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ANTI-ADHESIVE ACTIVITY OF PHENAZINE PRODUCED BY *P. AERUGINOSA* ISOLATED FROM RHIZOSPHERE PLANT ROOT FROM IRAQI SOIL AGAINST SOME WOUND AND BURN INFECTION BACTERIA

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ABSTRACT : The study including extraction and purification of phenazine produce from *P. aeruginosa* (PS 33) isolated from rhizospheric plantand studying its effect on wound & burn pathogenic bacteria isolated from locally hospitals in Baghdad, Iraq. The isolates involving *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Serratia marcescens*, *Streptococcus pyogens* and *Staphylococcus aureus.* PS33 was culturing on King's B broth and extracted crude phenazine with benzene, purified it with TLC (Thin layer chromatography), R_F- value obtained was 0.7. Then exposure to HPLC (High performance liquid chromatography) to detected the phenazine on wave length 248 nm. Single peak appeared on retention time 5.1, which related to phenazine. Antibacterial of crude and purified phenazine against Multi drug resistance (MDR) isolate of wound & burn infections. The results appeared that crude phenazine had greater activity than purifiedand the *Staphylococcus aureus* was more sensitive to crude phenazine. Antiadhesive activity of phenazine was distinguished and the results revealed that phenazine showed good activity as antiadhesive against pathogenic bacteria highly activity of phenazine against wound & burn bacterial isolates were reaching to 48.6% for *Acinetobacter baumanii*.

Key words : Phenazine produce, *P. aeruginosa*, *S. aureus*, *A. baumanii*.

INTRODUCTION

Pseudomonas aeruginosa could produce compounds including bacteriostatic and bactericide activity with potential use in the control of Multidrug resistant bacteria (Behnke *et al*, 2017). Multiple studies illustrated substances with bactericide and antifungal activity produce in the secondary metabolism, which could be used in the management of human, animal and plant diseases (Thi *et al*, 2016). Phenazines are a class of tricyclic aromatic molecules produced by *P. aeruginosa*, other Gram negative and Gram-positive bacteria (Saunders *et al*, 2018). Phenazines and their derivatives represent an important class of compounds and are known to possess broad spectrum of anti-bacterial activities. Examples of anti-bacterial phenazines are tubermycin B (a phenazine-1-carboxylic acid possess potent activity against *Mycobacterium tuberculosis*, *Bacillus cereus*), chlororaphine (an anti-bacterial agent), iodinin (possessed activity against gram-positive bacteria, several actinomycetes and some fungi), pyocyanin (showed broad spectrum bactericidial effect). Besides anti-bacterial potential phenazines and synthetic derivatives also possessed several other important biological activities such as antiparasitic, antimalarial and antitumor properties (Guttenberger *et al*, 2017). The aim of this study is to extracted of phenazine antibiotic from *P. aeruginosa* and more understanding of the impacts of phenazine (Phz) on wound & burn infection.

MATERIALS AND METHODS

Isolation and identification of *P. aeruginosa* isolates

The soil samples were taken from rhizosphere region from wheat root in order toisolation and identification of *P. aeruginosa* isolates (Omolola, 2007). Soil isolates were subjected to numerous cultural, biochemical tests and confirmed the identification with vitek 2 system proposed by Collee *et al* (1996).

Isolation and identification of wound & burn infection bacteria

Bacteria were isolated from swab samples collected from local hospitals in Baghdad city. Identification of isolated were done according to the morphological, biochemical test and VITEK 2 system were used to confirmed the identification of all isolates.

Extraction of phenazine

P. aeruginosa isolate (PS33) was grown at 37°C in King's B brothmedium. In a rotary shaker for 3 days at

110 rpm. Phenazine was extracted from the culture liquid depended on the method described by Kavitha *et al* (2005) as follows:

Five milliliters of culture was centrifuged at 5000 rpm for 30minutes and the supernatant was acidified to pH 2 with concentrated HCl. Added 5ml of benzene to samples mixed for 1 hour and centrifuged at 5000 rpm for 30 minutes. Four milliliters of the benzene layer was decanted and air dried. Samples were resuspended in 1ml of 0.1 NNaOH and the absorbance at 248 nm was determined.

