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Effect of Pomegranate Peel Extract on the Production of Some Enzymes in Proteus spp Isolated from Different Clinical Samples in Kirkuk city

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Abstract

The study investigates the effects of pomegranate peel extracts on some virulence factors such as the production of some enzymes produced by Proteus spp isolated from different clinical samples. Among 310 different clinical samples, 48 isolates of proteus were obtained, 14 isolates(29.16%)were obtained from urine, 15 isolates (31.25%) were obtained from stool, 7 isolates (14.58%) from ear swabs, 4 isolates (8.33%) were obtained from wounds, 5 isolates (10.41% (from burns and 3 isolates) 6.25%) of the high cervical. The study showed that from 48 isolates of Proteus spp 25 isolates (52.08%) identified as P.mirabilis, 15 isolates (31.25%)of P. penerri, while 8 isolates of P. vulgaris were obtained with a percentage of (16.66%) and it was found that 18 isolates (72%) of P.mirabilis isolates were positive for the production of beta-lactamase enzyme, 24 isolates (96%) are positive for proteases and 14 isolates (56%) produced lipase, while 13 isolates from P. penerri (86.66%) produced beta-lactamases and proteases and 10 isolates (66.66%) produced lipase, also found that 5 isolates (62.5%) of P. vulgaris produced Beta-lactamase and all the isolates (100%) produced protease while 7 isolates (87.5%) produced lipase. It was found that the hot aqueous extract of pomegranate peels inhibited the production of beta-lactamase enzyme in 4 out of 7 isolates that produced this enzyme, while all tested isolates did not produce lipase by using extracts of pomegranate peel extracts. The isolates lost their ability to produce protease enzyme, except for two isolates that were not affected by extracts of pomegranate peel fruits.

1. Introduction:

Proteus spp is Gram-negative bacilli that are highly motile most of the time, and their shape may change. They have the property of seizing the culture medium in successive concentric waves, and these waves form a membrane with which it is impossible to obtain single colonies [1]. The (Swarming) gives a weighted guess to determine the identity [2] and all species of proteus characterized by the production of highly effective urease enzym [3].

Proteus spp is present on the decaying tissues of animals. It is abundant in sewage, soil [4], and that it is a normal flora in the digestive system of humans and some animals [5] [6]. It is similar to the characteristics of Enterobacteriaceae. It ferments some sugars such as glucose, mannose, sucrose, and galactose. It also does not ferment lactose and is facultative anaerobic [3],[7].

Beta-lactamase enzymes have received great attention from researchers, as they are the cause of the failure of many antibiotics used to control various infections, especially among hospitalized patients. These enzymes attack the beta-lactam ring present in the nucleus of penicillins and cephalosporins it is breaking the amide bond in them to turn these antibiotics into an inactive compound[8].

Protease enzymes are one of the virulence factors for proteus[9]. One of these types of protein-degrading enzymes is the Metalloprotease enzyme, where this enzyme works to

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analyze proteins that contain metal ions in their composition, such as Na+, K+, which are essential in pathogenesis [10] Proteus secretes the enzyme lipase[11][12], which includes lipolytic enzyme (general) Lipases and specialized enzyme phospholipases. Lipase enzymes work to break down the ester bonds that bind fatty acids with glycerol, producing a molecule of glycerol and fatty acids [3]. The study aimed to investigate the ability of pomegranate peel extracts to influence the production of some enzymes produced by Proteus spp isolated from different clinical samples.

2. Materials and Methods:

2.1 Sample Collection and Culturing:

The study included the collection of urine samples, stool, high cervical swabs, ear swabs, wounds, and burns from patients admitted to Kirkuk General Hospital and Azadi Teaching Hospital in Kirkuk city. 310 samples were collected. Samples were transferred to the laboratory for isolation and diagnosis of bacteria, according to the methods approved in [3][13][14]. Samples were collected according to what was mentioned in [15]. The diagnosis was confirmed using the Rapid ONE system.

