Isolation and characterization of phenazine produced from mutant *Pseudomonas aeruginosa*

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Abstract

Hundred and four isolates of *Pseudomonas aeruginosa* were screening and isolate SP9 identified as belong to *Pseudomonas aeruginosa*, and was selected according to it's high phenazine production. That isolate was subjected to mutation by different concentrations of N-methyl-N-nitro-N-nitrosoguanidine (NTG).

In 250 µg/ml of NTG, the mutant strain produced noticeable amount of phenazine more than isolate of strain SP9 which named SPm9. separation and characterization of phenazine was done by using TLC, HPLC and IR spectrum. Gel filtration was used for purification of phenazine with sephadex G25.

Antimicrobial activity of phenazine was done by using disk method for numbers of Gram-positive and Gram-negative isolates.

The results indicate that phenazine which produced from mutant strain SPm9 is a broad spectrum antibiotic follows like that produced from isolate strain Sp9. it is effective against *Bacillus subtillus, Staphylococcus aureus* and *Staphylococcus epidermidis* as well as *Escherichia coli* and *Salmonella typhi*. Phenazine was also found to be effective against *Candida albicans*.

عزل وتشخيص الفينازين المنتج من عزلة طافرة لبكتريا *Pseudomonas aeruginosa*

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الخلاصة

فحصت 104 عزلة من بكتريا *Pseudomonas aeruginosa* وانتخبت العزلة SP9 لقدرتها العالية على إنتاج الفينازين. طفرت العزلة SP9 باستخدام تراكيز مختلفة من المطفر الكيميائي N-methyl-N-nitro-N-nitrosoguanidine (NTG). في التركيز 250µg/ml من المطفر NTG أظهرت العزلة الطافرة إنتاجية ملحوظة من الفينازين بكميات أكثر من العزلة الأصلية غير الطافرة. تم عزل وتشخيص الفينازين باستخدام تقنيات TLC،HPLC و IR. استخدمت طريقة الترشيح الهلامي لتنقية الفينازين بواسطة Sephadex G25. وتم دراسة الفعالية الحيوية للفينازين باستخدام طريقة الأقراص مع عدد من الأحياء المجهرية السالبة والموجبة لصبغة غرام. أشارت النتائج إلى ان للفينازين، المنتج من العزلة الطافرة، مضاد حيوي ذو مدى واسع وله تأثير ضد *Bacillus subtillus, Staphylococcus aureus* و *Staphylococcus epidermidis* الموجبة لصبغة غرام وبالإضافة إلى *Escherichia coli* و *Salmonella typhi* السالبة لصبغة غرام. ووجد أيضا ان للفينازين تأثير ضد المبيضات البيضاء *Candida albicans*.

Introduction

Phenazines constitute a large group of nitrogen containing heterocyclic compounds produced by a diverse rang of bacteria (1). Phenazine compounds produced by

fluorescent *Pseudomonas* competitiveness (2). The most widely studies the phenazine producing fluorescent Pseudomonad is *Pseudomonas aeruginosa*, a gram negative opportunistic bacteria of human, animals, insects, nematodes and plants (3,4,5). From 90-95% of *Pseudomonas aeruginosa* isolates produce pyocyanin (6) and which most of them characterized as phenazine compounds include phenazine-1-carboxylic acid (PCA), 1-hydroxy phenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (7, 6). More than 100 different phenazines structural derivatives have been identified in nature, and over 6000 compounds that contain phenazine as a central moiety have been synthesized (8), and these secondary metabolites have been studied intensively because of their broad spectrum antibiotic properties (9), against different bacterial and eukaryotic species. The side chain substituents on the phenazine backbone contribute to the biological activities of the compounds (10). Phenazine 1-carboxylate (PCA) secreted by *Pseudomonas fluorescens* contributes to biocontrol activity against fungal phytopathogens such as *Gaeumannomyces graminis* (11), and phenazine-1-carboxamide produced by *Pseudomonas chlororaphis* is able to inhibit the fungus *Fusarium oxysporum* (12). Previous studies showed that phenazine compounds also have antimicrobial activity against strains of *Bacillus subtilis*, *Candida albicans*, *Enterobacter aeruogenes*, and *Escherichia coli*, the phenazine also owing activity against *Mycobacterium tuberculosis* (13) and some plant pathogenic bacteria such as *Erwinia carotovora* and *Ralstonia solanacearum* (14). The objectives of the present work is to isolation and identification mutant *Pseudomonas aeruginosa* capable of phenazine production.