Purification of phenazine

Thin layer chromatographic method (TLC)

The technique of TLC was used as described by Genevive *et al* (2006) with some modifications as follows:

The sheet (silica gel 60f-254, 0.2 mm, layer thickness and aluminum support, size 20×20 cm, Spain) was used for analyzing samples. Slotting line was marked 1 cm from bottom edge of the plate. Twenty µm of sample were applied to thin layer chromatography plate coated with 250 mm layer of silica gel used to analyzed sampleand developed in Chloroform and methanol (9:1 v/v) for phenazine as solvent system. The plate was removed and left to dry examined under UV at 254 nm. Then the spot Scrubbed by the spatula and dissolved in benzene solvent finally dry in a desiccated vacuum at 40°C and then dissolved in methanol. The R_F value for each dye is then worked out using the formula:

R_F = space travelled by component/space travelled by solvent

High performance liquid chromatography (HPLC)

Partial pure of phz were investigated on preparative HPLC column and the chromatogram. 60% methanol was used as the mobile phase, the sample was loaded on a HPLC (Shimadzu YL9100, Kyoto, Japan) C18 reversed-phase column (Zorbax SB-C18, 5.0 µm, 4,6 mm*250 mm, Rockland Technologies Ind., Newport, DE, U.S.A.) and the column was eluted with methanol-water (60:40, v/v) at a flow rate of 0.8 ml min^{-1}. phz were detected by UV at 248 nm and determined the retention times of compound, which is specific value for each compound (Pelander *et al*, 2003).

Antimicrobial activity of crude and purified phenazine against wound & burn pathogenic bacteria

A. Effect of crude Phz against wound & burn pathogenic bacteria

The agar well diffusion method was used to distinguished the action of crude phenazine produce from *Pseudomonas aeruginosa* (PS33) against wound & burn pathogenic bacteria by spreading 0.1ml of each wound & burn pathogenic isolates were *Escherichia coli*, *Klebsiella pneumoinae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Streptococcus pyogens*on the Muller Hinton Agar, wells of 5mm diameter were punched in the agar lodded with 100µl of crude Phz, incubated in 37ºC for 24 hours. The resulting of zone of inhibitory was measured in millimeter (Colle *et al*, 1996).

B. Effect of purified Phz against wound & burn pathogenic bacteria

It was made as described previously in A put instead of crude Phz, the wells were filled with 100 µl of purified Phz

Anti-adhesive activity

The anti-adhesive activity of phenazine produced from *P. aeruginosa* against wound & burn pathogenic bacterial isolates were quantified by co-incubation test according to the procedure described by Ali (2012). Isolates of wound & burn infection causative bacterial suspensions in brain heart infusion with (2%) glucose (100 µl) were lodded to (96-well) flat-bottomed tissue culture plates together with 100 µl of the phenazine. Control hole contained (180 µl) of brain heart infusion with (2%) glucose and 20 µl of bacterial suspensions, the enveloped microtiter plate wasclosed with Parafilm incubation at 37ºC for 24 hours. Unattached bacterial cells were removed by rinse the holes with physiological Normal Saline (pH 7.2). After drying at room temperature for 15 min., 200 µl of crystal violet (1%) was lodded to the holes for (20 min). The dye attached bacterial cells were rinsed with physiological Normal Saline (pH 7.2), let to dry at room temperature for (15 min) and extracted two time with (200 µl) of (95%) ethanol and the absorbance of all holes was measured at 630nm by using ELISA Reader.

Inhibition of adhesion = $[1 - (A/A0)] \times 100\%$

A: refer to the absorbance of the holes with a phenazine.

A0 : refer to the absorbance of the control holes.

RESULTS AND DISCUSSION

Extraction and purification of phenazine from *P. aeruginosa* PS33

The isolate of *Pseudomonas aeruginosa* PS 33 which have high antimicrobial activity against pathogenic bacteria isolated from wound & burn, extracted with benzene as organic solvent as described by Morrison *et al* (2016). Then purification with TLC.

1. Purification by TLC

Separation of phenazine from crude extract of isolate PS 33 was done by TLC. Result in Fig. 1 showed that phenazine produced by isolate PS33 giving a band with R_f 0.7 based on mobility of the compound on the silica plate that is measured in terms of a retardation factor. The value R_f of 0.7 corresponded to phenazine antibiotic with Chloroform: methanol solvent system 9:1 v/v, as mentioned by Viviana *et al* (2015).

