Plant Archives Vol. 20 No. 1, 2020 pp. 1869-1875 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

MOLECULAR AND PHENOTYPIC STUDY OF SILVER NANOPARTICLE EFFECT ON PYOCYANIN PRODUCED FROM *PSEUDOMONAS AERUGINOSA*

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Abstract

Seventy two gram negative bacterial isolates were isolated from ninety eight burn wounds specimens obtained from Baghdad hospitals after culturing on macConkey agar medium. Fourty seven (47.9%) out of them were identified as *Pseudomonas aeruginosa* after culturing on ceramide agar medium and performing further biochemical tests, all these bacterial isolates were identified by VITEK2 compact system. They were individually screened for pyocyanin production by using mineral salt broth supplemented with 1% peptone, the results showed that nine bacterial isolates were pyocyanin producers and the isolate *P. aeruginosa* (Pa15) was the strongest in pyocyanin production. All 47 isolates went through the antibiotic susceptibility test, the results showed that 85.1% of total isolates were sensitive to imipenem and 60% were resistant to ceftazidime, while for the nine pyocyanin producers isolates, the results showed that 75% of isolates were sensitive to gentamicin while 88% were resistant to ciprofloxacin, levofloxacin and ceftazidime. Nine isolates which were resistant and gave the highest pyocyanin production were selected for this study. Then, the isolates were tested to determine the minimal inhibitory concentration for ciprofloxacin against *P. aeruginosa*, by agar dilution method, the results showed that the MIC distributed between 8 and 128 µg/ml. The strongest isolate in pyocyanin production *P. aeruginosa* (Pa15) was tested to determine the minimal inhibitory concentration for sliver nanoparticles against it, the minimal inhibitory concentration was 0.012 mg/ml . The control with three treatments, the sub MIC (0.006 and 0.003 mg/ml) for sliver nanoparticles and the sub MIC 6 mg/ml for ciprofloxacin against *P. aeruginosa* (Pa15) were taken to study pyocyanin expression. DNA of *P. aeruginosa* (Pa15) was successfully extracted from overnight cultures, and PCR was conducted for *P. aeruginosa* (Pa15) to amplify the constitutional genes 16srRNA, *phzM* and *phzS* and the bands were confirmed with gel electrophoresis, the results showed that genes 16srRNA (965 bp) and *phzM* (312 bp) were detected. RNA was extracted from the selected isolate after growing on cetrimide agar medium as control in addition to three treatments, the sub MIC of silver nanoparticle (0.006 mg/ml and 0.003 mg/ml) and sub MIC of ciprofloxacin antibiotic (6mg /ml), The results of QRT- PCR in this study showed that the expression of *phzM* gene in the presence of silver nanoparticles was decreased to 0.895025 fold compared to the control sample, But, exposure to ciprofloxacin antibiotic increased the expression of *phzM* gene by two folds.

Key words Silver nanoparticles, peptone, pyocyanin, *phzM* gene, *Pseudomonas aeruginosa*, gene expression.

Introduction

Pseudomonas aeruginosa is one of the most clinically and epidemiologically significant microscopic organisms. It is the major cause of nosocomial infections among non-fermenting Gram-negative bacilli and the main cause of opportunistic infections in immunocompromised patients (Kollef, 2013) and still is under continuous selective pressure in hospital settings (Boucher *et al.*, 2018).

P. aeruginosa is a frequent nosocomial microorganism that causes urinary tract infection. It is highly resistant to antibiotics and the resistance is enhanced by its capacity to form biofilms. The high resistance of *P. aeruginosa* led to the search for new methods to eradicate infections. Targeting virulence factors is one method that can improve the immune system to fight the infectious agent and keep away the emergence of antibiotic resistance at the same time (Richards *et al.*, 1999).

Silver is currently acquired interest for its famous antibacterial properties. It has been utilized since ancient times for treating a wide range of illnesses from burn

wounds, typhoid, anthrax to bacterial conjunctivitis in infants (Athirah *et al.*, 2012).

