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Study the Effect of Pomegranate (*Punica granatum*) Peel Extracts on Some Physiological Characters of *Pseudomonas aeruginosa*

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Abstract: ***A total of twenty isolates of Pseudomonas aeruginosa were gained after their diagnosis biochemically, APi 20 system and PCR technique. These isolates were isolated from patients with different infections. They were tested to detect their ability to form biofilm, and the strongest one was chosen (named N20) to complete this study which aimed to study the effect of pomegranate (Punica granatum) peel aqueous and alcoholic extracts in addition to Imipeneme antibiotic on biofilm, swarming motility and pyocyanin production. Results showed high decrease in biofilm values by using these extracts especially in case of biofilm formation rather than degradation. MIC values were detected in order to determine sub-MIC values that were used in this study. MIC was 25 mg/ml for aqueous extract, while alcoholic extract was more effective and it's MIC was 12.5mg/ml. Imipeneme MIC was 64µg/ml which is considered very high value and indicate N20 resistance to it. Both swarming motility and pyocyanin production were inhibited by addition of these extracts and antibiotic separately to swarming media and Nutrient agar respectively when comared with control plates.***

Keywords: *Pseudomonas aeruginosa* , Biofilm, Swarming motility, Pyocyanin

1. Introduction

Pseudomonas aeruginosa is a an organism that is must be focused on because of its serious role in disease. Numerous environmental niches can be exploited by this bacteria due to its flexible metabolic capabilities and relatively large genome. It is an opportunistic pathogen that begins its infection in the human host when the normal immune defenses are disabled (1). Once established in the patient, *P. aeruginosa* becomes difficult to treat making therapy against this pathogen particularly challenging due to the lack of novel antimicrobial therapeutics (2), in addition to that, the genome of this bacteria encodes a host of resistance genes, including multidrug efflux pumps (3) and enzymes conferring resistance to aminoglycoside and beta-lactam antibotics (4). This challenge is compounded by the ability of *P. aerugionsa* to grow in a biofilm, in which bacteria in organized communities are usually encased in an extracellular polymeric substances (EPS) matrix that hold microbial cells together to a surface which, in turn, protect bacteria from host defenses and chemotherapy and enhance its ability to cause infections (5,6). In order to face these limitations, the recent surge in multidrug-resistant bacteria necessitate the need for alternative therapeutic options to conventional drugs. It was indicated that pomegranates and their extracts can serve as natural alternatives due to their wide range antiviral and antibacterial potency. Antimicrobial activities of the fruit juice, peel, arils, flowers, and bark of pomegranate plant had been tested and many studies have utilized pomegranate peel with success (7).

Methanol extracts, especially of the peel, exhibit the broadest antibacterial activity (8,9), which can vary depending on the pomegranate variety tested (10). Methanol extracts of pomegranate are high in hydrolyzable tannins (punicalins and punicalagins), and gallic acid, a component of gallotannins, in addition to ellagic acid, which is a component of ellagitannins (11). Pomegranate showed positive effects on both pathogenic and probiotic bacteria, as well as shows protection against pathogenic bacteria that are responsible food poisoning that is, in turn, gives promise in preservation of food (7).

Besides, pomegranate exhibited wide bactericidal activity against numerous pathogenic bacteria including *Listeria monocytogene* , *Yersinia enterocolitica*, *Salmonella typhi*, *Vibrio cholera*, *Shigella* spp., *Staphylococcus* spp., *E. coli*, *Klebsiella* spp and others (12,13,14,15). So the aim of this study was to evaluate aqueous and alcoholic pomegranate peel extracts against some *P. aeruginosa* physiological properties.

2. Materials and Methods

1) Isolation and diagnosis of bacteria

Twenty isolates of *P. aeruginosa* were obtained from different local hospitals in Iraq from patients with different infections. All isolates were used after their diagnosis biochemically then confirmation by Api20E system and PCR technique were done before obtaining these isolates. The isolated bacteria used in the study were chosen according to their ability to give the strongest biofilm.

2) Ability of isolated bacteria to produce biofilm.

This method was applied on the obtaineded isolates according to (16). In brief; the bacterial cells were grown in Nutrient broth overnight at 37°C under aerobic conditions.

A suspension of bacterial isolate that equivalent to the McFarland No.0.5 turbidity standard were inoculated in Nutrient broth and incubated for 18-24 hours at 37ºC in individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plate in stationary condition.