Materials and Methods

- **Samples collection**: Hundred and four samples suspected to contain *Pseudomonas aeruginosa* were collected from different infections in human; Urine, Sputum, Ear and Wounds; from Al-Fallujah hospital and a clinical laboratory. *Pseudomonas aeruginosa* was enriched and then isolated by using cetrimid agar (Hi-Media, India) as selective medium which favor growth of *Pseudomonas aeruginosa* and inhibit other bacterial species. In addition to other media MacConkey's agar, King's A and B and Piocyanosel agar (Biomerix, France), which show a unique and easy characterize colonies. Cultures of *Pseudomonas aeruginosa* were grown on King's A medium at 37°C for 11 days, then 1ml of the suspension used to inoculate 100ml of Modified Pseudomonas P medium (PsP), consisted of the following: DL- alanin 2 g, Sodium citrate 10 g, Potassium sulfate 8.6 g, Potassium chloride 1.4 g, Magnesium sulfate 1.4 g, Dipotassium hydrophosphate 5 g. (15). All component were added to 1 liter of distilled water. pH was adjusted to 7.2-7.3 and sterilized by filtration through Millipore filters 0.45µm. The medium was suspended as 100ml in a 250ml Erlyenmeyer flask and incubated at 37°C on rotary shaker at 110 rpm for 3 days.
- **Test microorganism**: *Candida albicans*, *Escherichia coli, Klebsiella spp., Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus, Staphylococcus epidermidis* and *Bacillus subtillus* were used . These microorganisms were obtained from Baghdad university /College of science/ Biotechnology department and identified by using cultural properties and different biochemical tests as compared with schematic diagram of identification described by (16).
- **Standardization curve of Phenazine**: Standardization curve of Phenazine was done as follows:

1. **Wave length determination:** Maximum absorbance for phenazine was determined by scanning of wavelength using UV-visible spectrophotometer. Chloroform was used as reference sample, while 500 µg/ml of standard phenazine (sigma, U.S.A.) was used for scanning wavelength from (200-500 nm).

2. **Graf the standardization curve of phenazine antibiotic:** Standard phenazine were used. Different concentration of phenazine were used (50, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 µg/ml) for recording absorbance at 375 nm. (Fig. 1)

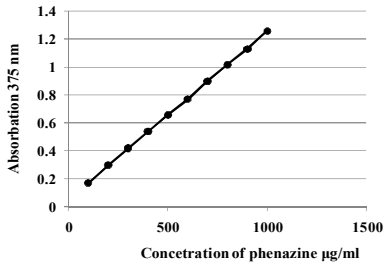


Fig (1): Standerization curve of phenazine

- **Measurement of pigment concentration:** Dark– blue chloroform extract was measured by UV-visible spectrophotometic analysis at 690 nm. Pure chloroform was used as blank. The following mathematical equation was used to caculate pyocyanin concentration: (17)

$$\text{Pigment concentration} = \frac{\text{Luminary density of pyocyanin suspension in wave length 690 nm}}{4.3 \times 106}$$

The result multiple with molecular weight of pyocyanin 210.2. The result measured with µg/ml.

Note: 4.3 × 106 is extinction coefficient of crystalline sample of pure pyocyanin in wave length 690 nm (18).

- **Mutagenesis:** The methylating compound N-methyl-N-nitro-N-nitroso guanidine NTG (Pharmacia U.S.A.) was used as mutagenic agent following the method described by (19):

1. An amount (10 ml) nutrient broth was inoculated with *Pseudomonas aeruginosa* and incubated untile count achieved about 5×10^8 cell/ml.
2. The culture centrifuged for 5 minutes at 5000 rpm to pellet the bacteria. Cells pellet of bacteria were then resuspended in equal volume of Tris-malic acid buffer prepared by Tris 6 g, Malic acid 5.8 g, $(NH_4)_2SO_4$ 1 g, $FeSO_4.7H_2O$ 0.25 mg. The volume was completed to 1 liter. pH was adjusted to 6 and autoclaved for 15 minute at 121°C. After medium has cooled to about 50°C aseptically $MgSO_4.7H_2O$ 0.1 g, KNO_3 5mg was added.
3. Different concentrations of freshly prepared NTG (100, 150, 200, 250, 300 µg/ml) was added and cultures were incubated at 37°C without shaking for 30 minutes.

The treated culture was centrifuged then the pellets were resuspended in an equal volume of M56 minimal medium containing Na_2HPO_4 8.2 g, KH_2PO_4 2.7 g, $(NH_4)_2SO_4$ 1 g, $FeSO_4.7H_2O$ 0.25 mg. Per liter. pH was adjusted to 7.2 and autoclaved for 15 minutes at 121 °C. After medium has cooled to about 50°C $Mg_2SO_4.7H_2O$ 0.1 g, KNO_3 5 mg was aseptically added and supplement with 10 ml of sterile 20% glucose solution.