The obtained results were found analogous to those detected by Saosoong *et al* (2009), who use TLC assessment to purify phenazine with R_f 0.70.

Hassanein *et al* (2009) used TLC technique for diagnose of phenazine produced by *P. aeruginosa* as antifungal compounds. And Aunchalee *et al* (2009) used TLC technique for detection of phenazine and its derivatives.

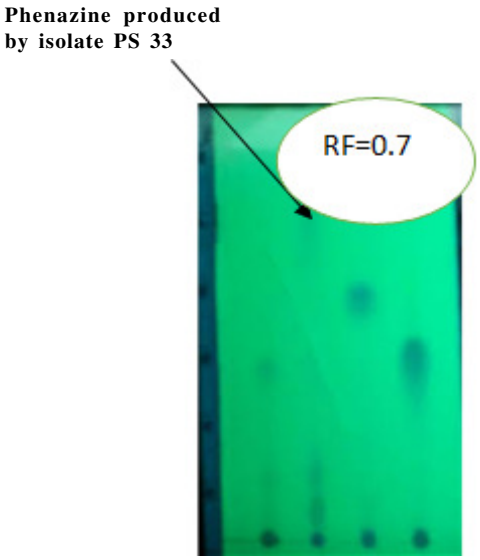

Fig. 1 : Purification by HPL.

2. Purification by HPLC (Figure 1) : Thin layer of chromatography test of phenazine produce from *Pseudomonas aeruginosa* isolate PS33

The spots found on TLC plate at R_f = 0.7 were scruped and loaded on a HPLC column. 20 µL of sample was loaded on a HPLC column. The chromatogram were shown in Fig. 2. When methanol-water (60:40 v/v) was used as the mobile phase, the peaks of purified Phz showed the retention time were 5.1 min. The result of peak agree with that described by Shanmugaiah *et al* (2010).

The result agree with Liu *et al* (2018), who demonstrated the ability of *P. aeruginosa* to produce phenazine and derivatives. Other studies by Xu *et al* (2015) reported that strains of *Pseudomonas aeruginosa* could produce a variety of redox-active phenazine compounds including pyocyanin, phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN), using HPLC in purification steps. Others studies found that antibacterial substance produced from *P. aeruginosa* culture was homogeneous and characterized by HPLC (Bilal *et al*, 2017). The retention times were differs according to the methods, conditions and solvents using in purification. Makarand *et al* (2007) used HPLC for purification of phenazine and showed retention time of 3.2 min.

Antimicrobial activity of phenazine

Antimicrobial activity of crude and purified phenazine on some bacteria causing wound & burn infection using agar well diffusion method. The results were summarization in Table 1. The results showed that highest

