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Bacteriological Study of *Pseudomonas Aeruginosa* Isolated from Different Infections and Study Antimicrobial Activities of Plant Extract *Solanum Nigrum* Against It

Ansam Dawod Salman*, Zainab Amer, Eman Sabah Abud_Rahman
Department of Biology, College of Sciences, Diyala University, Diyala, Iraq.

Abstract

This study was conducted for the period from 1/9/2016 to 30/3/2017 in Baquba city in Iraq. Eighty nine samples were collected from different infections from Baquba General Hospital and Al-Batool Hospital. Fifteen isolates (16.85%) were found to be *Pseudomonas aeruginosa* by using biochemical test. The bacterial diagnosis of all these isolates (100%) were confirmed by VITEK2, beside its possession of the 16s rDNA gene as determined by molecular detection.

The results of virulence factors that had *Pseudomonas aeruginosa* showed possession of all isolates many virulence factors and a high production of which increases the pathogenicity of it. All isolates were able to produce hemolysin (100%), protease (73.33%), and biofilm formation (52%).

The susceptibility test was applied on these isolates against (10) antibiotics. The results revealed that the highest resistances were for Ampicillin and Cefotaxime with 100% for each, while the lowest resistance were for Impineme (0.0%) and Ciprofloxacin (6.66%).

The effects of ethanolic extract of fruits *(Solanum nigrum)* against *P.aerugionsa* were studied. At a concentration of 6% mg/ml, *Solanum nigrum* caused a marked increase in zone of inhibition (mm) on this bacteria growth. Inhibition zones sizes were different and increased according to concentration of extract and again the growth was completely inhibited in the highest concentration.

Keywords: *Pseudomonas*, 16s rDNA, virulence factors and *Solanum nigrum*.

دراسة بكتريولوجية عن الزائفة الزنجارية المعزولة من الاصابات المختلفة ودراسة الفعالية المضادة للميكروبات للمستخلص النباتي لنبات عنب الذئب ضدها

انسام داود سلمان*، زينب عامر حاتم، ايمان صباح عبد الرحمن.

قسم علوم الحياة ، كلية العلوم ، جامعة ديالى، ديالى، العراق.

الخلاصة

أجريت هذه الدراسة للفترة من 2016/9/1 إلى 2017/3/30 في مدينة بعقوبة في العراق. جمعت 89 عينة من اصابات مختلفة من مستشفى بعقوبة العام و مستشفى ألبتول، 15 عزلة (16,85%) منها تعود الى الزائفة الزنجارية باستخدام اختبار الكيمياء الحيوية. تم تأكيد التشخيص البكتيري لجميع هذه العزلات (100٪) من قبل VITEK2، بجانب حيازتها للجين s16 كما هو محدد من قبل الكشف الجزيئي. وأظهرت نتائج عوامل الضراوة التي تمتلكها عزلات الزائفة الزنجارية ارتفاعا في معدل انتاج هذه العوامل مما يزيد من امراضيتها.

*Email: Ansamdawod83@gmail.com

وكانت جميع العزلات قادرة على إنتاج الهيموليسين (100٪) والبروتيز (73.33٪)، تكوين الفشاء الحيوي (52٪). اجري فحص الحساسية على العزلات ضد (10) مضادات حيوية. اظهرت النتائج أن أعلى مقاومة كانت للأمبيسلين وسيفوتاكسيم بنسبة 100٪ لكل منهما، في حين كانت أقل مقاومة للإمبينم (0.0٪) والسيبروفلوكساسين (6.66٪). تم دراسة آثار المستخلص الايثانولي من ثمارنبات (عنب الذئب) ضدالزائفة الزنجارية ، ان تركيز 6٪ مغ / مل من المستخلص اظهر زيادة ملحوظة في قطرمنطقة تثبيط (مم) على نموالبكتيريا. كانت أحجام مناطق التثبيط مختلفة وزادت تبعا لتركيز المستخلص ومرة أخرى تم تثبيط النمو تماما في أعلى تركيز.

Introduction

The *Pseudomonas* is gram negative, motile, aerobic rod some of which produce water-soluble pigments. Due to the fast growth adaptability to versatile environmental conditions (oxidative, nutritional, and other stresses), members of *Pseudomonas* are regarded as one of the most diverse and ubiquitous group, isolated from the variety of natural (e.g., soil, waters), clinical, and artificial niches (e.g., catheters, contact lenses, activated sludge). An important feature of *Pseudomonas spp.* is the production of a wide variety of extracellular products [1, 2].