200 µl of Nutrient broth containing *P. aeruginosa* which corresponds to an inoculum of approximately 1.5×10^8 cells/well was transferred to the assay wells. Each plate was covered with the lid supplied by the manufacturer. Subsequently, inoculated assay plates were transferred to an

incubator set at 37°C for 18–24 h. Negative control wells contained sterile Nutrient broth . After incubation, assay plates were uncovered and liquid culture was removed from each well, and non-adherent bacteria were removed by washing each well 2-3 times with D.W. Fixation of adherent cells was accomplished by methanol (125 µl) for 10 min.

Biofilms were stained by adding 125 µl of 0.5% crystal violet to each well for 15minutes. After the staining reaction has been completed, excess stain was removed by repeated washing (2-3 washes) with D.W. as described above. Afterwards, 125µl of 33% glacial acetic acid was added to each well for 10 minutes. All assays were done in triplicates.

The amount of crystal violet extracted by the glacial acetic acid in each well was directly quantified spectrophotometrically by measuring the OD_{630} using amicroplate reader (17).

3) Preparation of pomegranate peel extracts (PPE):
Pomegranate peel powder was obtained from local markets of herbes. 10 g of powder was extracted with 50 ml boiled distilled water at room temperature for 1 h. The clear extract obtained was centrifuged at 5,000 rpm for 10 min and sterilized by 0.22 µm filter (18). For alcoholic extract, absolute ethanol was used by adding 5g of powder to 25ml ethanol, left for 1 hour, also centrifuged and left in 60ºC until solvent drying. From each extract, 200 mg/ml was prepared as stock solution . The filtrates were used to determine antibacterial and antibiofilm activity.

4) Determination of minimal inhibitory concentration (MIC)
Double serial dilutions (16-2048µg/ml) were prepared form a stock solution of Imipeneme antibiotic according to directions wrote on antibiotic vial, in addition to positive and negative controls. 100 µl from10^8 CFU/ml bacterial suspension was added to all tubes except negative control tube and incubated at 37°C for 24hr. The lowest concentration that inhibit bacterial growth was considered as the MIC (19). Pomegranate peel extracts concentrations were also tested as serial dilutions by preparing 100, 50, 25,12.5, 6.25, 3.125 (mg/ml) in order to determine MIC of these extracts.

5) Detection the effect of extracts on bacterial motility
Both extracts were used to determine their ability to inhibit swarming motility of *P. aeruginosa*. Swarming medium was consisted from: 0.8% Nutrient broth, 0.5% agar and 0.5% glucose. This medium was sterilized by autoclave (without sugar which was sterilized by autoclave for only 10 min. and then it was added to that medium (20). Aqueous, alcoholic extracts and antibiotic (Imipeneme) were added to plates separately and were mixed with media before solidification. After period of time, these plates were approximately solidified and were cultured by spotting bacteria at the centre of media. All plates were incubated at 37ºC for 18- 24 hr.

6) Detection the effect of extracts on pyocyanin production
Extracts were used to detect their efficacy to inhibit pyocyanin production. Nutrient agar plates were prepared. Extracts and antibiotic were added separately to media and mixed together before their solidification. All plates were cultured by streaking and were incubated at 37ºC for 18- 24 hr.

7) Effect of extracts and antibiotic on bacterial biofilm.
Sub-MIC of extracts and imipeneme was added to bacteria both separately and mixed to evaluate their ability to form biofilm (by adding PPEs and antibiotic to bacteria in microtiter plate wells directly before incubation period) and ability to destroy the composed one (by adding PPEs and antibiotic after first incubation period which permit bacterial biofilm to form, and then reincubation of microtiter plate was done for the second period.

8) Statistical Analysis
The Statistical Analysis System- SAS (2012) program was used to effect of difference factors in study parameters. Least significant difference –LSD test (ANOVA) was used to significant compare between means in this study (21).

3. Results and Discussion

From current results, the strongest biofilm producer isolate had been chosen to complete this study which was named as (N20).

