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A Study on the Effect of Phenazine 1- Carboxylic Acid Extracted from Rhizosphoric *Pseudomonas putida* on the DNA of Some MDR Bacteria Causing UTI

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ABSTRACT

Pseudomonas putida considered one of the most important microbes were used in biological control against a number of soil borne diseases because of its ability to produce large number of antimicrobial agents against pathogenic bacteria and Fungi.

This study designed to isolate the *P. putida* from Iraqi soil of different plant roots rhizosphere, and identified the isolates by culturing as well as by morphological and biochemical testing , vitek 2 system using to confirmed the acquaintance of isolates, then extracted and purified active metabolites phenazine -1- carboxylic acid were produced from *P. putida* and measured the activity in vitro; via inhibition the growth of some pathogenic MDR bacteria causing urinary tract infection .

The effectiveness of phenazine -1- carboxylic acid was studied on DNA (deoxy ribonucleic acid) extracted from some pathogenic bacteria ; as well as the standard calf thymus gland double stranded ctds DNA.

The results obtained in this work showed That active compound phenazine -1- carboxylic acid had effect on the DNA extracted from calf thymus gland, caused (Hyperchromasia), referring to cleavage the double standard DNA and causing the (Hypochromasia) breakage DNA extracted from *Acinetobacter baumannii, Staphylococcus aureus*. This study concluded that the *P.putida* has inhibitory effect on the growth of pathogenic bacteria and the mode of action of its activity could be relevant due to the production of antibiotics such as phenazine -1- carboxylic acid and subpart to its direct effect on DNA.

Keywords: MDR bacteria; DNA; rhizospheric *Pseudomonas putida*

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INTRODUCTION

Presently, the exaggerative uses of antibiotics performed to the emergence of highly resistant strains of bacteria. The emergence of drug-resistant bacteria rises, and newly emerging infectious diseases have become a serious universal public health menacing specifically affecting the economics of developing country. There is a need for nouveau antimicrobial agents which are effective against multidrug-resistant bacteria (1) Microorganisms are a major provenance for the discovery of new drugs. The majority of microbial natural products have been isolated from terrestrial-borne microbes (2)

The results of inspection to nouveau antibiotics leads to acquired large number of antibiotic substances mainly produced by rhizosphoric bacteria and fungi. Such as Bacillus, Actinomyces, pancillium and pseudomonas (3,4) *Pseudomonas spp*, which is one of the most significant genus found in the rhizosphere of varied plant are ubiquitous bacteria that are ordinary inhabitant in soil.(5) In most cases the bio controlling ability of these genus was associated to the generative various numerous antibiotics such as 2-4-diacethyl- phloroglucinol (DAPG), Pyrrolnitrin (PRN), Phenazines (Phz), and derivative Phenazines and Pyoluteorin (PLT)(6)

Phenazines and its derivatives are qualified to donation and acceptation electrons, Consequent the relative redox potential to other electron transfer molecules. Formation toxic free radical is a significant mode of action of phenazines and can be advantage to the host, through supply toxic superoxide (O_2^-) and hydrogen peroxide (H2O2) could inhibition or delimitation the pathogenic organisms cell by an interference with normal cell functions.(7)

The aims of current study was to extracted , purified of active metabolites phenazine -1- carboxylic acid that were produced from *p.putida* isolated from rhizospher of plant and studied its activity on some pathogenic MDR bacteria causing urinary tract infection . additionally distinguished The effectiveness of phenazine -1- carboxylic acid on DNA that extracted from some pathogenic bacteria ; as well as from calf thymus gland double stranded DNA(ctds DNA) as the standard DNA.

MATERIAL AND METHODS

Isolation and identification of *p. putida*

p. putida isolated from soil sample of plant roots rhizosphere showed antimicrobial activity against UTI isolates (included *Escherichia coli*, *Klebsilla pneumonia*, *Pseudomonas aeruginosa* and *proteus mirabilis*, *Acinetobacter baumannii*, *Serratia marcescens*, *Enterobacter cloacae*, *Streptococcus agalactiae* *Staphylococcus aureus*) and identified by culturing as well as by morphological , biochemical testing .confirmed the identification with Vitek 2 system

Extracted, purified active compound phenazine 1-carboxylic acid (PCA)

The phenazine -1- carboxylic acid antibiotic production by *P. putida* was extracted as described by (8)

The *P.putida* was cultured at 28 ºC for 48 h in King' B medium in a rotary shaker at 110 rpm. Then centrifuged at 12000 rpm for 10 minutes, the supernatant mixed with the same volume of ethyl acetate, Separated the ethyl acetate layer, evaporated to dryness under reduced pressure then purified the extracted PCA with HPLC C18 reversed-phase column (Zorbax SB-C18, 5.0µm, 4,6 mm*250 mm, Rockland Technologies Ind., Newport, DE, U.S.A.), and the column was eluted with dichloromethan : methanol (9:1) flow rate of 1.0 ml min^{-1}. PCA were detected by UV at 248 nm and determined the retention

times of compound which is specific value for each compound.