4. The culture was recentrifuged and again pellets were resuspended in M56 minimal medium.
5. Serials dilutions were done of 10-1-10-10 which spreaded on nutrient agar and incubated in 37°C for 24 hours.
6. The treatment that gave Killing percentage 95%, was chose then count of residue bacteria was done using following equation:

$$\text{Killing percentage} = \frac{\text{Origin number of bacteria-residue number of bacteria}}{\text{Origin number of bacteria}} \times 100$$

7. Add 0.1 ml culture broth to PsP medium in dark condition at 37°C on rotary shaker at 110 rpm for 3 days.

- **Extraction of phenazine:** *Pseudomonas aeruginosa* mutant (SPm9) was grown at 37 °C in pigment production medium PsP, the cultures were incubated in rotary shaker at 110 rpm for 3 days. Phenazine antibiotics were extracted from the culture liquid depended on the method described by (20, 21) as follow:

Five milliliters of each culture was centrifuged (5000 rpm) for 30 minutes, and the supernatant was acidified (pH,2) with concentrated HCl. Then 5 ml of benzene was added. Samples were mixed for 1 hour and centrifuged at 5000 rpm for 30 minutes. Four milliliters of the benzene layer was decanted and dried under air. Samples were resuspended in 1 ml of 0.1 N NaOH, and the absorbance at 375 nm was determined.

- **Thin layer chromatographic method (TLC):** The technique of TLC was used as described by (22) with the some modification as follows:- The sheet (silica gel 60f-254, 0.2 mm, layer thickness and aluminium support, size 20×20 cm, Spain) was used for analyzing samples. Slotting line was marked 1 cm from bottom edge of the plate. A liquid of standard phenazine which dissolved with chloroform was spotted on the first position and the test samples spotted on other positions, the plate was left some minutes to dry in dark condition before development. The TLC plates were developed in chloroform-methanol (9:1 v/v) in an unlined and un-equilibrated glass tank, until the solvent front reached a mark distance from the spotting line. The plate was removed and left to dry. The plate examined under UV light and calculated Rf values.
- **High performance liquid chromatography (HPLC):** Analysis was performed with a Shimadzu LC-2010 AHT liquid chromatography (Japan) with Reodyne 7125, 20µl injector, a Shimdzu SPD-2010 A UV-visible detector set at 375 nm and the column of (250×4.6 mm) C18, 5 µm particle size was used. The mobile phase was 100% acetone nitrile. The flow rate was 0.5 $ml.min^{-1}$. The column temperature was maintained at 30 °C.
- **Gel filtration chromatography:** An amount (3 ml) of crude phenazine was add to the column, which packaged by sephadex G25, accurately and eluted with Tris 0.01M with pH 7.5 contain 0.1% of Triton X100 and 0.25M sodium chloride buffer which prepared by dissolving 1.2114g of Tris with 14.61g of sodium chloride in some distilled water, pH 7.5, then 1ml of Triton X100 was added then the volume was completed to 1 liter of distilled water. The flow rate was 1 ml/ 3min, phenazine in eluted fractions were detected using UV-visible spectrophotometer at a wavelength of 375 nm. After the complete elution, the column was rewashed by Tris 0.01M; pH 7.5, contain 0.1% of Triton X100 and 0.25M sodium chloride buffer for 24 hours. Results were ploted as optical density and fraction number.
- **Dialysis:** Dialysis was done opposite to Tris 0.01M; pH 7.5, contain 0.1% of Triton X100 and 0.25M sodium chloride buffer for several days with repeated changes every days for discard from salts.
- **Biological activity of phenazine antibiotic:** Determination of phenazine activity was done by using paper disk method: Filter paper (watman) discs 5.5 mm in diameter are prepared beforehand and sterilized by autoclave at 121°C for 15 min, then sterile paper disk was taken by using forceps, an amount (50, 100 and 200 µg/ml) of phenazine antibiotic were precipitated on the sterile paper disk and placed a top the Muller-Hinten agar which spreaded with different test microorganism and incubated for 24 hour at 37°C. Then inhibition zone around the disk was measured.

