

Effect of a new Quinoline-2-one Derivatives(Compound 3) on Purified DNA gyrase from clinical isolate *Pseudomonas aeruginosa* PA31

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Abstract:-Objective: The aim of this study to evaluate Study of new Quinoline -2-one derivatives (Q3) and application of the molecule on the DNA gyrase which purified from clinical *Pseudomonas aeruginosa* PA31. **Methods:** DNA gyrase enzyme was purified from clinical *Pseudomonas aeruginosa* PA31 with 70% saturation of ammonium sulphate then DEAE-cellulose column with final stage gel filtration. **Results:** Purified DNA gyrase showed activity on agarose gel against catenated DNA. Synthesized compound (Q3) was chosen for inhibition studies with purified enzyme, compound (Q3) showed full inhibition against purified DNA gyrase at concentration (1000 mg/ml) on agarose gel. **Conclusion:** *In vitro* showed that compound (Q3) has highest functional score against DNA gyrase.

Key words: DNA gyrase, Quinoline-2-one derivatives (Q3), Clinical *Pseudomonas aeruginosa* PA31.

I. INTRODUCTION

Pseudomonas aeruginosa's genome has uncovered and cleargenes that encoded DNA topoisomerase type II enzymes that work on DNA double strands. Where GyrA and GyrB genes are encoding DNA gyrase enzyme meanwhile ParC and ParE genes that encoded topoisomerase IV enzyme in gram negative bacteria^[1]. DNA Gyrase and topoisomerase IV modulate the topological state of DNA as these enzymes play essential roles in most nucleic acid processes, help control levels of DNA under- and over winding, and remove knots and tangles from the bacterial chromosome^[2]. In Gram-negative species, DNA Gyrase and topoisomerase IV both are comprised of two distinct functional subunits as A, B heterotetramers^[3]. To altercatenomic integrity during this process, the enzymes form covalent bonds between active site tyrosine residues and the newly generated 5'-DNA termini. These covalent enzyme-cleaved DNA complexes are known as "cleavage complexes".^[4] Despite their mechanistic and structural similarities, gyrase and topoisomerase IV have separate physiological functions^[5]. Gyrase is the only type II DNA topoisomerase that can actively introduce negative supercoils into DNA. On top of everything else, DNA gyrase is primarily responsible for removing the torsional stress that accumulates in front of replication forks and transcription complexes^[6]. Topoisomerase IV appears to play a lesser role than gyrase in maintaining chromosomal super helical density and alleviating torsional stress. Its major function is removing knots that accumulate in the bacterial chromosome^[4]. The aim of this study to evaluate Study of new Quinoline -2-one derivatives (Q3) and application of the molecule on the DNA gyrase which purified from clinical *Pseudomonas aeruginosa* PA31.

II. MATERIALS AND METHODS

2.1 Prepare of Schiff base compound (Q3):

General procedure:

To a mixture of coumarin compound(Q1) (0.002mol) and appropriate aldehyde (salicylaldehyde, 4-hydroxy salicylaldehyde, 5-chloro salicylaldehyde), (0.002mol) in absolute Ethanol (20ml), 3-4 drops of glacial acetic acid was added, then the mixture refluxed for 6-8 hours. On cooling the mixture, the precipitate formed was filtered off and recrystallized from Ethanol to give the required Schiff base.

1.2 Cells harvest and disruption^[7]:

Clinical *P.aeruginosa* isolation was activated by Brain and Heart Infusion agar for overnight at 37°C. Activated colonies were utilized for inoculate 1 liter of Luria agar on large petri dish plates. These plated allowed to incubate over night at 37°C. Cells were removed by scratching with sterilized spatula and wash what its left with Luria broth. Cells centrifuged with 5000 rpm for 15 minutes, re-suspended the cells with 9% NaCl,

and harvested them by centrifugation (net weight 17g). Cells were suspended at concentration of 0.5g/ml of 50mM Tris-HCL (pH 8.0), Cells got cell wall breaching by Ultra Sonication for 8 minute at (50 kHz) with cold conditions. The solution became quite viscous due to lyses cells.