Antibiotics are commonly used for the treatment of bacterial infections. With the broad spectrum occurrence of multi antibiotic resistant bacteria, it is becoming increasingly hard to treat bacterial infections with classical antibiotics (El-Fouly., 2015).

The antibacterial impacts of silver salts have been mentioned since antiq-uity (Silver and Phung, 1996), and silver is currently used to control bacterial growth in a assortment of applications, in-cluding burn wounds (Catauro *et al*., 2004).

Antimicrobial activity of silver nanoparticles is gaining significance, as the advancement of silver resistance in microorganisms is far-fetched due its action on wide range of targets in cell (Inoue *et al.*, 2002). Its mode of action is not yet completely understood. Several mechanisms have been proposed, such as a loss of the replication capacity of DNA and modification in membrane structure (Valappil *et al*., 2007).

Genus *Pseudomonas* produces a variety of extra-cellular pigments of which phenazines comprise the most important one. The most characteristic feature of *P. aeruginosa* is the production of soluble pyocyanin pigment: a water soluble blue green phenazine compound From the beginning, pyocyanin had been utilized as a reversible dye with a redox potential similar to that of menaquinone. Pyocyanin has various pharmacological impacts on prokaryotic cells; its biological activity is related to similarity in the chemical structure to flavoproteins, isoalloxazine, flavin adenine dinucleotide and flavin mononucleotide compounds (Ohfuji *et al*., 2004).

Previous investigations have proposed that PCN contributes to the capacity of *P. aeruginosa* to persist in the lungs of CF patients (Wilson *et al.*, 1988). Pyocyanin also changes the host immune response in several ways to establish chronic infection and aid evasion of the immune system. Evidence suggests that pyocyanin could prevent the development of an effective T-cell response against *P. aeruginosa* and prevents the activation of monocytes and macrophages (through inhibition of cytokine production) (Winstanley *et al.*, 2009).

To our knowledge, the effects of silver nanoparticles on the virulence genes of *P. aeruginosa* involved in pathogenicity and persistence have not been determined. Therefore, the objectives of this study were to evaluate the antimicrobial activity of $AgNO_3$ against *P. aeruginosa* and to deter-mine their effects on the expression of virulence genes of *P. aeruginosa*.

Materials and methods

Isolates Collection

Between August and December 2018, 98 burn wounds swab specimens were collected by laboratories at Al-Kindi hospital, Al-Yarmook hospital, and Teaching Hospital of Baghdad Medical city and cultured on MacConkey agar. Isolates were obtained from these laboratories by sub culturing on MacConkey agar, and kept at 4^0C during transportation, then incubated at 37^0C for 18-24 hours.

Identification of Bacteria

Cultural Characteristics

Isolates were inoculated on different culture media including MacConkey agar, Blood agar, Cetrimide agar, and Nutrient agar; the media were incubated at 37^0C for 18-24 hrs. Suspected colonies were identified morphologically and biochemically.

Microscopic Characteristics

The suspected colonies were tested by Gram staining, to identify the characteristic morphology of bacteria under the light microscope.

Biochemical Tests

Catalase Production Test (Benson, 2002), Growth at 4^0C and 42^0C (Holt *et al*., 1994) and Oxidase Test (Benson, 2002).

Identification of bacteria by Vitek -2 system

This device is used to diagnose bacterial isolates and confirm that is *Pseudomonas areginosa* after being confirmed by first biochemical test as well as testing their sensitivity to antibiotic.

Detection of pyocyanin production by *P. aeruginosa*

Mineral salts medium was prepared then This medium was inoculated with 0.1 ml of activated bacterial suspension and incubated in 37ºC for 3 days.

Antibiotic Susceptibility Testing

The modified Kirby-Bauer method (Vandeppitte *et al*., 2003) was used.

Determination of Minimal Inhibitory Concentration of Ciprofloxacin

The agar dilution method (Andrews, 2001) was used.