Antimicrobial activity of PCA on some bacteria isolated from UTI

Antibiotic susceptibility test were done to the isolates of UTI in order to determine the MDR isolates using the standard disc diffusion method and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (9).

using the method of agar well diffusion to distinguished the effectiveness of purified PCA produced from *P.putida* against uropathogenic bacteria on Muller Hinton Agar (6) MIC of purified PCA were determined using micro titration plate , The results of activity were measured in mm of formed inhibition zone .

Extraction of bacterial DNA

DNA of bacterial isolates were extracted using Mini Kits extraction Genomic DNA purification dependences on instruction of manufacturing company (Geneaid, Thailand)

Estimation of DNA Concentration and Purity

The DNA concentration of isolates were appraises using Nanodrop , 2µl of the extracted DNA were putting in the instrument to estimate concentration ,while purity retriever was finding using the ratio of O.D. 260/280, the acceded ratio of pure DNA is between 1.7-2 (10).

Effect of PCA on extracted DNA

Adding equal volume of purified phenazine 1- carboxylic acid(PCA) to the DNA extracted from some MDR pathogenic bacteria ; as well as the standard calf thymus gland double stranded DNA ,then exposures to the Ultraviolet scan at the weave length 260 nm in order to estimate the absorption .(11)

RESULTS AND DISCUSSION

The isolate of *p. putida* pp1 isolated from rhizosphoric Iraqi soil appeared good activity against the isolates of UTI bacteria.

Extraction, purification of PCA with ethyl acetate from the Isolate *P.putida* pp

In this experiment extracted PCA with ethyl acetate as organic solvent, produced from *P.putida* which appeared highly activity on filtrate against uropathogenic isolates. The organic phase was separated dried then re suspended in methanol, in ordered to obtained crude phenazine 1-carboxylic acid.

Purified PCA with HPLC

The retention time obtained of PCA with HPLC was 13.19 min in weave length 248 nm(figure 1) .this result very closely to result reported by other studies, the R.T obtained by (12) when purified PCA in HPLC was 14 min under same condition using in current study ,on the other said (13) showed that R.T of PCA depending on the orging solvent in mobile phase which using in elution of compound and weave length using to detect the absorbance . MIC were determined using micro titration plate, which was equal to 50 µg/ml the results of antibiotic sensitivity test of uropathogenic isolate appeared that all isolate of UTI had highly resistance on most antibiotics and showed multi drug resistant .the results were agreement with numerous researches, (14) confirmed that bacteria isolated from UTI was *E.coli, klebsiella, Enterococcus*, *Enterobacter* and these isolate showed resistance to antibiotic, Augmenten, Levofloxacine, cefoperazon, Ampicilline, Doxycytine, Gentamycine and Nalidix acid. The results in current study agreement with many others research which focusing to the importance of MDR bacteria and the roles in increasing risk factor of the UTI. (15) confirmed that all common bacterial causing UTI *Staphylococcus spp.*, *Streptococcus spp.*, *Enterobacter spp., Klebsiella spp.* and *E.coli* appeared MDR.

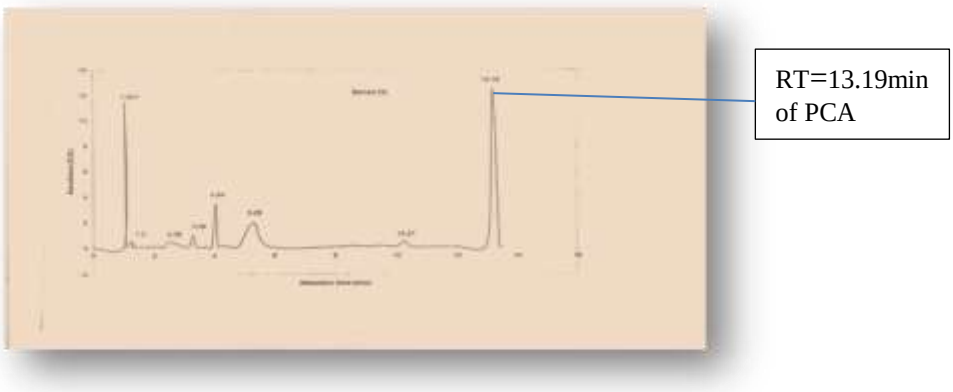

Figure 1: HPLC Chromatogram for PCA purification. Conditions: C-18 column (250 mm×4.6 mm, 5 µm); flow rate: 1 mL/min; detection wavelength: 248 nm; RT obtained was 13. Which belong to PCA

