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Synergism Activity of Pyoluteorin with Some Antibiotic Against Urinary Tract Infections Pathogens

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ABSTRACT

On some pathogenic bacteria isolated from the urinary tract, the antibacterial activity of pyroluteorin in combination with other antibiotics was studied. Pyoluteorin was extracted from *Pseudomonas aeruginosa* isolated from rhizospheric soil in Baghdad City. Fifty isolates belongs to Urinary Tract Infection were isolated, and the diagnosis was made using cultivars and biochemical tests, and confirmed using Viteck 2 system. Ten isolates each of Escherichia coli and Klebsiella pneumoniae, six of Proteus mirabilis, four of Acinetobacter baumannii, three of Serratia marcescens, and four of Enterobacter cloacae were among the bacterial isolates, gram-positive bacteria including *Streptococcus agalactiae* (3 isolates), *Staphylococcus aureus* (6 isolates) and *Staphylococcus epidermidis* (2 isolates). All isolates were tested for susceptibility test against 10 different antibiotics (Nalidixic acid, Tetracycline, Amoxicillin, Trimethoprin, Ampicillin, salbactam, Norfloxacin, Levofloxacin, ciprofloxacin and Amikacin). The outcomes indicated that 91% and 82% of resistance were reported for nalidixic acid and tetracycline, respectively, whereas 9% of resistance was found for amikacin.

Keywords- Pyoluteorin produce, *P.aeruginosa*, *S.aureus*, *A.baumanii*.

I. INTRODUCTION

Peudomonas aeruginosa is opportunistic and gram negative bacteria has the ability to create bacteriostatic and bactericide active chemicals, which could be used to kill multidrug-resistant bacteria., which can be used to treat illnesses in people, animals, and plants, are produced in the secondary metabolism. Among the rhizobacteria Pseudomonas fluorescens CHA0 and Pseudomonas sp., the polyketide antibiotic pyroluteorin is generated by specific strains of Pseudomonas spp.[12]Takeda isolated and identified pyroluteorin from Pseudomonas aeruginosa for the first time [16]. Pyoluteorin is a yellow crystal that dissolves entirely in organic solvents like methanol and chloroform. It is made up of a bichlorinated pyrrole bonded to a resorcinol moiety [11]. Moreover, pyolteorin occasionally enhances the generating strain's ecological competency within the rhizosphere [14].

II. MATERIAL AND METHODS

2.1 Isolation and Identification of P.aeruginosa isolates

In order to isolate and identify P.aeruginosa isolates, soil samples were collected from the wetland rhizosphere zone of the plant [10]Numerous cultural and biochemical assays were performed on soil isolates, and vitek 2 was used to validate the identification [2] [6].

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2.2 Isolation and Identification of urinary tarct infection bacteria

Urine specimens from local hospitals in Baghdad were used to isolate bacteria. Isolates were identified using morphological and biochemical tests, and the VITEK 2 system was utilized to confirm the identity of each isolate.

2.3 Extraction and purification of PLT

2.3.1 Extraction of pyoluteorin:

The pyoluteorin antibiotic production by isolate of *P. aeruginosa* PA40 was obtained as described by [17]A 15 ml aliquot of King B liquid broth was placed in a 150 ml conical flask, and the isolate PA40 was precultured for 12 hours at 28 oC. To produce PLT, a 150 ml aliquot of King B or PPM media was inoculated with a fraction (8 ml) of this culture in 500 ml conical flasks.For four days, the cultures were incubated at 110 rpm in a rotary shaker. Each culture broth was centrifuged at 12000 rpm for 10 minutes in order to extract Plt. The supernatant was then combined with an equal volume of ethyl acetate and acidified to pH 2 using 1N HCl. With distinct funnels, the topmost layer was gathered. After being dried in a desiccated vacuum at 40°C, the crude PLT was dissolved in one milliliter of methanol.