Determination of Minimal Inhibitory Concentration of Sliver Nanoparticle

The following concentrations of Ag per 50 ml (0.25 mg, 0.2 mg, 0.15 mg, 0.1 mg, 0.5 mg, 0.025 mg, 0.012 mg, 0.006 mg, 0.003 mg) were prepared, Each concentration was dissolved in 10 ml distal water by

sonicator (ultrasonic homogenizer) for at least 60 minutes, then Each concentration was added to the media (cetrimide agar + Agar agar), after Sterilized in oven the mix was poured (media + Ag) into petri dishes, agar plates were labeled for inoculation. Inoculums were prepared, by transferring 3-5 colonies into a tube of 5ml of normal saline to obtain culture with 1.5×10^8 CFU/ml, and adjusting to turbidity standard of McFarland 0.5, suspensions were used within 30 min. of preparation, then Inoculate the media with bacteria by Spreading on petri dish, Plates were inverted and incubated at $37^0C \pm 1$ for 18–24 hours.

Molecular Assay

Extraction of Genomic DNA

DNA was extracted from *P. aeruginosa* isolates using a commercial extraction kit (G-spin extraction kit, Intron, Korea), following the manufacturer's instructions, for DNA purification from gram negative bacteria.

PCR Amplification

The extracted DNA, primers (table 1), and distributed maxime PCR premix (intron, Korea), were thawed at 4 C, vortexed to have homogenous contents, a PCR mixture was made in a total volume as described in table 2.

PCR reaction tubes were placed in a thermo-cycler PCR instrument, DNA was amplified as in the conditions indicated in Table 2-7. The temperature and time of PCR program were optimized by using gradient PCR.

Electrophoresis

After PCR, agarose gel electrophoresis was adopted for confirmation. PCR was completely dependable on the extracted DNA criteria. An amount of 100 ml of 1X TBE was taken in a beaker, agarose gel (2%) was prepared by adding one gram of Agarose to the buffer, the solution was heated to boiling (using microwave) until all the gel particles were dissolved, 1 µl of Red Safe Nucleic Acid Stain (10 mg/ml) was added to the agarose, the agarose was stirred in order to be mixed and to avoid bubbles, the solution was allowed to cool down at 50-60°C.

Table 1: Primers sequences.

Gene	Primer	Sequences ´5 → ´3
pPhenazine-specific	PHZM	AACTCCTCGCCGTAGAACA
methyl-transferase (phzM)	FPHZM R	TAATTCGAATCTTGCTGCT
Flavine containing	PHZS	TGCGCTACATCGACCAGAGC
Monooxygenase (phzS)	FPHZS R	GGGTACTGCAGGATCAACT
16S rDNA	FR	ACCTGGACTGATACTGACACTGAG
		TGGACTACCAGGGTATCTAATCCT

Table 2: Reaction mixture.

Master mix components	1 sample
Master mix	10 µl
Forward primer	2 µl
Reverse primer	2 µl
Template	5 µl
Nuclease Free Water	1 µl
Total volume	20 µl

RNA Purification protocol procedure

RNA was extracted from *P. aeruginosa* isolates using GENE$_{zol}$™ TriRNA pure Kit, Japan.

Quantitative Real Time – Polymerase Chain Reaction Technique (qRT-PCR)

RT-PCR should be assembled in a nuclease-free environment. RNA sample preparation, reaction mixture assembly, PCR and subsequent reaction analysis should be performed in separate areas using RealMODTM Green qRT-PCR Mix.

Results and discussion

Sample Collection, Isolation , Identification, Cultural Characteristics and biochemical tests

Seventy two Bacterial isolates were isolated from (98) burn wounds specimens after culturing on MacConkey's agar medium, MacConkey agar that is indicator, a selective and differential culture medium for bacteria designed to selectively isolate Gram-negative and enteric (normally found in the intestinal tract) bacilli and differentiate them based on lactose fermentation (Anderson and Cindy 2013), The crystal violet and bile salts inhibit the growth of Gram-positive organisms (Anderson *et al*., 2013). The results showed that (55) isolates could not ferment lactose (lactose non fermenter) and characterized by grape-like odor, and re-cultured on cetrimide agar medium, this medium was used to determine the ability of an organism to grow in the presence of 0.03% cetrimide that acts as a quaternary ammonium cationic detergent (acetyl trimethyl ammonium bromide) and inhibits the growth of other microorganisms (Tang and Stratton, 2006), Most non-*Pseudomonas* species are inhibited on Cetrimide agar, and species of *Pseudomonas* may also be inhibited (Lilly and Lowbury, 1972) Cetrimide also enhances the production of *Pseudomonas* pigments such as pyocyanin and pyoverdine, which show a characteristic blue-green and yellow-green colour, respectively (Mahmudullah *et al*., 2018), the results showed that all isolates (47) grew on this medium, and recultured on blood agar, all isolates