Pseudomonas has a large number of virulence factors that make it responsible for many human infections, including the formation of biofilms and the production of toxins and enzymes that cause widespread tissue damage and thus reach the bloodstream, causing the spread of these bacteria in the body tissues [3].

Pseudomonas represents the phenomenon of resistance to antibiotics, as it is difficult to address the injuries caused by the drug resistance in this type of bacteria, as it possesses natural resistance, including changing the permeability of the outer wall, and the possession of several groups of stream systems, *Pseudomonas aeruginosa* also produces many enzymes responsible for the decomposition of many antimicrobials, especially Metallo ß-Lactamases (MßLs), Extended Spectrum Beta- Lactamases (ESßLs), and Ampler Molecular Class (AMPC) [4, 5].

The diagnosis of *Pseudomonas* began to decline due to the absence of a reliable diagnostic system. Therefore, many methods have been described for diagnosis of *Pseudomonas* including traditional methods including phenotypic diagnostics, biochemical tests, and direct molecular strategies such as PCR [6], and here the focus will be on these methods.

Solanum nigrum is an important plant in traditional medicines belongs to the family of solanaceae. It is used in hepatitis, fever, dysentery, and stomach complaint. The juice of the plant used on ulcers and other skin diseases [7].

According to World health organization approximately 80% world population use traditional medicines for the treatment of several diseases, because the herbal products safe in contrast to the synthetics, that are regarded as unsafe to human and environment. There are many plant/herbs having different medicinal value used against various

disease since early time. Among them *Solanum nigrum* L. (black nightshade "Makoi") is one of plant having great importance in Ayurvedic medication. Itis a member of family *Solaneace.* The member of this family known for the presence of natural products of medical significance mainly steroidal lactones, glycosides, alkaloids and flavonoids. This herb is antiseptic, anti-dysenteric and diuretic in nature and used in the treatment of cardiac, skin disease, psoriasis, herpi virus and inflammation of kidney. The medicinal plant have antimicrobial agent, that kills or inhibit the growth of microorganisms such as *Echerichia coli, Bacillus subtillus, K. pneumonia, V. cholera, Micrococcus luteus, Salmonella typhiurium, Aspergillus niger, A.fumigatus, Candida albicans,* etc [8].

Materials and Methods

Samples collection

Eighty nine different clinical samples (urine, ear swabs, wound and burn) were collected from patients in Baquba General Hospital and Al-Batool Hospital over period from 1/9/2016 to 30/3/2017.

Isolation and Identification of *Pseu*domonas

The collected samples were inoculated on the Maconkey and blood agar, incubated at 37ºC for 24 hours. The isolates were examined for their shape, size, color, pigments, and hemolytic activity. Then transferred and streaked on Pseudomonas base agar. All plates were incubated at 37ºC for 24 hours

then a single pure isolated colony was transferred to Nutrient agar medium for the preservation and to carry out other biochemical tests that confirmed the identification of isolates.

The isolates were identified according to the Bergey's Manual [9]. as the following: gram stain and biochemical tests which included (catalase test, oxidase test, and pigment production test).

The device uses the VITEK 2 to conduct biochemical tests for bacterial isolates. This device includes 48 tests of biochemical tests that are used in the diagnosis of germs, so that the accuracy of diagnosis in this device is 99%.

DNA Extraction

Boiling method was used for extraction of DNA from *Pseudomonas* isolates as the method described by Ahmed *et al* [10].with follow: bacterial cells were harvested in one ml of Tris-EDTA (TE) buffer and centrifuged at 12000g for 2min. Pellet was re-suspended in 100 µl of sterile distilled water and boiling at 100 C° for 10 min, cooled on ice then centrifuged at 10000g for 10min. The supernatant was stored at -20C° until use.

PCR amplification

16s rDNA gene was detected by PCR using specific primers Table-1. The PCR mixture set up in 20 µl total volume consisting of 5 µl of premix Accupouer (Bionear, Korea), 10 pico/ µl of each primer and 100 ng/ µl of DNA template. The thermal programmed was optimized and performed in master cycle (Eppendrof, Germany) as follows: 5 min at 95C° , then 30 cycles of 45sec at 95 C°, 40sec at 56 C° as well as 45sec at 72 C° then final elongation step at 72 C° for 3min.

Table 1-The sequence of primer used in PCR for detection of 16s rDNA gene in *Pseudomonas*

Primer	Sequence	Product size (bp)	references
16s rDNA	F/5-CAACGAGCGCAACCCTT-3	375	(9)
	R/5- GGTTACCTTGTTACGACTT-3		
	R/5-AATTTGGGCTTAGGGCAGAA-3		

Detection of virulence factors

The *Pseudomonas* ability to produce some of virulence factors (enzymes and toxins) was recognized and tests were applied on 15 isolates that identified. It included: Hemolysin production, Protease production and biofilm formation.

