Add absolute alcohol then centrifugation for 5 min then remove DNA then collect the supernatant having protein in fresh tube. Add acetone precipitation then mix at vortex. Store at 20°C for at least hour then centrifugation 5 min. Discard. Discard the supernatant and dry the pellet Reconstitute with phosphate buffer. But in ice 20°C overnight

Statistical analysis

Statistical analysis was performed using SPSS software, version 8.0 (SPSS, Chicago, IL). The significance level was tested using ANOVA(Multiple-comparisons) value < 0.05 was considered statistically significant

Result

Table.1 Antibiotics susceptibility pattern of *E. coli* and *Acinetobacter*

Average inhibition diameter mm		
	Types of bacteria	
Antibiotics	***E.coil***	*Acinetobacter*
AMC	0(R)	0(R)
OFX	28 (S)	0(R)
CRO	0(R)	0(R)
F	0(R)	0(R)
NA	0(R)	0(R)
IMP	28 (S)	26 (S)
CN	0(R)	21 (S)
SXT	0(R)	0(R)
AK	9 (R)	0(R)
CTX	0(R)	0(R)
DO	12 (R)	0(R)
CEP	0(R)	25 (S)

Sensitive, S; I, Intermediate; Resistant, R; Nalidixic acid, NA; Ciprofloxacin, CIP; Nitrofurantoin, F;Trimethoprim-sulfamethoxazole, SXT; Amoxicillin-clavulanic acid, AMC; Cefotaxime, CTX; Ceftriaxone, CRO;Imipenem, IPM.Ofloxacin, OFX; Amikacin, AK. Gentamicin, CN; Doxycycline, DO.

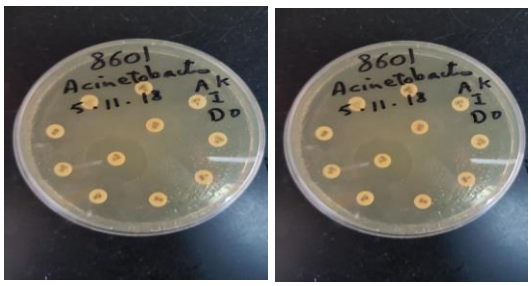
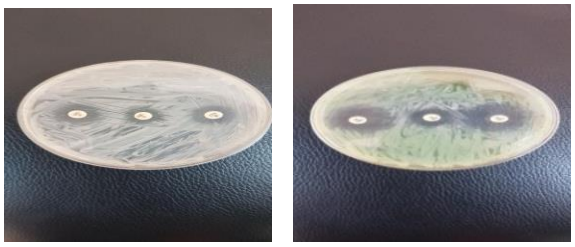


Fig 1: Antimicrobial susceptibility testing with an isolate of *E.coil* and *Acinetobacter*



(a) **(b)**

Fig2: Screening for ESBL producing isolates *(a)E.coil* and (b) *Acinetobacte*

Table3: Diameters of inhibiting of bacteria growthwith treatmentof *Punica granatum* L and *Oregno. Vulgare L.*Extraction

Average inhibition diameter cm						
Types of bacteria	*Punica granatum* L			Oregano. Vulgare L.		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Escherichia coli	25 (S)	15 (S)	11 (I)	0(R)	0(R)	0(R)
Acineto-bacter	23 (S)	15 (S)	3 (R)	0(R)	0(R)	0(R)

Sensitive, S; Intermediate, I; Resistant, R; all isolates sensitive to *Punicagranatum* L **compared with control** Oregno. vulgare L

Table4:Turbidity test methodfor bacterial isolates with treatment of *Punica granatum* L.Extraction at595nm

Types of bacteria	***Punica granatum* L extract**				**F**	**P-value**
	10^{-3}	**10^{-2}**	**10^{-1}**	**Control**		
E.coli	0.389*	0.286*	0.2*	0.487	58.6	0.000
Acineto bacter	0.384*	0.297*	0.219*	0.472	53.73	0.000