MIC values of extracts and Imipeneme.
Results showed that no bacterial growth was observed at concentrations 100, 50 and 25 (mg/ml) for aqueous extract and sub-MIC was 12.5 mg/ml. While, for alcoholic extract, *P. aeruginosa* N20 did not grow in concentrations 100, 50, 25, 12,5 (mg/ml), and sub-MIC would be 6.25 mg/ml. Finally, MIC for Imipeneme antibiotic was 64µg/ml and sub-MIC was 32 µg/ml. These results can be shown in table 1.

Table 1: MIC and sub-MIC values for extracts and antibiotic against *P. aeruginosa*

Treatments	MIC values	Sub-MIC values
Aqueous extract	25 mg/ml	12.5 mg/ml
Alcoholic extract	12.5 mg/ml	6.25 mg/ml
Imipeneme	64µg/ml	32 µg/ml.

From these results, it appears that alcoholic extract was more effective than the aqueous extract because of the values of MIC and sub-MIC of alcoholic extract was lower than those of aqueous extract against *P. aeruginosa* N20. This can be due to that methanol extracts of pomegranate are high in hydrolyzable tannins (punicalins and punicalagins), gallic acid, a component of gallotannins, in addition to ellagic acid which is a component of ellagitannins (11). Pomegranate peel extracts are rich sources of alkaloid, tannins, saponins, phenolic, flavonoids, resinsins which is considered a phytochemical, polyphenols, cyanidins and some anthocyanins as delphinidines, in addition to glycosides. In a local study done by Al- Wazni and Hadi (2015), it was found that the saponins and flavonoids were absent in aqueous extract, but during ethanol extraction, only saponins were not extracted (22, 23).

The active material in the pomegranate peel needed the polar solvent in order to be extracted in a good way and that is, in

turn, mean that most of these materials were extracted in good amount by the ethanol more than their amount extracted in distilled water. Type and amount of the active material that extracted depends on several extraction conditions such as type and concentration of the solvent, time and temperature for the
extraction process (24) which are responsible for the antibacterial activity of plant extracts. On the other hand, the mode of action of these compounds such as polyphenols is generally attributed to polyphenol-protein interactions, though different mechanisms have been suggested including microbial enzymes inhibition and action on membranes
or deprivation of substrates were required for microbial growth (25). Current results can be agreed with those obtained by (22) from view of that alcoholic extract had more antibacterial efficacy than the aqueous one, but disagreed with them in MIC of extracts, which were 60 mg/ml for aqueous extract and 40 mg/ml for alcoholic extract against *S. aureus* and *E. coli*.

Results of this study showed that the pomegranate peel extracts had more effectiveness against *P. aeruginosa* N20 than antibiotic Imipeneme for which bacteria showed high resistance against it, and MIC was 64µg/ml that is considered to be very high value when compared with values of CLSI that says bacteria are considered as resistance when MIC is ≥16 for Imipeneme antibiotic (26).

Effect of PPEs and Imipeneme on *P. aeruginosa* N20 biofilm.

Both PPEs showed high effect in biofilm formation inhibition when compared with +ve control and reduced biofilm more than ten times, while in biofilm degradation, it was appeared that aqueous extract of pomegranate peel wasn't effective when compared with activity of alcoholic extract on biofilm degradation as shown in table 2.