Results and Discussion

- **Identification of the isolates:** A number (104) of isolates suspected to be *Pseudomonas aeruginosa* were isolated from different infections in human included Urine, Sputum, Ear, Wounds and burns which collected from patients in Al-Fallujah hospital and clinical laboratories, the percentage of prevalence rates of *Pseudomonas aerugiosa* infection were 28.8% of burns, 19.2% of sputum, 21.1% of urine, 17.3% of ear and 13.4% of wounds. The isolates were identified as *Pseudomonas aeruginosa* using cultural and biochemical tests and grown on different media along with their ability to produce pyocyanin pigment. These tests were compared with schematic diagram proposed by (16).
- **Screening of the isolates:** Screening of the isolates was done according to their production of pyocyanin on Pseudomonas P medium as measured by (17). Isolate SP9 was selected as the highest producer which produce as much as 0.174 mg/ml. The SP9 strain then mutated by subjecting the wild type to (0, 100, 150, 200, 250, 300 µg/ml) of NTG. The results indicated that 250 µg/ml was the concentration which produce 95% killing ratio. This concentration was used to develop the mutant which designated as SPm9. Results indicated that as much as 496 µg/ml of phenazine was produced by mutant SPm9 as compared with wild type Sp9 281µg/ml.
- **Identification of phenazine:** Prior separation of phenazine from cultural medium was done by TLC with the solvent system chloroform: methanol (9:1 v/v). A band 2 with Rf 0.85 was characterized as related to phenazine as long as it gives biological activity when tested as shown in (Fig 2).

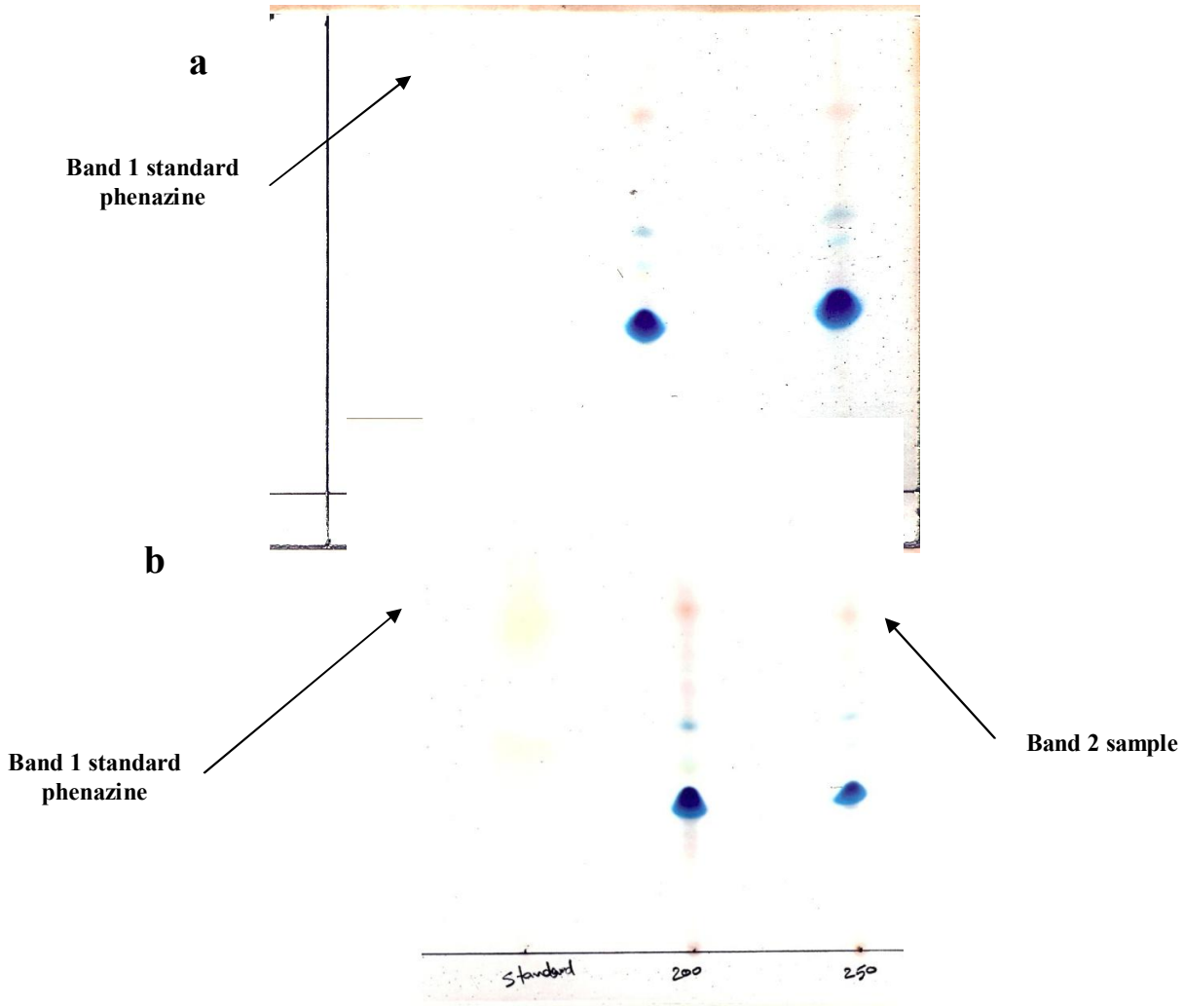


Fig. (2) Identification of phenazine by TLC a- concentration of NTG 100 and 150 µg/ml. b- concentration of NTG 200 and 250 µg/ml.