All subsequent operations in the purification of the enzyme were carried out at 4C. Cell debris and DNA were removed by centrifugation for 40minutes at 15,000 rpm. The supernatant fluid was decanted and treated with 10% solution for Gentamycin prepared in water, 1ml of Gentamycin solution was added to supernatant, the mixture was stirred for 20min, and precipitate which formed was separated by centrifugation for 30 min at 15,000rpm. The precipitate was discarded, and the supernatant fluid was dialyzed overnight against 1.5 Liter of 50 mMTris-HCL buffer (pH8.0) containing 0.1mM EDTA, 10% glycerol.

1.3 Precipitation by ammonium sulfate ^[8]:-

A specific weight of ammonium sulfate crystals was added to the crudeenzyme gradually in an ice bath with continuous stirring for 30 min to get saturation percentage of 0-25, 25-50 and 50-70 %, then centrifuged at 10000 rpm at 4°C for 30 min, the precipitation which formed was collected and dialyzed overnight against 1 liter of Buffer B.

2.4 Preparation of ion exchange column (DEAE-cellulose) gel:

The DEAE-Cellulose was prepared according to the method suggested by Whitaker and Bernhard ^[9].

2.5 Separation with ionic exchange by using DEAE-cellulose

Ten ml solution was loaded on ion exchange column, the separated fractions were collected at flow rate 30 ml/hour, the wash was obtained of by using buffer A (the same buffer used in equilibration), the elution was achieved by the same buffer with gradual increase in concentration of sodium chloride, the flow rate was 30ml/hour too, the protein concentration of the fractions was measured at wavelength 280nm to the washed and eluted fractions, protein concentration then was calculated.

2.6 Gel filtration Sepharose -6B

Sepharose 6B column (87 x1.5cm) was prepared and packed according to the instructions of the manufacturing company (Pharmica Sweden). The column was equilibrated with 10mM Tris- HCl (pH 7.4). (2ml) of partially purified DNA gyrase enzyme was added to the column, carefully using pasture pipette. Elution of Tris- HCl (5ml) fraction was collected of enzyme activity then protein contents were estimated by measuring the absorbance at (280nm).

2.7 Determination of Molecular Weight of DNA gyrase By Gel Filtration Chromatography

The method of filtrating gel on a column Sepharose 6B was followed to estimate the molecular weight of the DNA gyrase enzyme, using a standardProtein, by drawing the relationship between the logarithm of a standard protein molecular weight and the size of recovery/ size of Void (V_e/V_o), molecular weight was calculated as shown in the following steps: -

2.7.1 Determination of the void volume of the column:

Use column sepharose 6B with dimensions (1.5 × 87) cm used for purification of the enzyme, carry out its using Tris-HCl solution concentration of 10mM pH 7.4, and Void volume determined by using blue dextran to recovered parts the same budget buffer. Absorbance was measured in separate parts (5ml) at a wavelength of 600 nm.

2.7.2 Determination elution volume for standard protein

Gel filtration was carried out for four of standard proteins table (3-6), absorption was measured at 280nm in separated volumes to determine elution volume (V_o) for each standard protein. The relationship between elution volume percentages was blotted for each standard protein to the elution volume of blue dextrin (V_e/V_o) against molecular weight logarithm. This way helped measure enzymatic molecular weight.

Table (3-6):Molecular Weight of Standard Proteins.

Standard proteins	Molecular weight (Dalton)
Casien	31000
Pepsin	34000
Alkaline phosphatase	80000
Urease	409000
α - hemolysin	102000

Table (4-10): Purification steps of *Pseudomonas aeruginosa* DNA-gyrase enzyme.

Steps	Volume (ml)	Protein (mg/ml)	Enzyme activity (Unit/ml)	Specific activity (Unit/mg protein)
Cleared lysate	27	0.446	ND	---
Ammonium sulphate 75%	27	0.425	ND	--
DEAE-Cellulose	11	0.384	25	65.104
Gel-filtration	5	0.220	50	227.273