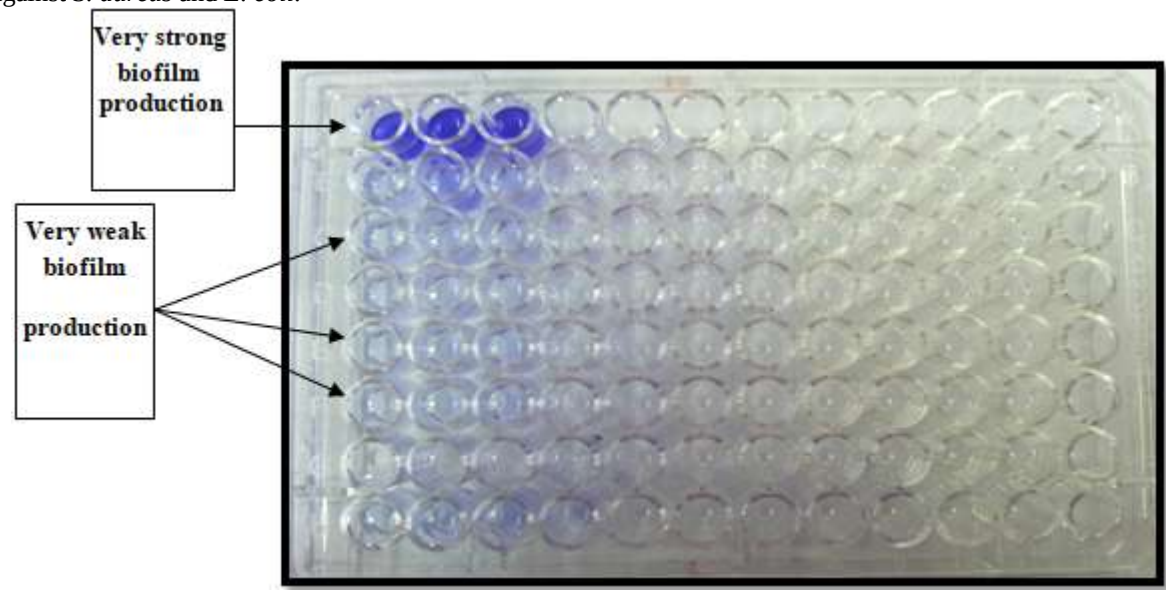

Figure 1: Detection of *P. aeruginosa* N20 in microtiter plate with addition of PPEs and Imipeneme

Table 2: Effect of treatments on Biofilm results

Treatments	O.D for formation	O.D for Degradation	LSD value
1- Bacteria + Nutrient broth (+ ve control)	0.760 ± 0.08 A	0.368 ± 0.04 A	0.109 *
2- Bac+ Aqueous extract	0.089 ± 0.007 B	0.254 ± 0.03 A	0.064 *
3- Bac+ Alcoholic extract	0.068 ± 0.005 B	0.070 ± 0.005 B	0.029 NS
4- Bac+ Antibiotic	0.061 ± 0.005 B	0.069 ± 0.003 B	0.022 NS
5- Bac+ Aqu+ Anti	0.068 ± 0.004 B	0.070 ± 0.008 B	0.026 NS
6- Bac+ Alco+ anti	0.067 ± 0.00 B	0.062 ± 0.005 B	0.031 NS
7- Nutrient broth (- ve control)	0.084 ± 0.007 B	0.080 ± 0.007 B	0.027 NS
LSD value	0.183 *	0.138 *	----

* (P<0.05), NS: Non-Significant.
Means having with the different letters in same column differed significantly.

It is obvious from table 2 that addition of sub-MIC of extracts to bacteria gave results nearly similar to that obtained from addition of sub-MIC of Imipeneme, and also addition of antibiotic to mixtures of bacteria and extracts didn't gave a significant effect in both biofilm formation and degradation. This is may be due to it's less effect on the studied bacteria which showed high resistance towards this antibiotic, which return to several mechanisms like multidrug efflux pumps (3) and enzymes conferring resistance to aminoglycoside and beta- lactam antibotics (4), in addition to ability of *P. aerugionsa* to grow in a biofilm, in which bacteria in organized communities are usually encased in an extracellular polymeric substances (EPS) matrix that hold microbial cells together to a surface which, in turn, protect bacteria from host defenses and chemotherapy and enhance its ability to cause infections (5,6).

As a comparison, ethanol extract was the best anti biofilm factor while the aqueous extract had the less effect

especially in biofilm degradation which showed significant decrease on biofilm formation when compared with it's activity on degradation. This may return to the weak ability of distilled water to extract the active materials from plant peel in affected amounts in compare with alcohol solvent. Effectiveness of PPEs in biofilm inhibition can be referred to their anti –adhesive ability, because of the large amounts of the flavonoids, saponins, tannins, alkaloids, phenolic, glycosides and resinsin, which are considered to be directly responsible for the anti-adhesive activity against pathogen (27). The effect of PPEs is return to their ability to inhibit cell attachment, which is the first step in bacterial biofilm formation (28). Researches found that bacterial exopolysaccharides have always been suggested to play crucial roles in the bacterial initial adhesion and development of complex architecture in the later stages of bacterial biofilm formation (29, 30). Therefore; pretreatment of the body surface with plant extracts produced an unfavorable film which can prevent and reduce the surface adhesion of pathogenic bacteria (28).