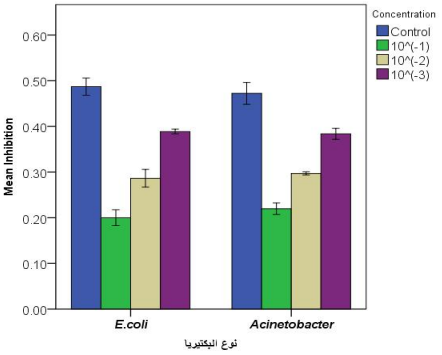


Fig:3 Turbidity test methodfor bacterial isolates with treatment of *Punica granatum* L.Extraction at595nm

Table5:Turbidity test method for bacterial isolates with treatment of *Oregno. vulgare* L.Extraction at595nm

Types of bacteria	***Punica granatum* L extract**				**F**	**P-value**
	10^{-3}	**10^{-2}**	**10^{-1}**	**Control**		
E.coli	0.424*	0.486	0.429*	0.526	9.850	0.005
Acineto bacter	0.427	0.495	0.531	0.489	4.673	0.036

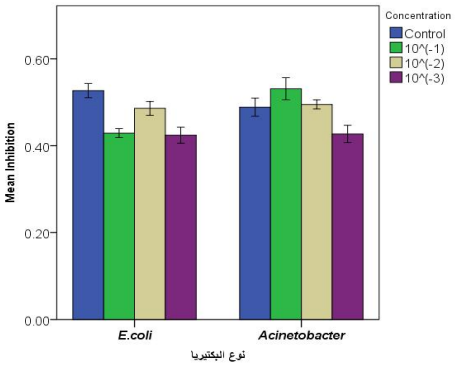


Fig4: Turbidity test method for bacterial isolates with treatment of *Oregno. vulgare L.*Extraction at595nm

Table6: Effect of *Punica granatum* L.extract on the number of colonies

Types of bacteria	***Punica granatum* L extract**				**F**	**P-value**
	10^{-3}	**10^{-2}**	**10^{-1}**	**Control**		
E.coli	246.7*	140.7*	6.67*	300>	127.9	0.000
Acineto bacter	300	291.7*	201*	30>	455.9	0.000

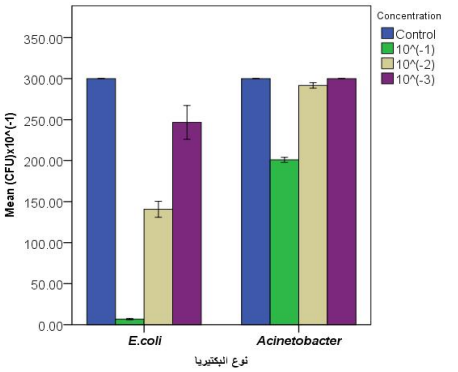


Fig5: Effect *Punica granatum* L. extract on the number of colonies.

Table7: Effect of *Oregno. vulgare* L.**extract on the number of colonies**

Types of bacteria	***Punica granatum* L extract**				**F**	**P-value**
	10^{-3}	**10^{-2}**	**10^{-1}**	**Control**		
E.coli	260.7	300	194.3*	300>	8.606	0.007
Acinetobacter	300	287.7	240	300>	3.560	0.067

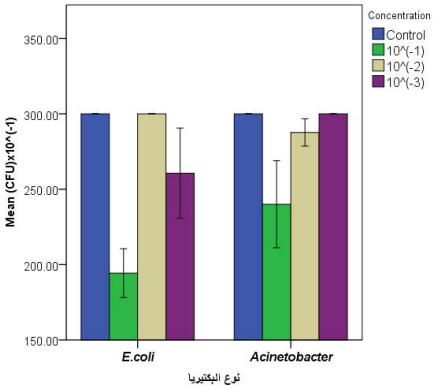


Fig6: Effect of *Oregno. vulgare* L.extract on the number of colonies

Table8:Effect of *Punica granatum* L extract on the level of protein concentration in bacterial isolates treated with different concentrations