2.2 Beta-lactamase Production:

The test was used to detect the ability of Proteus spp to produce this enzyme, the iodometric method used as stated in [15]. (0.1) of Penicillin G solution at a concentration of (6)mg/ml was added to test tubes containing the bacterial suspension at the age of (18-24) hours. The tubes were shaken and the mixture was left for one hour at a temperature of 37°C. Two drops were added to each tube of the prepared starch solution, then a drop of iodine reagent solution was added to each tube. The tubes were mixed well and left for (5) minutes at laboratory temperature. The disappearance of the dark blue or violet colour within less than 10 seconds indicates the positive result for the production of b-lactamase enzymes.

2.3 Protease production:

The isolates were cultured on a medium of fermentation and oxidation medium. The dishes were incubated at a temperature of (37) C for (24) hours. The appearance of a clear zone around the developing colonies indicates the production of the protease enzyme[15].

2.4 Lipase production:

Lipase production medium inoculated with young colonies of Proteus isolates and incubated at (37) oC for (24-48) hours. The production of lipase enzyme was detected by immersing the medium in a sufficient amount of CuSO4 saturated solution for (20) minutes, then the dish was dried after removing the excess of the solution by placing it in the incubator for a short period. The appearance of blue-green colour around the colonies indicates the activity of the lipase enzyme [15].

2.5 Collection and preparation of pomegranate peel:

Pomegranate fruits were obtained from the local markets in the Kirkuk city, the peels were separated from the rest of the fruit parts, and then the peels were sterilized with a solution of sodium hypochlorite at a concentration of (1%) free chlorine.[16] was then washed with sterile distilled water, dried with a dry paper, and dried using an electric oven at a temperature of 50 °C for 48 hours, then it was ground using an electric grinder until a fine powder was obtained and kept in plastic containers until use.

2.6 Pomegranate peel extracts preparation:

2.6.1 Alcoholic extract:

The alcoholic extract was prepared using ethanol alcohol 98% as a solvent [17]. The extract was prepared when fruit was purchased from the local market at Kirkuk City. One kilogram of fresh peel was cut into small pieces then mixed in a mechanical blender with 98% ethanol and left at room temperature for 72 h. The extract was filtered through a filter paper and concentrated to dryness under reduced pressure in a rotary evaporator at 40–50oC.

2.6.2 water extract:

The cold and hot aqueous extract was obtained according to what was mentioned in the previous paragraph with the replacement of alcohol with water

2.6.3 chloroform extract:

The chloroform extract was prepared as mentioned in the (previous) paragraph, except for using chloroform 98% as a solvent instead of water, and after mixing the extract with a magnetic stirrer it was left for several hours at room temperature. Three separated parts appear. These parts were then dried in an electric oven at a temperature of 50°C, and the sensitivity of the isolates was tested for each of them.

2.7 Detection of alkaloids:

Dissolve 5 g of the sample in 200 ml of a mixture of acetic acid and ethanol in a ratio of (15:1) and gradually add ammonium hydroxide until the precipitate is formed, filter the solution, dissolve the precipitate, filter again and separate the precipitate, which represents the alkaloid[18] .

2.8 Detection of flavonoids:

10 g of the plant sample was added to 100 ml of 80% methanol alcohol and filter the solution with filter paper. Whatman NO 42 extract evaporated and the weight of the precipitate representing the flavonoids present in the sample [18].

2.9 Sensitivity test to pomegranate peel extracts:

Agar well diffusion method was used in this test [19]. The agar plate surface is inoculated by spreading a volume of the microbial inoculum equivalent to 0.5 MacFarland tube over the entire agar surface. Then, a well with a diameter of 6 mm is punched with a sterile cork borer and a volume (70

mL) of the extract is added to the well. Then, agar plates are incubated in an incubator.

2.10 Effect of pomegranate peel extracts on the production of beta-lactamase enzyme:

The sub MIC of pomegranate peel extracts mixed with the nutrient broth medium and inoculated with the isolates under study and placed in the incubator at a temperature of (37°C) for (24) hours, Then the production of beta-lactamase enzymes was detected.