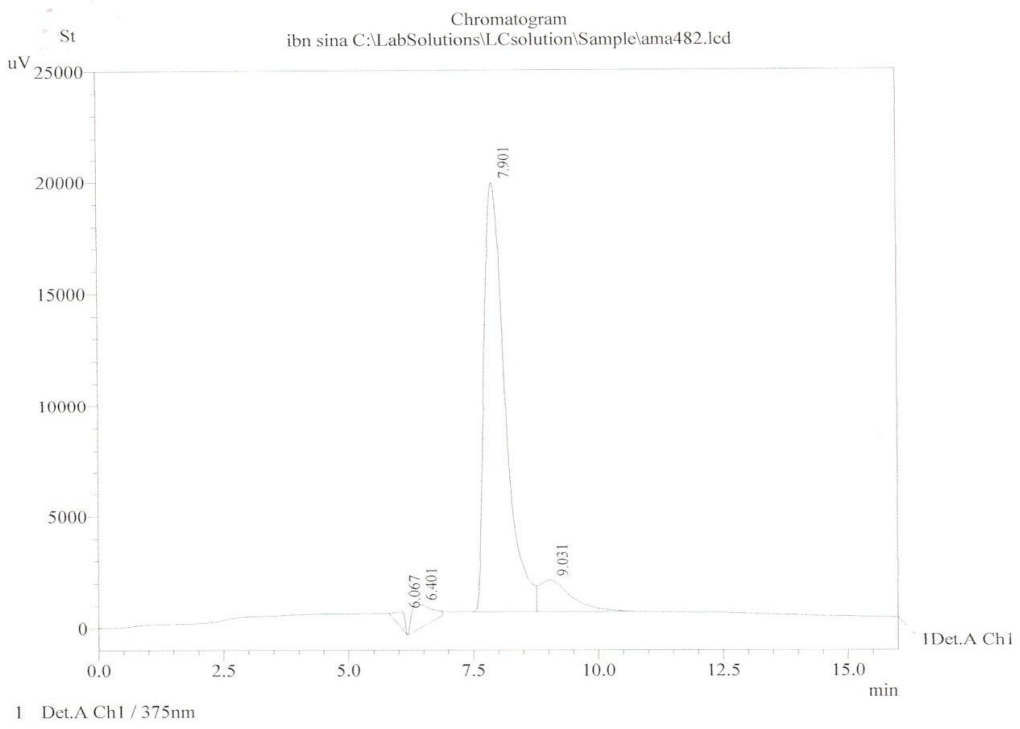
- **Identification of phenazine by HPLC**: A typical extraction of phenazine was done by using HPLC. Results shown in (Fig 3) which includes:

a. Standard phenazine represented by a peak with retention time of 7.9 min. HPLC analysis of phenazine from *Pseudomonas aeruginosa* isolate SP9 revealed the presence of typical peak of maxima at 375 nm. This result agree with that described by (23).

b. Presence of three peaks with a retention time 7.049 min, 7.792 min, 8.667 min compared with standard phenazine. These results agree with that of (24) who demonstrated the ability of *Pseudomonas aeruginosa* to produce many phenazine derivatives beside pyocyanin. Other studies reported that strains of *Pseudomonas aeruginosa* could produce a variety of redox-active phenazine compounds included pyocyanin, phenazine-1-carboxylic acid (PCA), 1-hydroxy phenazine (1-OH-PHZ), and phenazine-1-carboxamide (PCN) (7, 6).

The concentration of phenazine was calculated in the diagram as 496 µg/ml as compared with standard curve of phenazine antibiotic (Fig 3).