Antimicrobial activity of phenazin

Activity of crude and MIC of purified PCA on MDR bacteria causing urinary tract infection using agar well diffusion method ,results obtained were summarized in(table1)

Table 1: Antimicrobial activity of crude and purified PCA on UTI isolates measured the inhibition zone in mm

Bacterial isolates	Crude PCA	Purified PCA 50 µg/m
Klebsiella pneumonia	16	12
Escherichia coli	10	20
Pseudomonas aeruginosa	15	12
Serratia marcescens	12	20
Enterobacter cloacae	8	14
Acinetobacter spp baumanii.	12	20
Proteus mirabilis	15	17
Morganella morganii	12	14
Staphylococcus aureus	29	34
Streptococcus agalactiae	28	24

The results showed that highest activity of crude PCA recorded on *Staphylococcus aureus* 29 mm and the lowest activity was recorded on *Enterobacter cloacae*(8mm) compared with purified PCA the inhibition zone of PCA on *Staph.aureus* 34 mm and for *E.coli* the inhibition zone was 10.

Numerous studies reported that *P.putida* could synthesis various substances as secondary metabolite which could played significant role in dominating pathogens and could synthesis abroad spectrum antibacterial and ant fungal antibiotics , one of these compound phenazine -1- carboxylic acid (16) The phenazine -1- carboxylic acid antibiotics action as reducing agents generating toxic intracellular superoxide radicals radical (O_2^-) and hydrogen peroxide H_2O_2 which are disadvantage to the organisms .and considered impotent mode of action in dominating pathogenic agent (17) .

Effect of phenazine -1- carboxylic acid on extracted DNA :calf thymus gland double stranded ct ds DNA : the effect of mixing 50 µg/ml of purified PCA with equal volume of extracted ct ds DNA and measured the absorebtion on weave length 260 nm , caused Hyperchromasia , referring to cleavage the double standard DNA ,and this effect depending on the concentration of PCA on the solution and it was ejective proportionally with it , figure 1 .

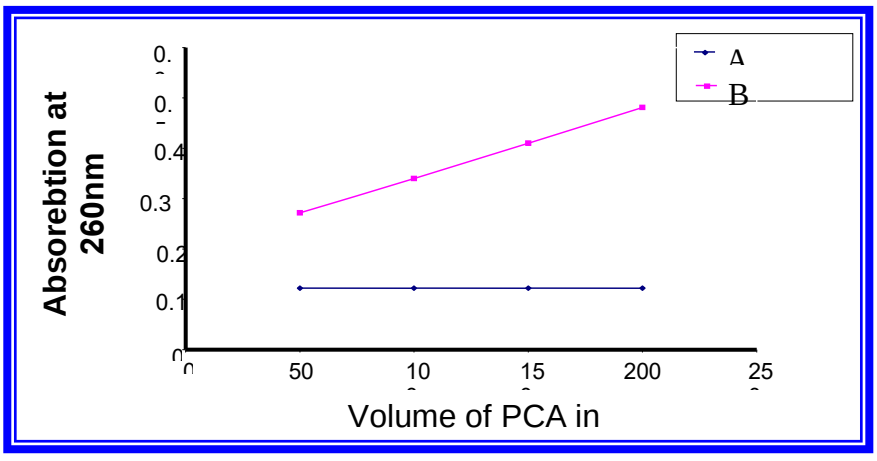

Figure 1: Effect of PCA on ds calf thymus DNA
A / before adding PCA B / after adding PCA

While the effect of PCA on DNA extracted from *Acinetobacter baumanii.* as gram negative bacteria and on DNA extracted from *Staph .aureus* as gram positive bacteria .The PCA also showed effect in breakage DNA extracted from this isolates of bacteria *S.aureus* and *Acinetobacter baumanii* causing the (Hypochromasia).figure 2 ,3