2.3.2 Purification of pyoluteorin:

The first step in purification of extracted PLT was carried out using gel filtreration chromatography 1ml of extracted PLT created from *P.aeruginosa* PA40 was placed on silica gel column(1.5*25 cm) the column was eluted using solvent Chloroform and aceton from 9:1 (v/v) elution flow rate was 1ml/min [15]. The surface of the silica gel column was coated with the crude separate that was to be fractionated, and the extract was adsorbed on top of the silica gel. The second step in purification PLT is TLC was used as described by [5] with some modifications as follows: - Sample analysis was conducted using a sheet (silica gel 60f-254, size 20x20 cm, Spain), with a layer thickness of 0.2 mm and an aluminum support. One centimeter from the plate's bottom border was the positioning line. Twenty microlitre from the fraction of gel filteration of silica gel column samples was applied to thin-layer chromatography plates coated with a 250 ml layer of silica gel and developed in Chloroform and aceton (9 :1 v/v) for pyoluteorin as solvent system.The spots were visualized by spraying with diazotized sulphanilic acid or under UV at 254 nm in an UV illuminator. Next, the spot Scrubed dissolved in ethyl acetat solvent after being scraped with a spatula, dried in a desiccated vacuum at 40°C, and then dissolved in methanol.

2.4 Biofilm formation bacteria

As stated by[1], the production of biofilms was evaluated using the Microtiter Plate method. the steps as follow:

20 µl of bacterial suspension equal to 0.5 Mcfarland standards tube are used to inoculate microtiter wells containing 180 µl of B.H.I. broth with 2 % sucrose. Each well consist of 200 µl (isolated bacteria+ media), In triplicate for each isolate. incubation at 37°C for 24 hrs.200µl of crystal violet solution was added let stand for 15 min. washed wells to remove the unbounded dye, allowed to dry at room temperature. At 630 nm, the absorbance of each well was measured using an ELISA reader, the O.D value for well control has been deducted. The adherence capability of the tested isolates has been categorized into four Categories Isolates were classified as follows:

- (OD < ODc) …… .. non-adherent.
- (ODc < OD < 2×ODc) …….. Weakly-adherent.
- (2×ODc < OD < 4×OD) ……. Moderately-adherent.
- (4×OD < OD) ……. Strongly-adherent

2.5 Synergistic effect between purified pyoluteorin combination with some antibiotics:

Minimum Inhibition Concentratio (MIC) of some antibiotic against uropathogenic isolates, which showed highly resistans to number of antibiotics were determind using varying concentrated of antibiotics (Tetracyclin, Nalidixic acid, Amoxicillin clavulanic acid) in broth microdilution assay.

An overnight growth of (*Streptococcus agalaciae, Staphylococcus aureus, Enterobacter cloacae, Acinetobacter baumannii, Morganella morganii* and *Kkebsiella pneumoniae*) Was inoculated in to N.B in 96 well of microtitration plates and serial dilution of antibiotics solution with approximately (1×10^8 CFU) of each isolates were added, incubated at 37ºC for 24 h. Minimum Inhibition Concentration (MIC) were confirmed according to and considered the least concentration of antibiotic which prevent the visible growth of bacteria was MIC[6] The antibacterial activity of purified pyoluteorin in combination with sub MIC of antibiotics was carry out against some uropathogenic isolates by mixing 50µl of sub MIC of antibiotics (Tetracyclin, Nalidixic acid, Amoxicillin clavulanic acid)with 50µl of purified pyoluteorin in agar well diffusion method described previously by spreading each isolates on surface of Muller Hinton Agar and using cork borer to make 3 wells and lodded with 100µl of (50µl sub MIC of antibiotic + 50µl purified pyoluteorin) and second lodded with 100µl with sub MIC alone and last was control filled with D.W.