2.11 Effect of pomegranate peel extracts on production of Protease:

The sub MIC of pomegranate peel extracts mixed with the medium to investigate the production of protease enzyme and inoculated with the isolates under study and incubated at a temperature of (37°C) for (24) period, the production of protease enzyme was detected in the presence of clear areas around the colonies

2.12 Effect of pomegranate peel extracts on the production of lipase enzyme:

The sub MIC of pomegranate peel extracts mixed with the medium for detecting lipase production and inoculated with the isolates under study and incubated at a temperature of (37 C) for 24 hours. The medium was lled with a suf cient amount of a solution saturated with copper sulfate $CuSO_4$ for (20) minutes, then the plate was dried after removing the excess of the solution by placing it in the incubator for a short period. The appearance of greenish-blue colour around the developing colonies indicates the activity of the lipase enzyme.

3. Results:

310 samples were obtained from people who were suffering from various diseases, from those who were admitted to Kirkuk General Hospital and Azadi Teaching Hospital, from both sexes. The samples included. Urine, stools, ear swabs, wounds swab, burn swabs and high cervical dried swabs, as shown in Tabel 1.

Table 1. numbers and percentages of samples.

samples			
	type	Numbers	%
1	urine	143	64.12
2	Stool	25	8.06
3	Ear swab	58	18,70
4	wounds	26	8,38
5	Burns	23	7,41
6	high cervical	35	11,29
	Total	310	

Various tests were carried out on the isolates under study, which showed the characteristic of swarming on blood and non-lactose fermenting on McConkey medium, which produces the smell of rotting sh, and it appeared under the microscope in the form of the gram-negative bacillus of varying lengths.

To con rm the diagnosis, a RapID ONE system was used supplied by (Remel company) which is characterized by ease and speed of diagnosis, and the results were identical and con- rmed to the results of biochemical tests. from 310 samples 48(15.45%) Proteus spp isolated for different clinical samples as shown in Table 2 , whereas in another study the percentage of Proteus by 4% were isolated[20].

Table 2. Number and percentages of Proteus spp in different clinical samples.

	Samples	No.	%
1	Urine	14	29.16
2	Stool	15	31.25
3	Ear swab	7	14.58
4	Wound	4	8.33
5	Burns	5	10.41
6	high cervical	3	6.25
	Total	48	

After the diagnosis, it was noted that the isolates of P.mirabilis were the most common as its number reached 25 isolates with a percentage (52,08%) of the total 48 Proteus isolates. As for P. penerri isolates, it reached 15 isolates (31,25%), and the number of P.vulgaris isolates reached 8 (16,66%).) as in Figure 1.

Figure 1. Percentages of the Proteus spp isolates understudy.

The results of this study showed the prevalence of the Proteus mirabilis in urine, stool and vaginal swabs compared to P. penneri and P. Vulgaris, as shown in Table 3. As the percentage of this species in urinary tract infections was about (64.28%), while P.penneri and P.vulgaris percentages were about (21.42%) (14.28%), respectively, and these results are very close to the results of [21], who indicated that P.

mirabilis more common than the P. vulgaris and P.penneri in clinical samples.

Proteus was isolated in a percentage (31.25%) of the diarrhoea cases, and this result was higher than the one obtained from the study of [22], where isolated this bacterium from diarrhoea cases with a percentage of (7.11%). The most common proteus species of cases of diarrhoea are P. mirabilis, which represents about (53.33%), the high percentage of Proteus in cases of diarrhoea may be attributed to being part of the normal ora found in the digestive system, as P. mirabilis is present in a (17%) and P. vulgaris in a percentage (5%) in the stooles of healthy people [23] and that the Alkaline environment of the intestine helps to multiply and increase the normal ora in it, but the excessive reproduction of bacteria as a result of the weakness of the body may lead to the occurrence of infections of the gastrointestinal tract and thus the occurrence of diarrhoea which maybe because of the entrance of proteus with foods or drinks in the absence of personal and general hygiene.