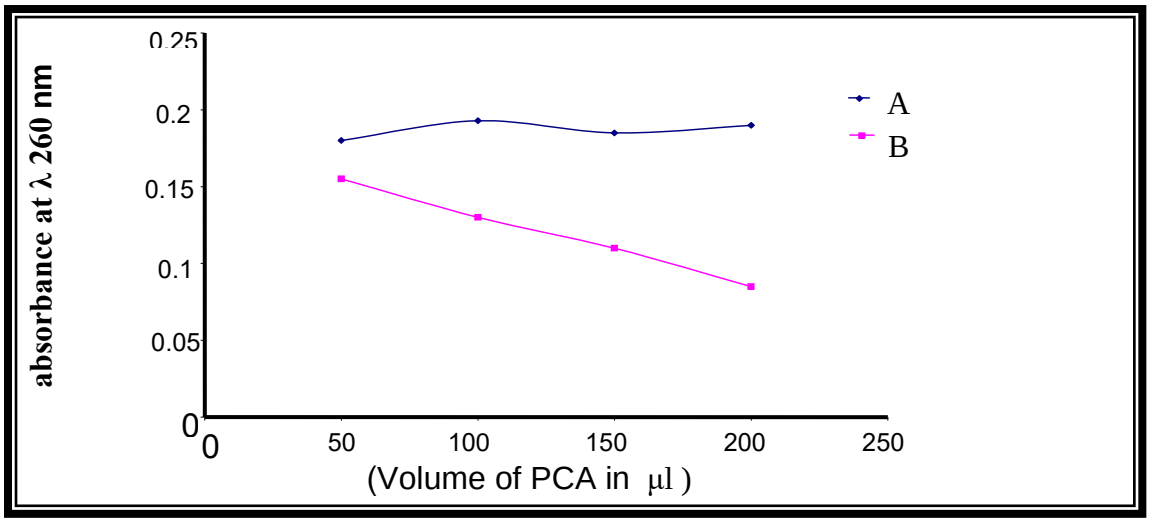

Figure 2: Effect of PCA on Staph. Aureus DNA
A / before adding PCA B / after adding PCA

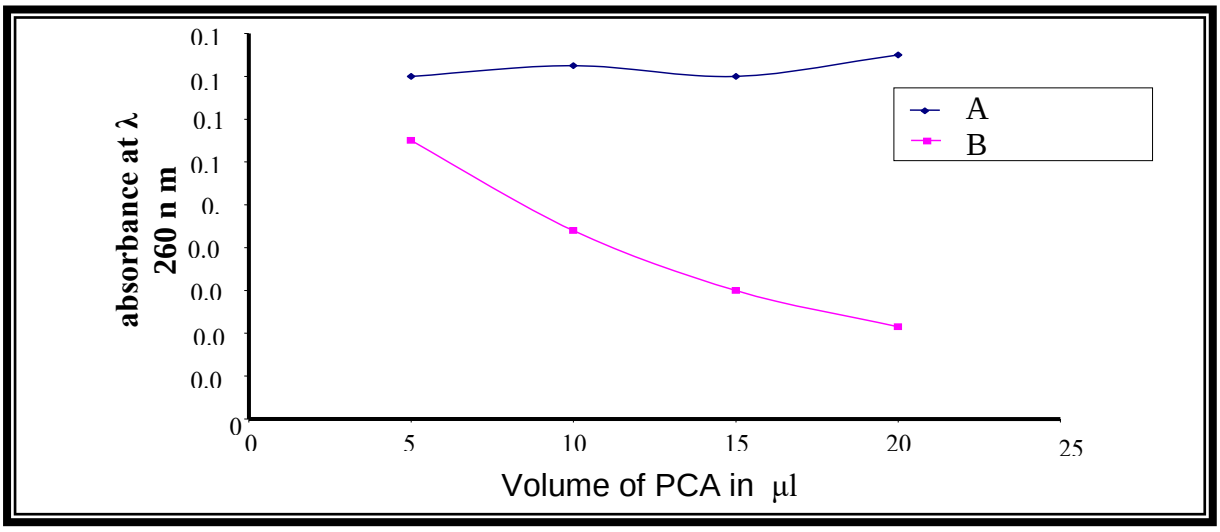

Figure 3: Effect of PCA Acinetobacter baumanii DNA
A / before adding PCA B / after adding PCA

As clear from the results obtained that antibiotic phenazine -1- carboxylic acid (PCA) had various effected in DNA extracted from different cells ranged from separated of DNA stranded or breakage the stranded of DNA (Hyperchromasia and Hypochromasia) respectively ,and since the experiment achieve in vitro , the effectiveness of PCA was directly and there was no intermediate of any biological interaction correlated with activated or inhibited of any enzymes ,this agreement with maney other studied (19. 18)

The PCA acted in breakage the hydrogen bound found between DNA stranded of double stranded calf thymus gland ct ds DNA and this action is reversible (20) .

While the PCA action on bacterial DNA was Hypochromasia and this action is irreversible (18) .numerous studies pointed that derivatives of phenazein could binding in DNA bases causing destroyed it .(21,22) .on the other said (23) ascertained the action of these compound in vivo could destroyed the DNA via Topoisomerase II enzyme , whereas the producing bacteria could be protected from the action of these compound through inhibitors the isoleucyle – tRNA synthatase and avoided self – suicide (24,25,26)

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