a



b

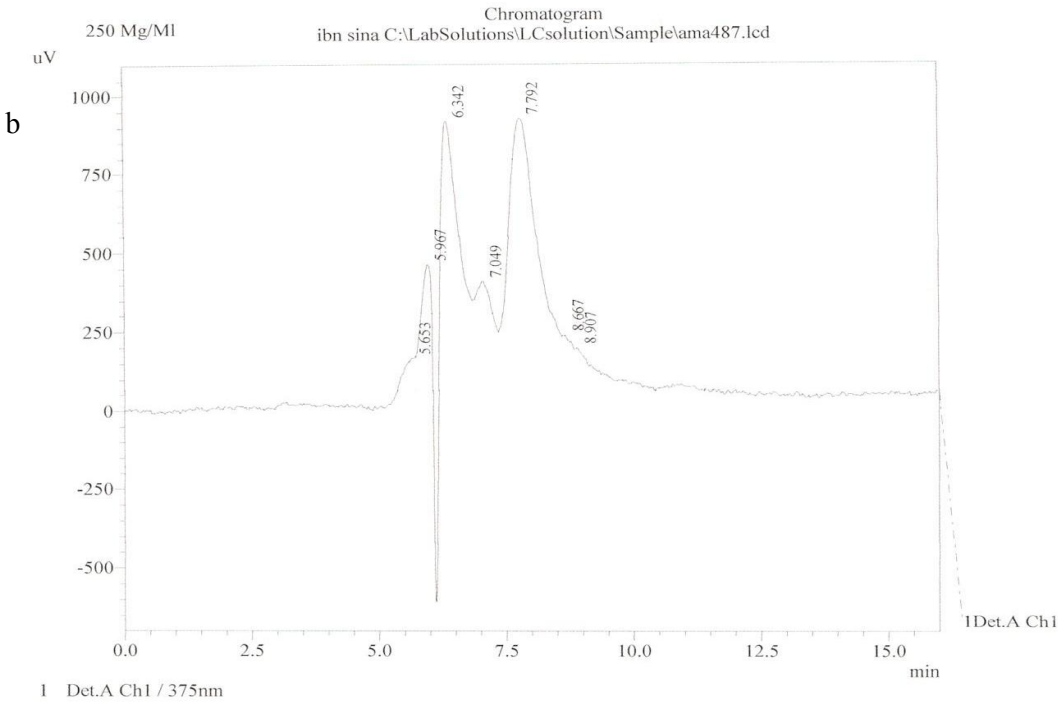
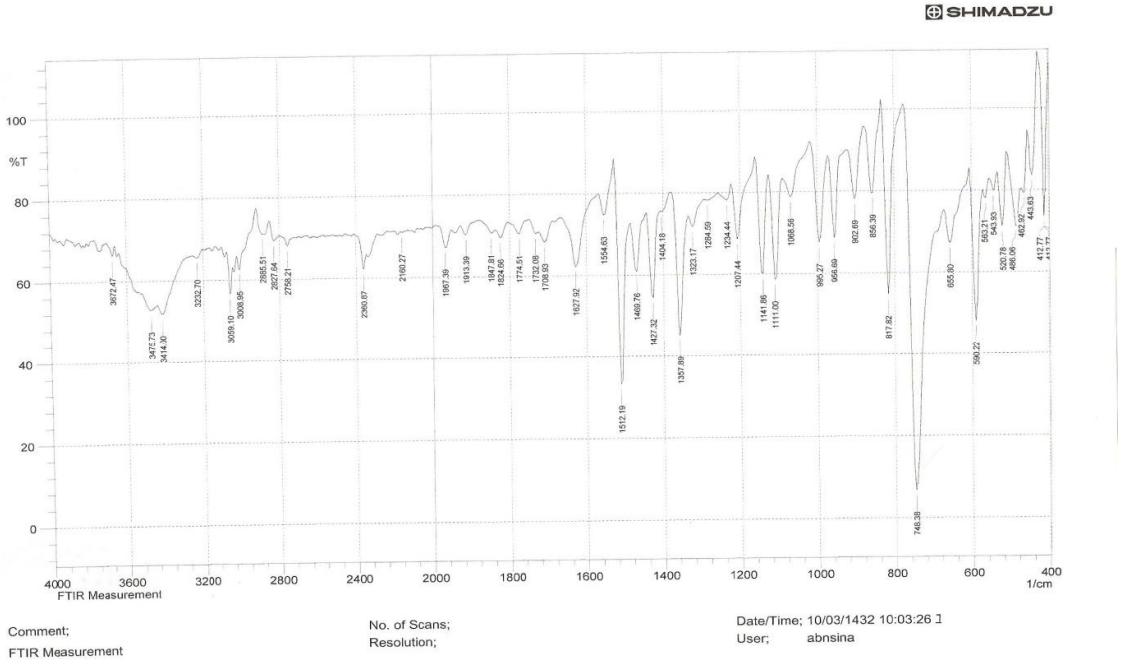


Fig (3) Identification of phenazine by HPLC. a- standard phenazine. b- Sample of extract phenazine separation from SPm9 strain

- **Identification of pheazine by IR:** Further identification of the metabolite separated from culture of SP9 was done by IR. The spectrum shown in (Fig 4) indicate the presence of phenazine as specified by side chains of the molecule as compared with standard which their results shown in(Table 1).These results agree with that of (13).