III. RESULTS & DISCUSSION

3.1 Extraction and purification of phenazine from P.aeruginosa PS 40

The Pseudomonas aeruginosa PS40 isolate, which was extracted using benzene as an organic solvent, exhibited strong antibiotic activity against uropathogenic bacteria. Crude pyoluteorin was the substance obtained after the organic phase was separated using a saperated funnel, dried, and then resuspended with methanol.

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3.2 Purification of pyoluteorin

3.2.1 Column chromatography:

A silica gel column was utilized to test the crude solvent extract (1.5 X 25) cm and 60- 120 mesh size) 1ml/min flow rate. A mixture of chloroform and aceton in a 9:1 ratio was utilized to extract the compound, [15]. The crude extract was separated on the surface of the silica gel column and then absorbed onto the gel. The fractions extracts were collected by class tube and the Partial purification of antibiotic compound were eluted on HPLC**.**

3.2.2 High Performance Liquid Chromatography:

On a preparative HPLC column, the elution conditions of the partially purified chemical Plt were examined, and the chromatogram was displayed in Figure. (3-1) When methanol-water (70:30, v/v) was used as the mobile phase, the results obtained showed there was two peaks appeared on fig (3-8) first one belong to the solvent on retination time 3.85. While the scond in Rt= 7.04 belong to PLT with low concentration 8µg/ml.

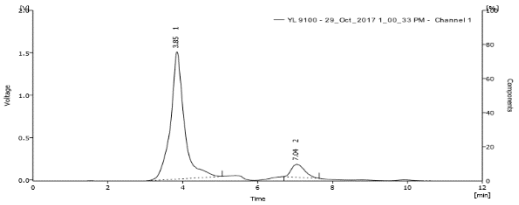

Figure (3-1) preparative HPLC for separation pyoluteorin on Conditions: C-18 column (250 mm×4.6 mm, 5 µm)

3.3 Antimicrobial activity of pyoluteorin:

Antimicrobial activity of crude and purified pyoluteorin on some UTI bacteria using agar diffusion method against one selected isolates of each uropathgenic isolates that showed higher resistance to antibiotic the result sumemarized on table. The outcome showed that highest activity of crude pyoluteorin appeared on bacteria 34 mm and the lowest activity was recorded on Enterobacter cloacae (8mm) compared with purified pyoluteorin.

PA40, included (50, 100 and 200 µg/ml) were prepared to the activity determine of pyoluteorin against certain microbes, such as : Gram-positive such as Streptococcus agalactiae, Staphylococcus aureus. Gram-negative such as Escherichia coli, Proteus spp, Acinetobacter spp and Enterobacter spp and yeast as Candida albicans Results indicated the antibiotic pyoluteorin is effective against all kinds of microbes. Researchers discovered that a concentration of pure pyoluteorin at 200 µg/ml had a greater effect on isolate PA40. The PLT inhibition zone for Staph. aureus was 34, 32, and 30 mm at 200, 100, and 50 µg/ml, respectively. For E. coli, the inhibition zone was 20, 10, and 10 mm at 200, 100, and 50 µg/ml, respectively. Many studies reported that P.aeruginosa could produced various secondry metabolic which could plyed important role in controlling pathogens and could produced abroad spectrum bacterial and fungicidal and pyoluteorin was one of these compound[4][13].

Table (3-1) inhibition Zone of crude and purified pyoluteorin extracted from *P.aeruginosa* PA40 agains UTI isolates

Bacterial isolates	Zone of inhibition(mm) Crude pyoluteorin	Zone of inhibition of purified PLT		
		50	100	200
Staphylococcus aureus	30	30	32	34
Streptococcus agalactiae	34	15	20	24
Escherichia coli	10	10	10	20
Serratia marcescens	12	12	17	20
Acinetobacter spp	12	0	12	20
Proteus mirabilis	15	12	15	17
Enterobacter cloacae	8	0	12	14
Morganella morganii	12	10	12	14
Klebsiella pneumonia	16	0	0	12
Pseudomonas aeruginosa	15	10	10	12

3.4 Biofilm production

Bacterial biofilms play an essential role in UTIs as they responsible for persistent of infections and important to recurrences and relapses [8].