P-value	F	*Punica granatum* L extract				Types of bacteria
		10^{-3}	10^{-2}	10^{-1}	Control	
0.000	22.58	0.319*	0.207*	0.111*	0.482	*E.coli*
0.000	24.95	0.366*	0.333*	0.169*	0.470	*Acinetobacter*

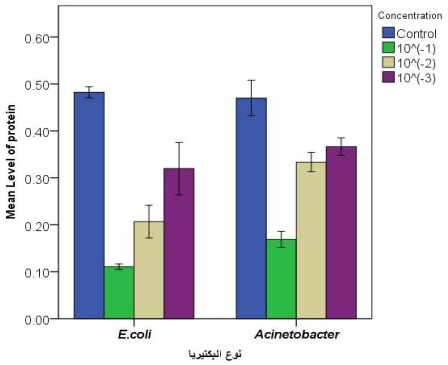


Fig7: Effect of *Punica granatum* L extract on the level of protein concentration in bacterial isolates treated with different concentrations

Discussion

ESBL-producing bacterial are frequently renitent to many of antibiotics making them difficult to treat their infections . They are resistant to penicillin ,first, second and third generation antibiotic such as cephalosporin and aztreonema (15,16). The susceptibility of bacterial isolated in this study were tested Bauer disk diffusion method which include ; Nalidixic acid, Ciprofloxacin, Nitrofurantoin, Trimethoprim-sulfamethoxazole,; Amoxicillin-clavulanic acid, Cefotaxime, Ceftriaxone, Imipenem, Ofloxacin, Amikacin,. Gentamicin, and Doxycyclinethe resent in (Fig1,Table1) showed that *E.coli* isolated are resistant to Nalidixic acid, Ciprofloxacin, Nitrofurantoin, Trimethoprim-sulfamethoxazole,; Amoxicillin-clavulanic acid, Cefotaxime, Ceftriaxone, Imipenem,. for all tested except Imipenem,.Ofloxacin,; Amikacin. *Acinetobacter* isolated are resistant to most antibiotic was tested except Imipenem. The studied UTi isolate are resistant to to all cepholospne and canbopenum antibiotic and considered to be probable produced of B-Lactamasemclude different types of ESBLs. In this study tested ethanolic extracts of *punica granatum* peels and *Oregano. vulgare* L for their antibacterial activity against multi drug, ESBLs strains of UTI isolated. Agar diffusion assay table showed that (Table2) had antibacterial activity against all tested UTI isolated. This potent activity was concentration dependent. Decrane in diameter of inhibition zone was observed by decrease of plant extract concentration from 10^{-1}.,10^{-2},10^{-3}. Maxim inhibition of bacterial growth was recorded by 10^{-1} concentration (25cm and 23cm) for *E.coil* and *Acinetobacter* respectively (Table3). The result showed also that Oregano ethanolic plant extract has no effect on bacterial growth. The antibacterial aexivity *punica granatum* was confirmed by turbidity test which showed decrease in O.D595 in bacterial culture treatment with high plant extract concentration. O.D595 were *E. coli* 0.2, 0.286, 0.389 *Acinetobacter* 0.219, 0.297, 0.384 for treatment concentration 10^{-1}.,10^{-2},10^{-3} respectively as table 4 showed

In this paper the plant extracts of *punica granatum* had a negative effect on the level of proteins in the treated buffer at all tested concentrations 10^{-1}.,10^{-2},10^{-3} were result, *E. coli* 0.111,0.207,0.319 *Acinetobacter*. 0.169,0.333,0.366 respectively as table 4 showed, plant extracts interaction with target such as Inhibition of ribosome 50s of Gram negative bacteria same mechanism of lincosamide and Some others are linked with ribosome 30s who produce Purine as gentamicin or prevent aminoacylt-RNA access to ribosome thus, inhibition Protein(15). This may be due to the inclusion of these extracts on flavonoids and phenols at high levels which play a role in the protein denature

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