a



b

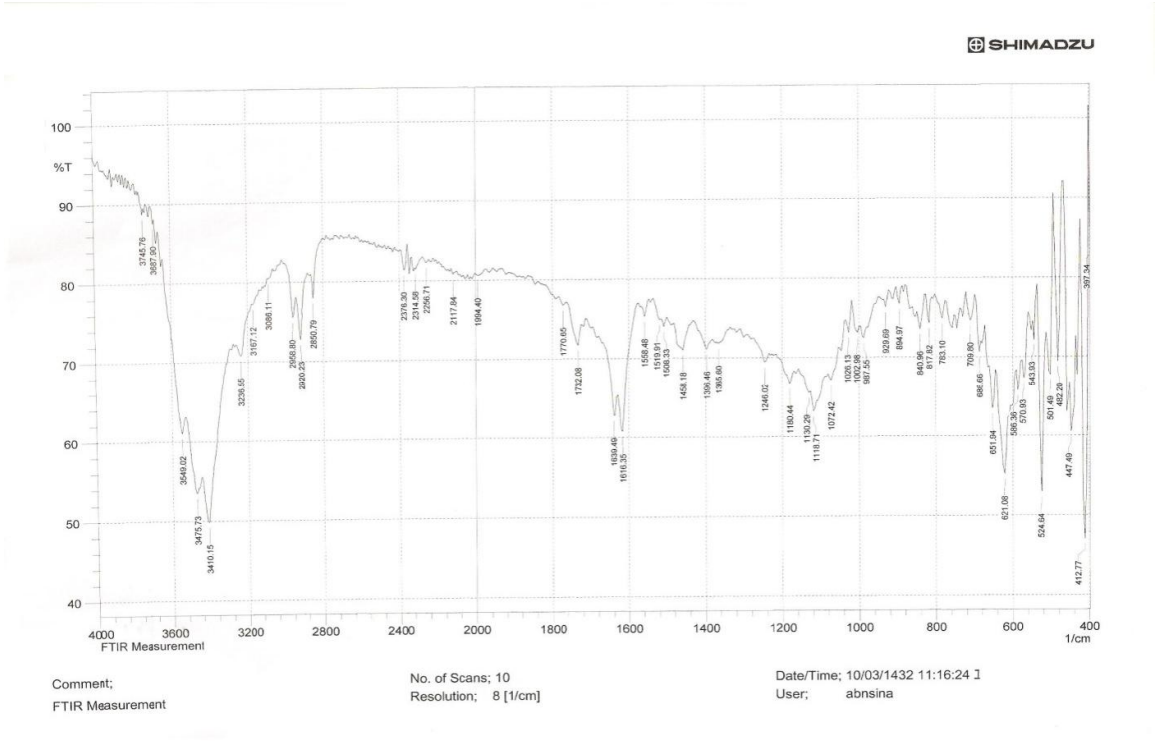


Fig (4) FTIR measurements for: a- standard phenazine b- sample

Table (1) Absorption bands of FTIR for cycle compounds of phenazine antibiotic

compoun ds	H-O	C-H aromatic	C=N	C=C aromatic	C=O	C-O	C-N	ip O-H	ip C-H aromatic	oop C-H aromatic
Standard phenazine	3475.73	3059.10	1627.90	1554.63	1469.76	1323.17	1284.59	1207.44	856.39	748.38
Sample with 250 µg/ml NTG	3475.73	3086.11	1639.49	1558.48	1458.18	1365.60	1246.02	1180.44	840.96	709.80

- **Mutagenesis:** Mutation experiment was conducted to obtain more productive strain. Survival curve for isolate SP9 was done to specify the most effective concentration of mutagen NTG . Results indicate that 250 µg/ml of NTG reducing the number of cells to 95% as shown in (Fig 5). This concentration was used to develop the mutant strain.

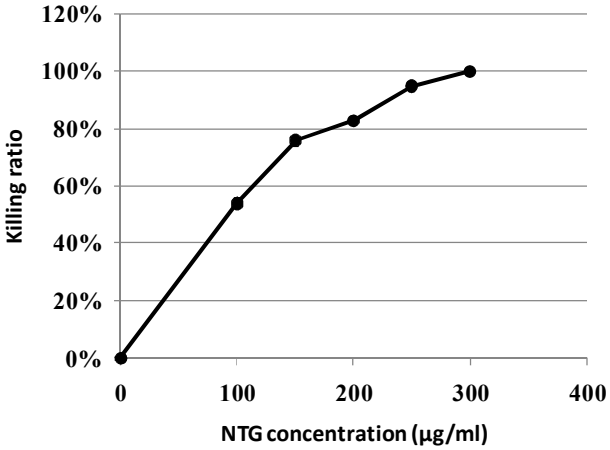


Fig (5) The effect of mutagen NTG on growth of *Pseudomonas aeruginosa* SP9

A maximum amount of 496 µg/ml of phenazine was obtained from mutated strain SPm9 as compared to that of wild type Sp9 (281 µg/ml) which indicated an increases in production of phenazine. The methylating compound NTG is one of the most potent mutagens yet discovered for bacteria. It induces primarily base transition mutation of the GC to AT type, although AT to GC transitions, transversions, and even framesshifts arise (19). So that mutagen used in many studies for increase the production of secondary metabolites of bacteria. A duplication in production was obtained when Al-Tememi 2000 (23) mutanted *Pseudomonas aeruginosa* by using NTG. Byng and Turner, 1976 (25) reported the ability of mutants *Pseudomonas phenazinium* to synthesize nine other phenazines.