Table 3: Reaction mixture of QRT-PCR.

Reagent	Volume	Final concentration
RealMODTM Green qRT-PCR mix (2X)	10 µl	1 x
qRT-PCR Enzyme mix (50x)	0.4 µl	1 x
Forward primer (6µM)	1 µl	300nM
Reverse primer (6µM)	1 µl	300nM
Template RNA or Poly(A)+ mRNA	variable	2 pg - 0.2 µg/rxn 0.01 pg – 2 ng/rxn
DNase/RNase free water	Up to 20 µl	-

Table 4: Program of QRT-PCR.

QPCR Steps	Tem.	Times	Cycle(s)
cDNA Synthesis	42 ºC	15 minutes	1
Enzyme Activation	95 ºC	10 minutes	1
Denaturation	95 ºC	15 seconds	30-50
Annealing / Extension	60 ºC	60 seconds	
Melting Curve	Refer to specific guidelines for instrument used		

showed positive hemolysis activity.

All bacterial isolates which grew on this medium were tested for their oxidase and catalase activity, the results showed that all bacterial isolates were oxidase and catalase positive, then tested for their capability to grow at 42ºC, all isolates were capable to grow at 42ºC but not at 4°C. This is a very important character to distinguish *P. aeruginosa* from other *Pseudomonas* spp. like *P. putida* and *P. fluoresences* that grow at 4°C, but not 42°C (Govan, 2005). The forty seven bacterial isolates (47.9%) which were obtained are expected to be *P. aeruginosa*.

Identification of bacteria by Vitek -2 compact system

All isolates (47) which grow on cetrimide agar were identified by Vitek -2 compact device, the results showed that all the isolates were *P. aeruginosa*.

Production of Pyocyanin

Nine bacterial isolates which produced pyocyanin (blue green pigment) on cetramide agar were cultured on Minerals salt Medium with and without 1% peptone, the results showed that all the bacterial isolates produced pyocyanin on minerals salt medium with 1% peptone, while they did not produce the pigment on mineral salts medium without peptone. The production of pyocyanin was noticed for three days, the results showed that Pa15 was very effective in pyocyanin production. Many synthetic media are previously recommended for *P. aeruginosa* proliferation and pyocyanin production such as; king's A medium, glycerol supplemented nutrient broth (GSNB) and mineral medium (Kavitha *et al.*, 2005). The growth of selected *P. aeruginosa* strains and pyocyanin pigment production was determined after 4 days. Results revealed that the three tested media enhanced the growth of both *P. aeruginosa* strains (R1 and U3) regard less to type of aeration condition (shaking or static). The highest increase in the growth yield for *P. aeruginosa* (R1) strain is achieved by mineral medium; then king's A medium and finally GSNB medium. On the other hand, king's A medium supported the highest increase in the growth yield for *P. aeruginosa* strain (U3).To reduce the cost of pyocyanin production, the low-cost raw materials and wastes were aseptically added to basic mineral salt medium containing potassium sulfate (10g/l), and magnesium chloride (3g/l). Peptone, yeast extract and glycerol were individually added to cotton seed meal medium to enhance pyocyanin production according to their percentages in GSNB medium (control). Pyocyanin pigment production was estimated after four days. In another experiment, the cotton seed meal medium was supplemented with different concentrations of peptone (2.5, 5, 7.5, 10 and 12.5g/l) (El-Fouly *et al.*, 2015).