Biofilms production was detected in 26 Uropathogenic organisms by Tissue Culture Plate (TCP) method of two species 12 strain of *Staphylococcus aureus* and 14 isolate of *Klebsiella pneumoniae.* According to the TCP method results, biofilm production was detected in 10 (80%) of the 12 staphylococcal isolates, albeit at varying intensities; 6 (40%) of the isolates were strong biofilm producers, 3 (30%) of the isolates were moderate producers, and 1 (10%) of the isolates was a weak producer. Where as 2 (20%) were non biofilm

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producers.and biofilm production of *Klebsiella* by TCP method was detected in 12 (83%) of the 14 *Klebsiella* isolates with different intensities 6 (33.33%) isolates were strong producers, 3(25%) isolates were moderate and 3 (25%) isolates were weak biofilm producers, where as 2 (16.66%) were non biofilm producers.

Table (3-2) The percenrage of Biofilm production of *S. aureus* and *K.pneumoniae* isolates in microtiter plate.

Biofilm formation	*S.aureus* No.=12	Percent (%)	*K.pneumon* No.=14	Percent (%)	Total (n=26)	Percent (%)
Strong	6	40	6	33.33	12	36.36
Moderate	3	30	3	25	6	27.27
Weak	1	10	3	25	4	18.18
None	2	20	2	16.66	4	18.18

3.5 Synergism activity of pyoluteorin with some Antibiotic against Urinary Tract Bacteria

The combination of antibiotics with pyoluteorin were determind against UTI bacteria. MIC were determind to 3 antibiotics (Ttetracyclin, Nalidixic acid, Amoxicilline clavulanic acid), against higher resistance of UTI bacteria, one isolates of each (*Staphylococcus auerus, Klebsiella pneumonia, Acinetobacter baumannii, Enerobacter cloacae and Morganella morganii*). To detect the influencs of PLT produced from PA 40 isolates in combination with sub MIC of the 3 antibiotic separatelly, The Agar Well Diffusion Method was used to determine the antibacterial activity. The results showed the combination of PLT with sub MIC of Tetracyclin get higher diammeter of inhibition was (30 mm) against *staphylococcus auerus*, then the effect of combination PLT with Nalidixic acid (26 mm) and the Amoxicillin clavulanic acid combination with PLT had the lowest effect against *staphylococcus aureus* (21 mm). While the combination of PLT with Amoxicillin clavulanic acid had the lower activity on bacterial isolates (7 mm) on *Enterobacter cloacae.*

Table (3-3) Inhibition zone (mm) of synergistic antimicrobial against g+ve and g-ve UTI.

Synergistics antimicrobial drug	*Staphylococcus aureus*	*Sstreptococcus agalaciae*	*Klebsilla pneumoniae*	*Enterobacter cloacae*	*Acinetobacter baumannii*
	Inhibition zone (m m)				
Nalidixic acid	26	24	13	12	13
Tetracyclin	30	42	20	14	16
Amoxicillin clavulanic acid	21	28	12	7	8

Many studies reveld that randomly used of common antibiotics made it useless against numerous bacteria and development several mechanisums of resistance, so new antibacterial agent are required for treatment such infection[3]. in his report pointed that they could treatment of MDR gram negative bacteria (*Acinetobacter spp., Pseudomonas spp., Klebsiella spp.*) infection by using combination of new molecules and antibiotic in stead old antibiotics. [9].mentionted that becaused of increasing antibiotc resistance, alternative therapy foe treatment infectious disease using different compoundin combination with antibiotics to controle some bacteria (*E.coli, Pseudomonas spp., Acinetobacter spp.*) herble tea with antibiotics of some *E.coli, Pseudomonas spp., Acinetobacter spp., Staphylococcus aureus.*

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