ND: Not Detected

2.8 Detection of DNA gyrase Activity [10]:

DNA gyrase has ability to catalyze the decatenation of intact double-stranded DNA. By utilizes the kinetoplast DNA (KDNA) from *Crithidia fasciculata*, since it forms a large network of interlocked (catenated) circles, DNA gyrase decatenates these circles from the network. While the catenated circles are unable to enter an agarose gel, upon decatenation the free circles are detected as a discrete band on the gel. By using "A Decatenation-Supercoiling Assay Using Kinetoplast DNA" provided by TopoGene company. This assay as following :- DNA gyrase reaction buffer, Substrate: kinetoplastDNA^[11] Purified DNA gyrase or cell extract 5 × loading dye and 0.9% agarose gel and gel photography. Add 20 µl of DNA gyrase reaction buffer and 200 ng kinetoplast DNA (10 µl of a 20 µg/ml stock) to (1 ml) microcentrifuge tubes. Added 20 µl of purified DNA gyrase protein or cell extract to the tube, then incubate 30 min at 37°C. At enzyme concentration, DNA gyrase will catalyze the catenation of circular DNA. Add 5 µl of 5× loading dye to each tube and load contents on a 0.9% agarose gel. Run gel 60 minutes at 50 volts. Stain gel with ethidium bromide, and photograph the gel illuminated with a U.V. transilluminator.

2.9 Definition of Enzyme Activity Unit : One unit of DNA topoisomerase II of *P. aeruginosa* is defined as the amount of enzyme required to fully decatenate 0.1 µg of KDNA in to open circular or covalently closed minicircles under standard reaction conditions.

III. RESULTS AND DISCUSSION**3.1 Chemistry Studies:**

Since quinoline-2-one are very important organic compounds to having a wide spectra of biological activities, the target of present work is synthesize new quinoline-2-one derivatives which name (salicyl aldehyde) containing biological various moieties with expected biological activity. The physical properties of this compound (Q3) like melting point 158-160 °C and the colour is yellow and Purification solvent is ethanol with yield 80%.

3.2 Purification DNA gyrase enzyme

Many purification steps were proceeded to obtain highest purity of DNA gyrase enzyme from clinical isolations. *P. aeruginosa* isolation No (31) were chosen as it was the most isolate affected by prepared compounds. These steps were minimized as long as quantity of enzyme is low and could be lost during process of purification. Main purification steps are follows:

3.2.1 Cells harvest and disruption:

After activation of Clinical *P. aeruginosa* isolation, the activated colonies were utilized for inoculate 1 liter of Luria agar on large petri dish plates. Incubation, scratching and harvesting were done in sterilized conditions, total gain of harvest process of these activated colonies were (net weight 17g). Cells were suspended at concentration of 0.5g/ml of 50mM Tris-HCL (pH 8.0), Cells got cell wall breaching by Ultra Sonication for 8 minute at (50 kHz) with cold conditions. The solution became quite viscous due to lyses cells. Cell debris and DNA were removed by centrifuged and The supernatant fluid was decanted and treated with 10% solution for Gentamycin prepared in water for prevent the contamination, Dialyzed overnight against 1.5 Liter of 50 mM Tris-HCL buffer (pH8.0) containing 0.1mM EDTA, 10% glycerol^[7]. The total volume was (27 ml) and protein concentration (0.446 mg/ml) as showed in table (1). The detection of enzymatic activity of dialyzed cell lysate were done by incubate (20µl) of sample with (200 ng) of KDNA (substrate) in reaction buffer for 30 minutes at 37°C. figure (1) showed that band in (A) lane succeed to migrate through the agarose gel (0.9%) after electrical running at 50 volts for 60 minutes, this indicated that DNA gyrase enzyme at concentration succeeded to decatenation of the substrate (catenated DNA) and separated it into relaxed DNA monomers that

able to pass through agarose gel, meanwhile substrate in (S) lane could not pass into gel because its big size (2.5 kb)^[11].

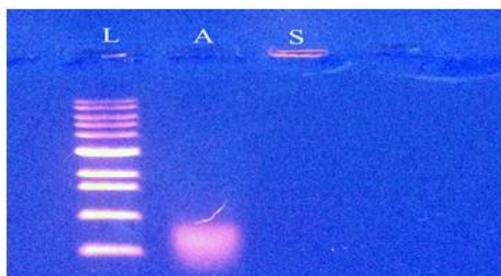


Figure (1): Enzymatic activity detection of dialyzed cell lysate by topogen kit. Where L: ladder, A: sample with substrate and S: KDNA substrate