Antibiotic Susceptibility Testing for *P. aeruginosa*

Forty seven isolates went through the susceptibility test, for eight different antibiotics (Imipenem, Amikacin, Gentamicin, Ciprofloxacin, Ceftazidime, Azteronam, Levofloxacin, Pipercilin) by the disc diffusion method recommended by the clinical and laboratory standards institute (CLSI, 2013) guidelines**,** the results showed that 85.1% of total isolates were sensitive to imipenem and 60% were resistant to ceftazidime. the Susceptibility of

Table 5: The production of pyocyanin in mineral salts medium with and without 1% peptone during three days of incubation at 37ºC.

Pyocyanin production						Bacterial ISOL-ATES
3rd DAY		2nd DAY		1st DAY		
Without peptone	With 1% peptone	Without peptone	With 1% peptone	Without peptone	With 1% peptone	
No	Strong	No	Moderate	No	Moderate	Pa18
No	Strong	No	Strong	No	Moderate	Pa20
No	Strong	No	Strong	No	Strong	Pa15
No	Strong	No	Moderate	No	Weak	Pa12
No	Moderate	No	Weak	No	Very weak	Pa10
No	Strong	No	Moderate	No	Moderate	Pa16
No	Strong	No	Strong	No	Moderate	Pa8
No	Moderate	No	Weak	No	Weak	Pa17

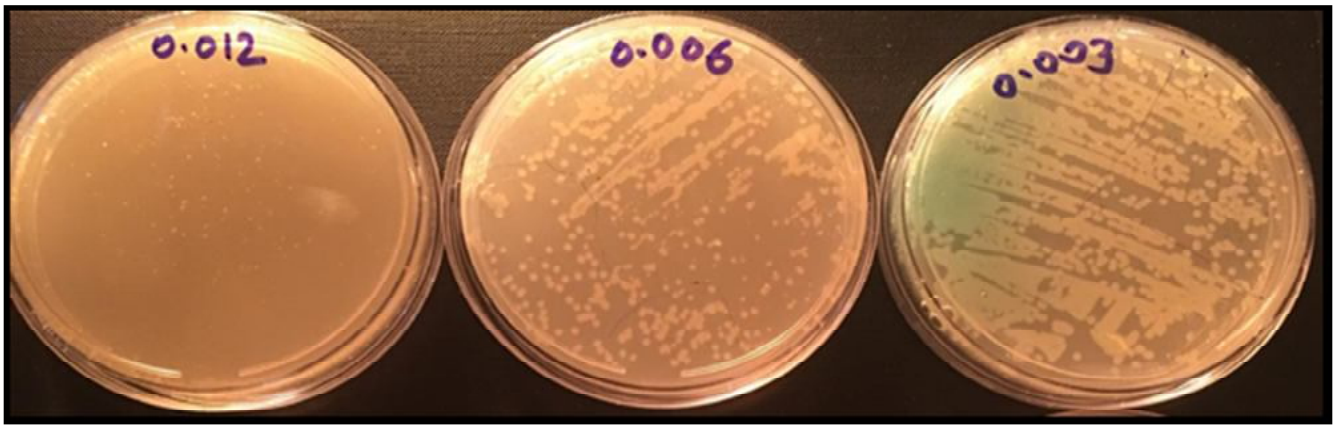

Fig. 1: Shows the MIC of silver nanoparticles against *P. aeruginosa* (Pa15).