On the other hand, it is known that flavonoids such as quercetin, kaempherol, naringenin and apigenin reduce biofilm synthesis because they can suppress autoinducer-2 activity which is responsible for cell-to-cell communication and regulation of quorum sensing (31). Also, it was found that pomegranate peel extracts led to the loss of curli fimbrae, and can repress flagella that associate in bacterial biofilm (32). As a conclusion, the results obtained in our study suggest that anti-biofilm effect of plant extracts can be caused by modifications in the bacterial surface structures responsible for binding to the occupied surface.

Effect of extracts on swarming motility of *P. aeruginosa* N20

Current results showed that addition of extracts gave a result similar to the addition of Imipeneme and that all of them showed negative result of swarming, at the same time they led to distribution of growth all over the plate (as shown in figure 2 and 3), which may be referred to partially liquification of swarming media and growth distribution of N20. Inhibition of swarming motility could be because partial repression of flagella function that is essential for swarming motility (20). It was found that type IV pili are required for swarming motility in *P. aeruginosa* (33) which can be repressed by current additions. D´eziel et al. (2003) revealed that one of the virulence factors in *Pseudomonas*, which is rhamnolipid biosurfactant has the ability to act as a swarming modulator (34).

From other side of view, swarming inhibition of N20 by the PPEs and antibiotic can be explained by the suppression the production of surface materials including exopolysaccharide and lipopolysaccharide, which are probably involved in swarming motility in *P. aeruginosa* (20).

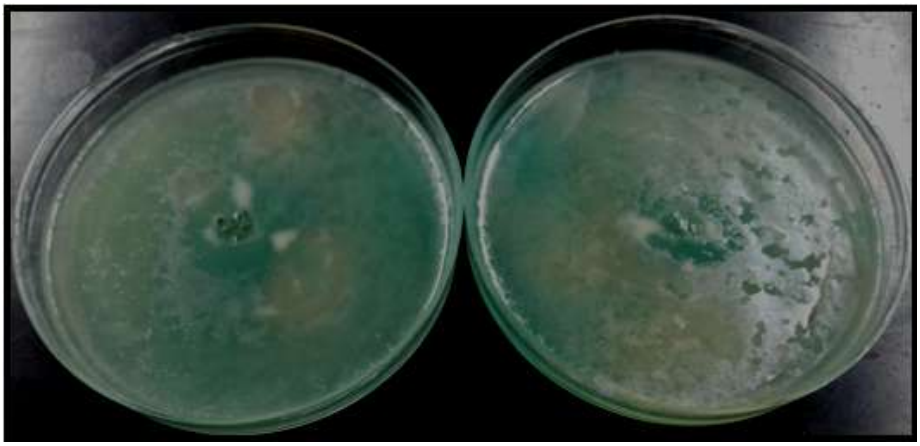

Figure 2: Inhibition of swarming motility by addition of aqueous extract on the left and alcoholic extract on the right, (antibiotic showed similar effect)

Figure 3: Swarming motility of N20 without additions.

Effect of extracts and antibiotic on pyocyanin (PCN) production.

It was appeared from our results that addition of PPEs and Imipeneme separately to nutrient agar medium resulted in inhibition of pyocyanin production and no changes in color of this medium was shown when compared with control plate that is empty from any treatments except bacteria. These results can be shown in figure 4.

Figure 4: Inhibition of pyocyanin production by addition of tested materials (all of them gave the same result) on the left, and control plate on the right

Recently, pyocyanin had emerged as an important virulence factor which is produced by *P. aeruginosa*. The redox-active tricyclic zwitterion had been shown to have a number of potential effects on various organ systems *in vitro*, including the respiratory, cardiovascular, urological, and central nervous systems. It had been shown that a large number of the effects to these systems are via the formation of reactive oxygen species (ROS) (35). So, addition of the tested materials in current study may lead to inhibition of reactive oxygen species production which can reduce bacterial toxicity and inhibit PCN production. Because of little studies on PCN production, it is important to study the factors that inhibit PCN production *in vitro* and *in vivo* and be highlighted because of it's virulence.

4. Conclusion

As a conclusion, PPEs can be used in treatment of *P. aeruginosa* infections especially that caused by biofilm formation which results in multidrug resistance, in addition to inhibit motility of this bacteria which is important for bacterial infection distribution, and finally, this study recommends to use these extracts to prevent pyocyanin production which is considered as a toxin, and that in turn, can reduce the virulence of this bacteria.

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