Antimicrobial susceptibility test

The sensitivity and resistance of *Pseudomonas* to antimicrobials agents was tested by the disc diffusion method on Mueller-Hinton agar using antibiotic discs according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[11]. Ten antibiotics were tested: Amikacin (30µg), Amoxicillin (25µg), Gentamycim (10 µg), Ampicillin (10 µg), Co-trimoxazole (25 µg), Cefotaxime (30µg), Piperacillin (100 µg), Tobramycin (10 µg), Ciprofloxacin (5µg) and Imipenem (10µg).

Interpretation of inhibition zones was carried out based on the manufactures and CLSI guidelines .

Preparation of plant extract and determination the antimicrobial activity:

The following step are conducted based on Harbome (1973) for detecting the phenolic compounds for the studied species: First The fruits of the plants have been taken from *Solanum nigrum* and have been grinned by using electric grinned, 50 (gm) from the specimen are weight, and added 500 ml of ethyl alcohol (70%), left in the room temperature for 24- 48 hour, later filtration has been done by using ederol filter paper (medium pores filtering).The extract has been concentrated to a suitable volume to discard the ethyl alcohol by using air dryer in a moderate temperature. As many as the extract volume, petroleum ether (with boiling point 40-60°C) has been added to the extract, the mixture was shaken well, then has been put in separating funnel, and has been left till separate in to two obvious layer, in this point discarding take place from a large part of the chlorophyll that dissolved in the petroleum ether and floated above because it has less density than the aqueous extract of the phenolic compound and then withdrawn from funnel bottom. The extract of the phenolic compounds were concentrated to half of its volume by leaving it in dry air current.

The antibacterial activity of *Solanum nigrum* leaf extract the first dissolved in a distilled water solvent, and then varying concentrations of the extracts (2%, 4%, 6%) were soaked on autoclaved discs of Whitman filter paper. These filter paper discs were placed on a streaked Muller-Hinton agar

plate surface. The plates incubated overnight at 37° C for 18-24 hours. The antimicrobial activity was detected by measuring of inhibition zones.

Result and Discussion

Isolation and identification

Eighty nine samples were collected from patients, the samples comprised from (urine, ear swab, wound and burn).

Fifteen isolates (16.85%) have the ability to grow on the Pseudomonas base agar which considered selective and differential media for genus *Pseudomonas*. All 15 isolates had ability to form large metallic green colonies.

Microscopic examination was used to all 15 isolates after staining by gram stain and cells appeared as Gram-negative. For further identification some of the biochemical tests were performed on 15 isolates, included: catalase test was all 15 isolated gave positive results. The 15 isolates gave the positive result to the oxidase test. Also all 15 isolates were negative to Voges-Proskauer (VP) test, methyl red test and Indol production test.

The assay of the isolates was performed using the VITEK 2 device. After diagnosis, it was ascertained that all the bacterial isolates included in the study were of the type *Pseudomonas aeruginosa*.

The results of the detection of the presence of the gene 16s rDNA in the isolates included in the study were all 100% carrier of the gene, as the results of PCR for the gene 16s rDNA has a volume of 375 pair base when comparing the multiplication packets with the volumetric guide (DNA Ladder) and observed that the size of packages similar to the expected size and that when coupled with the findings [12, 13].

The results of the diagnosis showed that 100% of the isolates were classified as *Pseudomonas aeruginosa*. This result came in line with what Jaafar and others [14] found in 2014, where 90.7%.

The method of diagnosis in the VITEK 2 device is fast and less effort than common biochemical [15].

The results of the molecular diagnosis were consistent with the results of the study of Abdullah and Mehdi in 2016, with the percentage of *Pseudomonas aeruginosa* isolates carrying the gene 16s rDNA 100 %[16], and the current study coincided with the results of both Jaafar and Zainab [13, 14]

Pseudomonas aeruginosa is the most important and reliable method for diagnosis of *pseudomonas aeruginosa*. This gene is an important gene in which the genetic factor of the germ can be identified. of dermis and that depending on 16s ribosome DNA [16].

Detection of some virulence factors:

Results in Table-2 revealed that the detection rate of some virulence factors of *Pseudomonas aeruginosa* .

Hemolysin production

The results showed that highest in production of hemolysin (100%), many studies reported results consistent with present results in highest production of hemolysin [13, 17].