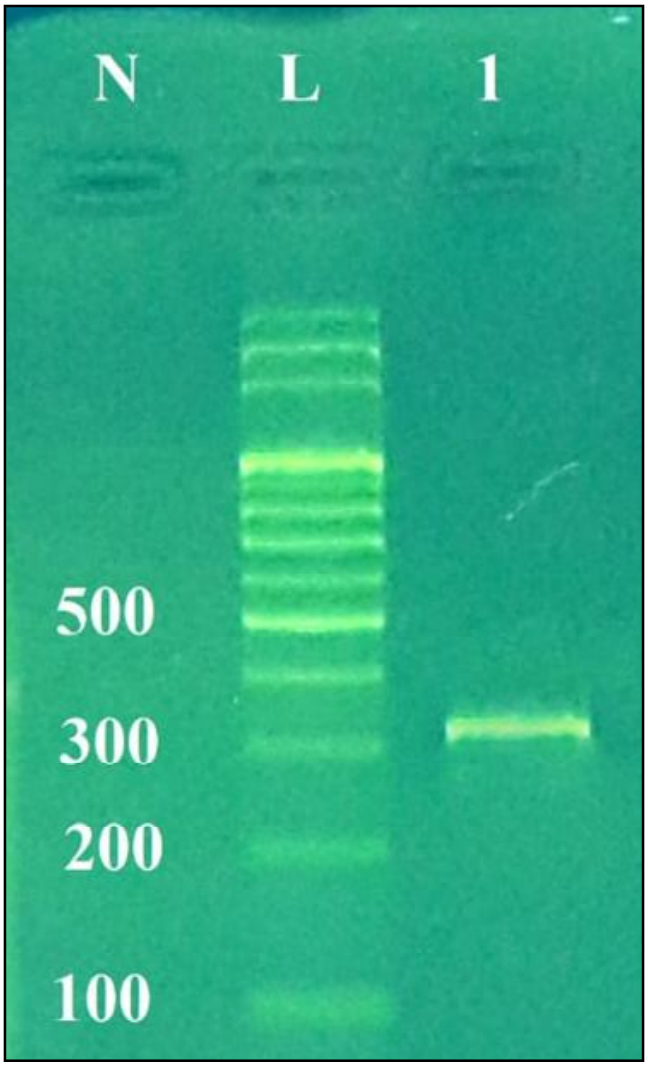

Fig. 2: Agarose gel electrophoresis of *16SrRNA* gene of *P. aeruginosa*, L: 50bp DNA ladder, *16SrRNA* amplicon (956bp). The electrophoresis was done in 2% agarose gel, TBE 1X, 90volt for 60 min stained with red stain. N denotes to the negative control, R1: control, R2, R3: Pa15 treated with AgNo3 (0.006, 0.003 mg/ml respectively), R4: Pa15 treated with sub MIC of ciprofloxacin 6 µg/ml.

isolates for antibiotics were (85.1%, 78.7%, 78.7%, 55.3%, 25.50%, 61.7%, 29.5%, 63.8%) for (Imipenem, Amikacin, Gentamicin, Ciprofloxacin, Ceftazidime, Azteronam, Levofloxacin, Pipercilin) respectively. Antibiotic Susceptibility Testing for pyocyanin producing *P. aeruginosa* Nine isolates (pyocyanin producers) went through the susceptibility test, for eight different antibiotics (Imipenem, Amikacin, Gentamicin, Ciprofloxacin, Ceftazidime, Azteronam, Levofloxacin, Pipercilin) by the disc diffusion method recommended by the clinical and laboratory standards institute (CLSI, 2013) guidelines, the results showed that 75% of isolates were sensitive to gentamicin while 88% of isolates were resistant to Ciproflxacin, Levofloxacin and Ceftazidime.

Minimal Inhibitory Concentration Test for *P. aeruginosa*

Nine isolates were tested to determine the minimal inhibitory concentration for ciprofloxacin against *P. aeruginosa*, by the agar dilution method recommended by the clinical and laboratory standards institute (CLSI, 2013) guidelines, the results showed that the MIC of ciprofloxacin against the nine isolates were (64, 64, 8, 128, 64, 16, 32, 32, 32 µg/ml) for (Pa18, Pa20, Pa15, Pa12, Pa10, Pa16, Pa8, Pa17, Pa22) respectively the MIC distributed between 8 and 128 µg/ml.

Minimal Inhibitory Concentration of Silver Nanoparticles against *P. aeruginosa*

One isolate, Pa15 was tested to determine the minimal inhibitory concentration for sliver nanoparticle against *P. aeruginosa* (the strongest isolate in pyocyanin production as our results showed) the results showed that the MIC was 0.012 mg/ml. the effect of silver nanoparticle on pyocyanin production, the results showed that in 0.003 mg/ml there was growth with pyocyanin pigment while in 0.006 mg/ml the bacteria did not produce the pigment, because bacteria pyocyanin is controlled by quorum

sensing.