Concerning ear infections, the percentage of Proteus spp isolates that appeared in this study is (14.58%) of the total 48 isolates of Proteus spp and this result is close to the results of [24], as the Proteus spp were isolated from ear infections with percentages (12.9%), that the high percentage of isolation that was obtained may be due to the presence of several factors that contribute to ear infection, including infection of the upper respiratory tract with viruses that lead to blockage of the Eustachian Tube and thus uid collects inside the ear, and any small wound resulting from excessive cleaning of the ear canal and using a cotton swab or any sharp tool prepares the situation for bacterial growth in addition to the use of earplugs and headphones continuously leads to an increase in the incidence of infection

The results showed the isolation of Proteus spp from the female reproductive system with a percentage of (6.25%), which was represented by isolation of P. mirabilis and P. vulgaris with percentages (33.33%) and (66.66%) respectively, because this bacterium is one of the bacteria present as a normal ora in the human digestive system, due to the proximity of the anus to the female genital, which leads to exposure to infections by the contaminating bacteria for that region, just as the vagina and cervix are in a normal acidic environment due to the presence of the normal bacterial ora in them, but as a result of the menstruation, the PH changes to alkaline, which increases the chance of infection by opportunistic bacteria, and these results are higher than the results obtained by [25], which isolated Proteus spp from the female reproductive system with a percentage of (4.9%).

The results for wound infections showed isolation of Proteus spp with a percentage of (8.33%), which belongs to the two species P. mirabilis and P.penneri, and this result is almost close to what was reached [26] which isolated Proteus by (13.6%), and these percentage differences may be due to the difference in the geographical location of the source of the samples. The incidence of wound infection may be attributed to the high sensitivity of the exposed area of the wound to microbial invasion, whether the wound was spontaneous or resulting from a surgical operation. Just as the wounds become more susceptible to infections by not following the correct methods in terms of hygiene, and by not paying attention to sterilization rules by hospital staff, as well as other factors related to the personal hygiene of the patient himself and the medical staff.

About burn infections, Proteus bacteria were isolated with a percentage of (10.41%), and this result is less than the result obtained by [27] which isolated Proteus bacteria from burns with a percentage of (24%).

The production of the enzymes is one of the important mechanisms of resistance to beta-lactam antibiotics in members of the Enterobactericeae family. Table 5 showed the ability of most isolates of Proteus to produce beta-lactamase enzymes, as the number of isolates reached P . mirabilis isolates producing 18 (72%) isolates. As for P. penneri isolates, 13 (86.66%) were enzyme-producing, while the number of P.vulgaris isolates producing beta-lactamase was about 5 (62.5%). The results of the detection of protease enzyme production by the Proteus spp an understudy showed that all P.vulgaris were produced this enzyme, and 24(96%) of the P.mirabilis Enzyme-producing while P.penneri produced protease in 13 (86.66%). Table 4 and Figure 2.

Figure 2. A non-protease producing enzyme.
B producer of the protease enzyme.

Table 6 and Figure3 and Figure4 showed lipase production. 14(56%), 10(66.66%), and 7(87.5%) belonging to P.mirabilis,

Table 3. Number and percentages of Proteus spp in different clinical samples.

Proteus spp.	Urine		Stool		ear swabs		Wound swabs		Burns		Vagina	
	No.	%	No.	%	No.	%	No.	%	N0.	%	No.	%
P. mirabilis	9	64.28	8	53.34	3	42.85	2	50	2	40	1	33.34
P.penneri	3	21.42	4	26.66	3	42.85	2	50	3	60%	0	0
P.vulgaris	2	14.28	3	20	1	14.28	0	0	0	0	2	66.66
Total	14		15		7		4		5		3	

Table 4. Number and percentages of the isolates of the proteus spp distributed according to the ability to produce protease enzyme.

Samples	P.mirabilis		P.penneri		P.vulgaris	
	Protease +ve	Protease -ve	Protease +ve	Protease -ve	Protease +ve	Protease -ve
Stool	8(32%)	0	4(26.66%)	0	3(37.5%)	0
Urine	10(40)	0	2(13.33%)	1(6.66%)	2(25%)	0
Ear	3(12%)	0	2(13.33%)	1(6.66%)	1(12.5%)	0
Wound	2(8%)	0	1(6.66%)	0	0	0
Burns	1(4%)	0	4(26.66%)	0	0	0
Vaginal	0	1(4%)	0	0	2(25%)	0
Total	24(96%)	1(4%)	13(86.66%)	2(13.32%)	8(100%)	0
	25		15		8	

P.penneri, P.vulgaris respectively produced lipase.