- **Purification of phenazine by Gel filtration:** Purification of phenazine was done by using gel filtration using sephadex G25 due to its low molecular weight (26). Results shown in (Fig 6) indicate the presence of fraction having activity at 375 nm. When these fraction pooled, the concentration measured spectrophotometrically, 200 µg/ml an amount of was obtained as compared with standard phenazine.

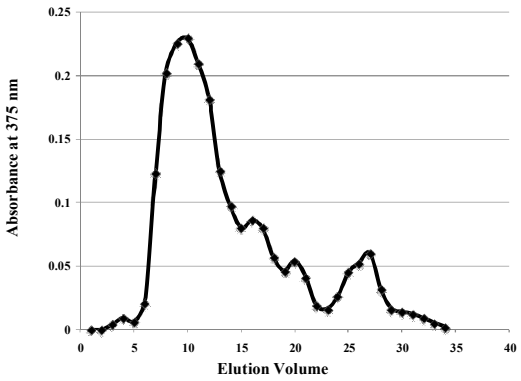


Fig **(6) Gel filtaration of Phenazine extraction from Spm9 strain by sephadex G25**

- **Biological activity of phenazine antibiotic:** Different concentration volumes of phenazine antibiotic (50, 100 and 200 µg/ml) were prepared for specify antimicrobial activity of phenazine against different microorganisms included: Gram-positive such as *Bacillus subtillus, Staphylococcus aureus* and *Staphylococcus epidermidis*; Gram-negative such as *Escherichia coli, Klebsiella spp*., *Salmonella typhi* and *Pseudomonas aeruginosa*, and Yeast as *Candida albicans* . Results shown in (*Fig* 7) indicate that phenazine antibiotics shows high antimicrobial activity against all mentioned organisms except *Pseudomonas aeruginosa* and *Klebsiella spp*. MIC for phenazine was found to be 200µg/ml when purified phenazine was tested. This concentration was found to be effective against gram-positive bacteria specially *Staphylococcus aureus* and *Staphylococcus epidermidis* more than *Escherichia coli* and *Salmonella typhi* and have antimicrobial activity against *Candida albicans* more than *Escherichia coli* and *Salmonella typhi* but show less than that observed against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Results shown in (Fig 7) indicate that, all concentration of phenazine gave antimicrobial activity against all microorganisms tested except *Klepsiella pneumonia* and *Pseudomonas aeruginosa*, and these results indicated that the purified phenazine have biological activity against gram negative as well as gram positive bacteria. We notice that the phenazine produced from mutant *Pseudomonas aeruginosa* (SPm9) have biological activities mimic that produced from isolate *Pseudomonas aeruginosa* (SP9). It was also have antimicrobial activity against *Staphylococcus aureus* and *Staphylococcus epidermidis* more than *Escherichia coli* and *Salmonella typhi* and have antimicrobial activity against *Candida albicans* more than *Escherichia coli* and *Salmonella typhi* but less than that observed against *Staphylococcus aureus* and *Staphylococcus epidermidis*. These results are in agreement with that results of (13) who reported that phenazine antibiotic have antimicrobial activity against strains of *Bacillus subtilis*, *Candida albicans*, and *Escherichia coli*. However others demonstrated that activity of phenazine antibioties are concentration dependent (27).

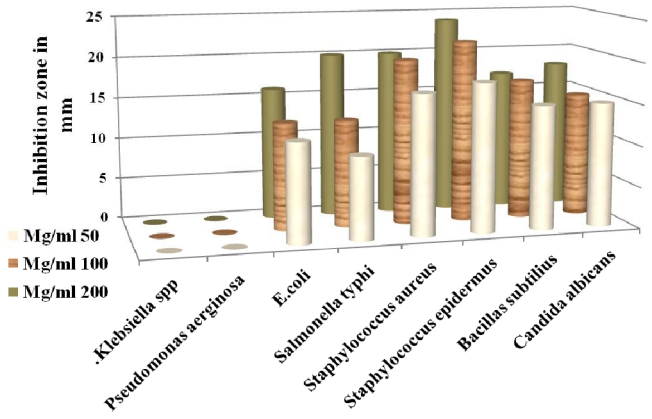


Fig (7)*:* **Antimicrobial activity of phenazine extraction from Spm9 against positive and negative bacteria and yeast**

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