Molecular Analysis

The control with three treatments, the sub MIC (0.006 and 0.003 mg/ml) of silver nanoparticles and the sub MIC 6 µg/ml for ciprofloxacin against *P. aeruginosa* (Pa15) were taken to study pyocyanin expression.

DNA Extraction

DNA of *P. aeruginosa* (Pa15) was successfully extracted from overnight cultures of isolate, the Concentration was confirmed with biodrop which was 105 ng/µL.

Detection of 16srRNA gene

The result showed that the isolates harbored this gene (956 bp).

Detection of *phzM* and *phzS* gene

PCR was conducted for one isolate, using the, *phzM* and *phzS* primers to amplify the constitutional genes *phzM* and *phzS*, and bands were confirmed with gel electrophoresis as shown in Figure. The result revealed that *phzM* gene (312 bp**)** was detected.

Real Time-qPCR

RNA Extraction

RNA was extracted from the selected isolate *P. aeruginosa* (Pa15) which grown in cetramide agar as control in addition to these were treated with silver nanoparticle (sub MIC 0.006 mg/ml and 0.003 mg/ml) and MIC of ciprofloxacin 6 µg/ml. Total RNA of samples were extracted by using GENE$_{zol}$ ™ TriRNA pure kit, the concentration ranging between 150-264 ng/µl and the purity fluctuated from 1.96 to 2.1.

Effect of silver nanoparticle and ciprofloxacin on the expression of *phzM* gene in *P. aeruginosa* Isolate

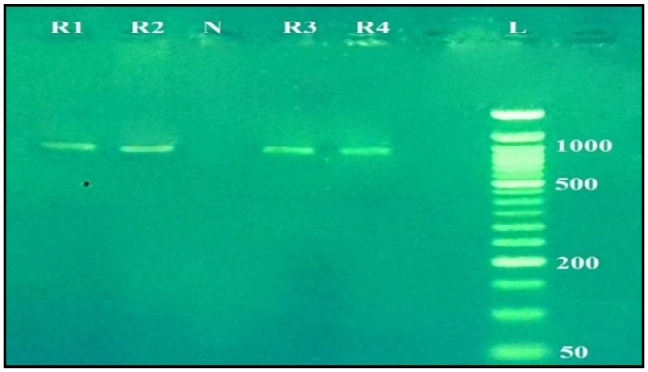

Fig. 3: Agarose gel electrophoresis of *phzM* gene of *P. aeruginosa* (Pa15), L:100bp DNA ladder , *phzM* amplicon (312 bp). The electrophoresis was done in 2% agarose gel, TBE 1X, 90 volt for 60 min stained with red stain. N denotes to the negative control.

Quantitative real-time PCR was carried out using SYBR green though a one-step RT-PCR method in order to determine the effect of sub MIC of silver nanoparticle on the expression of *phzM* gene in *P. aeruginosa* (Pa15) isolates. The results showed decreasing in the expression level of the *phzM gene* under silver nanoparticles effect, while the effect of ciprofloxacin increase the expression of pyocyanin. Dosunmu *et al.*, (2015) mentioned that the molecular effects of AgCNTs against the mucoid and nonmucoid strains of *P. aeruginosa*, virulence genes involved in resistance, stress response, motility, pathogenicity, attachment, and redox regulation were selected for quantification of their transcriptional expression using qRT-PCR, and then compared to the molecular effects of gentamicin. Their expression was significantly down regulated in the nonmucoid strain, but remained unchanged in the mucoid strain. By contrast, the expression of the mexT gene was down regulated in the mucoid strain, but showed no significant change in the nonmucoid strain. As expected, the presence of AgCNTs significantly downregulated expression of the outer-membrane porin oprD gene by ten- and elevenfold in the mucoid and nonmucoid strains, respectively. The gentamicin-treated samples of both strains showed upregulation of gene-expression patterns for all genes analyzed, except for down regulation of oprD gene expression.

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