Figure 4. not produces lipase.

Figure 3. produce lipase.

(+: enzyme-producing, -: non-enzyme-producing) Figure 5 showed the effect of aqueous (cold and hot water) and alcoholic extracts of pomegranate peels extracts (at a concentration of 100 mg/ml) on proteus spp. The results showed a difference in the effectiveness of the extract from one isolate to another in the three extracts. The zone of the inhibition appears to be (21-33 mm) in the hot aqueous extract against several bacteria, while the diameters of the inhibition zone in the cold aqueous extract and alcoholic extract ranged from (20-32 mm) and this result is greater than the study by [28] and three isolates showed resistance to the three extracts. The results indicate that the active substance of this plant

Table 5. Number and percentages of Proteus spp in different clinical samples.

1	P.mirabilis		P.penneri		P.vulgaris	
	enzyme producer%	Non-enzyme producing%	enzyme producer%	Non-enzyme producing%	enzyme producer%	Non-enzyme producing%
Stool	7(28)	2(8)	5(33.33)	0	3(37.5)	0
Urine	7(28)	2(8)	1(6.66)	0	1(12.5)	1(12.5)
Ear	2(8)	1(4)	4(26.66)	0	1(12.5)	0
Wound	1(4)	1(4)	0	1(6.66)	0	0
Burns	1(4)	0	3(20)	1(6.66)	0	0
Vaginal	0	1(4)	0	0	0	2(25)
	25		15		8	

Table 6. Number and percentages of isolates distributed according to sample type and ability to produce lipase enzyme.

Samples	P.mirabilis		P.penneri		P.vulgaris	
	Lipase+ve	Lipase-ve	Lipase+ve	Lipase-ve	Lipase+ve	Lipase-ve
Stool	6(24%)	2(8%)	3(20%)	1(6.66%)	5(62.5%)	0
Urine	5(20%)	5(20%)	3(20%)	0	0	0
Ear	1(4%)	2(8%)	2(13.33%)	1(6.66%)	0	1
Wound	2(8%)	0	0	1(6.66%)	0	0
Burns	0	1(4%)	2(13.33%)	2(13.33%)	1(12.5%)	0
Vaginal	0	1(4%)	0	0	1(12.5%)	1(12.5%)
Total	14(56%)	11(44%)	10(66.66%)	5(33.34%)	7(87.5%)	1(12.5%)
	25		15		8	

Table 7. Number and percentages of isolates distributed according to sample type and ability to produce lipase enzyme.

	Isolates	hot aqueous extract	cold aqueous extract	alcoholic extract
1	+	-	+	+
2	+	-	+	+
3	-	-	-	-
4	-	-	-	-
5	+	+	+	+
6	+	-	+	+
7	+	-	+	+
8	+	+	+	+
9	+	+	+	+
10	-	-	-	-
11	-	-	-	-
12	-	-	-	-

has a solubility in water, as the diameter of the growth inhibition zone ranged from (20-32 mm) in the cold aqueous extract (at a concentration of 100 mg/ml) and the diameter of the growth inhibition zone ranged from (21-33 mm) in The hot aqueous extract (at a concentration of 100 mg/ml), the results of the current study agreed with what was indicated [29] by the effect of hot water extracts on some bacterial isolated from patients with pharyngitis, tonsillitis, wounds, some skin infections and bronchitis, the bacteria isolated from those cases included Micrococcus spp. Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae), all of which showed sensitivity to the extracts of pomegranate peels used and with different degrees of effect from one species to another. Also, it appears that the hot aqueous extract of pomegranate peel had an inhibitory effect on the growth of P.aeruginosa and E.coli [30].

Lime (2012)[31] pointed out that the phenolic compounds present in the pomegranate peel interfere with the function of the cell membrane in bacteria and affect the synthesis of DNA and RNA and inhibit the synthesis of proteins, while alkaloids, some of them affect the DNA of bacteria by getting stuck in the strands of bacterial DNA.

As for the alcoholic extract (ethanol 98%) of the peels of pomegranate fruits also had a great effect in inhibiting the growth of proteus, as the diameter of the inhibition zone