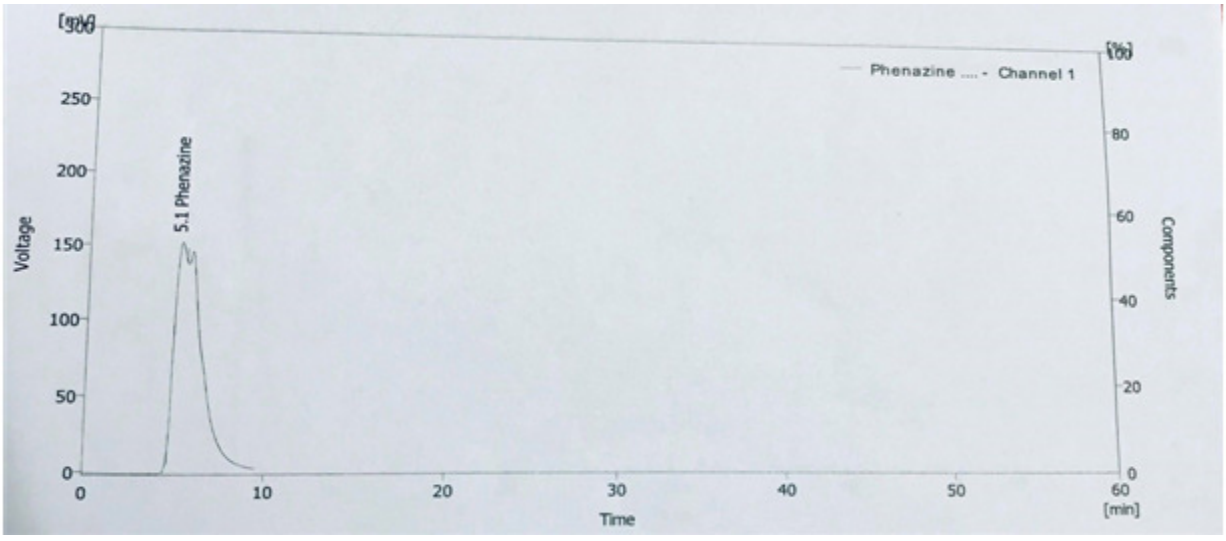

Fig. 2 : Preparative HPLC Chromatogram for Phz purification. C-18 column (250 mm×4.6 mm, 5 µm); flow rate:0.8 mL/min; detection wavelength: 248 nm; 60% methanol.

Table 1 : Antimicrobial activity of crude and purified phenazine against wound & burn isolates

Bacterial isolates	Zone of inhibition	
	Crude phenazine	Purified phenazine
Klebsiella pneumonia	10	12
Escherichia coli	14	13
Pseudomonas aeruginosa	8	0
Serratia marcescens	13	10
Acinetobacter baumanii	14	12
Staphylococcus aureus	18	16
Streptococcus pyogens	15	0

Table 2 : Antiadhesive percentage of phenazine against wound & burn isolates

Bacterial isolates	OD of phenazine	OD of Control	Antiadhesive percentage%
Escherichia coli	0.232	0.182	-27.4
Serratia marcescens	0.157	0.097	-61.8
Acinetobacter baumanii	0.353	0.687	48.6
Pseudomonas aeruginosa	0.137	0.161	14.9
Klebsiella pneumonia	0.087	0.080	-8.7
Staphylococcus aureus	0.086	0.136	36.7
Streptococcus pyogens	0.144	0.138	-4.3

activity of crude phenazine appeared on bacteria *Staphylococcus aureus* was 18 mm and the lowest activity was recorded on bacteria *Pseudomonas aeruginosa* was 8 mm.While purified phenazine appear highest activity on bacteria *Staphylococcus aureus* was 16 mm and there were no effect recorded on bacteria *Pseudomonas aeruginosa* and *Streptococcus pyogens*.

Many studies reported that *P. aeruginosa* could produce various secondary metabolic, which could acted important role in controlling pathogens and could produce abroad spectrum bacterial and fungicidal compound phenazine was one of these compound (Vinay *et al*, 2016). The mechanism for action of phenazine was assumed that they diffuse across or insert into the membrane and act as a reducing agent, resulting in the uncoupling of oxidative phosphorylation, generation of toxic intracellular superoxide radicals and hydrogen peroxide which are harmful to the organisms (Jaaffar *et al*, 2017). These toxic molecules are thought to control the growth of microorganism and may enhance the ability of these pathogens to colonize human tissues. The crude had highly activity compared with purified and these could be happened because the crude had numerous compound like phenazine, pyoluteorin and other compound (Mussa and Ziayt, 2018).

Antiadhesive activity of phenazine

The antiadhesive activity of phenazine was evaluated against wound & burn pathogenic bacteria.The phenazine showed antiadhesive activity against all tests bacteria. The highest antiadhesive percentage was observed for *Acinetobacter baumanii* by percentage 48.6%. The results showed in Table 2.

Positive percentage indicate the reduction in bacterial adhesion when compared to the control and negative percentage indicate increase bacterial adhesion. Adhesion is first stage for biofilm development, Suleman and Mussa (2018) showed that the highest antiadhesive percentage were obtained for *Staphylococcus aureus* from different compound isolated from *P. aeruginosa*, while, Sakhtah *et al* (2016) appeared that phenazinehad hailey inhibition activity on biofilm formation for *Escherichia coli*. Numerous study focusing on the mode of action by which antimicrobial compound could controlling pathogenic agents (Arseneault *et al*, 2013). Phenazine could be consider one of novel antibiotic in treatment various bacterial infections. Phenazine modify the physicochemical characteristics of the outer membrane of biofilm-forming organisms through generation of toxic intracellular superoxide radicals and hydrogen peroxide (Saunders *et al*, 2018).

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