Production of protease

Test ability of all isolates of *Pseudomonas aeruginosa* on production of protease enzyme by using skimmed milk, as it was given 11(73.33%) isolates positive results. The results of the present study are in line with the findings of researchers (Al-Tikriti 2009) [18] reported 73%, 80.1% respectively.

Biofilm production

The current study showed that 52% of the isolates had the ability to form the biofilm. These results coincided with the findings of Jakribettu and others in 2013 [19] , the percentage of isolates formed for biofilm 54%. The results of the present study also disagreed the results of Zainab in 2017 [13] with 75% of the isolates of *Pseudomonas aeruginosa* forming the biological membranes.

Table 2- Some of virulence factors of *Pseudomonas aeruginosa*

Virulence factor	Positive results (%)	Negative results (%)
Hemolysin	100	0
Protease	73.33	27.77
biofilm	52	48

Susceptibility test of *Pseudomonas aeruginosa*:

The sensitivity of 15 isolates was tested against 10 antibiotics. The susceptibility test was applied according to the Kirby-Baure Method (antibiotic disc diffusion method).

Table 3- Percentage sensitivity and resistance of different antibiotics for *Pseudomonas aeruginosa*

Antibiotics	(%) of Resistance	(%) of sensitivity
Ampicillin	100%	0.00%
Amoxicillin	93.44%	6.66%
Co- trimoxazole	86.67%	13.33%
Cefotaxime	100%	0.00%
Piperacillin	66.67%	33.33%
Gentamycin	60%	40%
Tobramycin	73.34%	26.66%
Amikacin	60%	40%
Ciprofloxacin	6.66%	93.44%
Imipenem	0.0%	100%

The results in Table-3 showed that all isolates (100%) were resistance to Ampicillin and Cefotaxime antibiotics and this result was agreed with local studies by AL-Saady [20] and Abdullah, *et al*. [16]. 100% reported." This increased in penicillin resistance isolates among *Pseudomonas aeruginosa* strains can be explained in most cases to production of β- Lactamase enzyme that destroyed the β- Lactam ring and inactivated the penicillin and this enzyme was encoded by plasmid that easy to transfer among strains [1].

Pseudomonas aeruginosa was resists to Amoxicillin (93.44%), this result agreed with others studies [16, 13].

The resistance of Co- trimoxazole was (86.67%), while *Pseudomonas aeruginosa* resists Piperacillin, Amikacin, Tobramycin and Gentamycin with 66.67%, 60%, 73.34% and 60% respectively. The results showed that resistance to other antibiotics such as Ciprofloxacin (6.66%), Imipenem (0.00%), this results that constant with other studies[13, 20] In general the resistance to different antibiotics may be due to the type of antibiotics and how much that used among the patients in the community. In addition to that the resistance to ward any antibiotics was depended on the amount of PBP2a or β-Lactamase enzyme that produced by each strain of *Pseudomonas aeruginosa*. All these reasons could create variations in the rate of resistance.

Antimicrobial potential of extracts of *Solanum nigrum* fruits

In our study the result showed the antimicrobial activities of ***S. nigrum*** (alcohol) extracts against the *Pseudomonas aeroginosa*, and their potency, were assessed by the presence or absence of inhibition zones and zone diameter. The results are given in Table-4 showed the antimicrobial activities of ***S. nigrum*** ethanol extracts against *Pseudomonas aeroginosa* and the diameter of inhibition zone of plant extract.

Table 4- The antimicrobial activities of *S. nigrum* ethanol extracts against *Pseudomonas aeroginosa*

Diameter of inhibition zone (mm)			
Concentration / No. of Isolate	2%	4%	6%
1	10	12	20
2	8	10	16
3	-	-	10
4	13	15	19
5	7	9	10
6	11	14	20
7	14	20	25
8	-	-	-
9	18	20	28
10	13	19	22
11	-	-	-
12	20	20	22
13	14	14.5	17
14	10	10	12
15	20	19	21

The bacterial resistance to several different antibacterial agents constitute significant problem. The antimicrobial activities of *Solanum nigrum* ethanol extracts against *Pseudomonas aeroginosa* examined in the present study .the presence or absence of inhibition zones and zone diameter. This result agrees with the study of [7, 8] Different concentrations of *Solanum nigrum* ethanolic extract of were used in agar well diffusion assay, caused different degrees of zones of inhibition against .The results showed that concentrations of ethanol extracts have different antimicrobial effects. Three different concentrations of ethanol extracts were used in our study. It was not surprising that more condensation of ethanol extracts resulted in more effective antimicrobial activity. If more concentrations of extracts are used, antimicrobial effect will increase.

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