3.2.2 Precipitation of DNA gyrase by ammonium sulphate:

The crude extract of enzyme and to avoid much as could the water molecules, ammonium sulphate was utilized at (0-25, 25-50, 50-70)% saturation, the saturation ratio (70%) was chosen to precipitate DNA gyrase enzyme. In this step, water molecules came out as salts molecules allowed that, as ammonium sulphate has ability to maintenance and neutralize the charge at the surface of the enzyme and cause disruption in water layer surrounding the enzyme, leads to minimize the solubility of enzyme which turn off to precipitate of enzyme^[12]. Ammonium sulphate is used because of availability of ammonium sulphate, high solubility, low cost and it stabilizes the protein^[9].

3.2.3 Partial purification of DNA gyrase by Ion-Exchange chromatography:

Ion-exchange chromatography (DEAE-cellulose) was utilized for partially purification of DNA gyrase. Figure (2) showed elution of DEAE-cellulose column for dialyzed sample

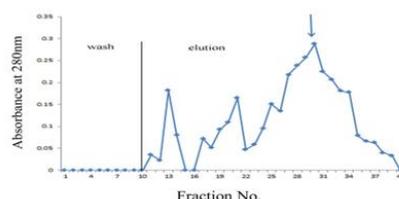


Figure (2): Purification of GTF Enzyme by Ion-Exchange Chromatography DEAE- Sephacel

column 12 x 3 cm. The Column Was Washed by Using 50mM Phosphate Buffer pH 7.5 and then Eluted by Using a Gradient of 0.25 M to 1 M NaCl . No protein detected in wash step, meanwhile in the eluted fractions revealed. Results of absorbance at 280nm indicated the presence of three peaks (fraction 13, 21 and 30) and these results was harmonized with Robert^[13]. For enzymatic activity detection of DEAE-cellulose fractions was done by take (40µl) of sample was incubated with (200 ng) of substrate KDNA in reaction buffer for 30 minutes at 37°C. And loading the samples into wells on agarose gel 0.9% and running at 50 volts for 60 minutes. However, only fraction (30) showed enzymatic activity against substrate KDNA that detected by Topogen kit as shown in figure (3).

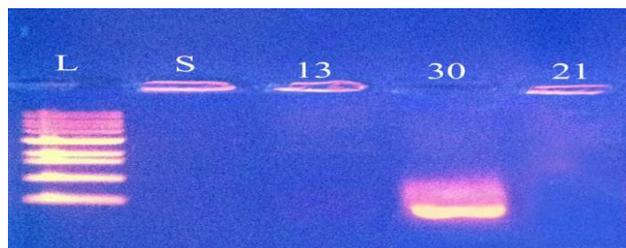


Figure (3): Enzymatic activity detection by Topogen kit. Where L: ladder, S: KDNA substrate and fractions (13, 30, and 21).

3.2.4 Purification of DNA gyrase A by gel filtration chromatography:

Purification of DNA gyrase by gel filtration chromatography were done by sample passed through Sepharose – 6B. Column was washed by buffer C and then sample passed, fractions were collected up to 45 fractions. DNA gyrase enzyme was present in fractions 24-26. Enzymatic activity was conducted to by using Topogen Kit. The results of purification showed the presence of one peak as indicated of pure enzyme as showed in figure (4).

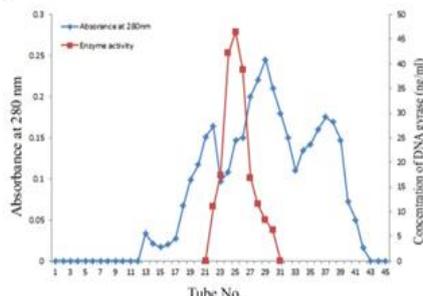


Figure (4): Purification of DNA gyrase by using gel filtration chromatography (Sepharose CL- 6B column, 87 x1.5cm, 10mM Tris-HCl buffer eluent, pH7.4) at a flow rate 50ml/hour.

Finally, gel filtration product enzyme was detected on their enzymatic activity by incubated (20µl) of sample with (200ng) of substrate (KDNA) and reaction buffer for 30 minutes at 37°C, after loading the sample in agarose well gel and power on at 50 volts for 60 minutes. Band of sample was clearly noticed as showed in figure (5).

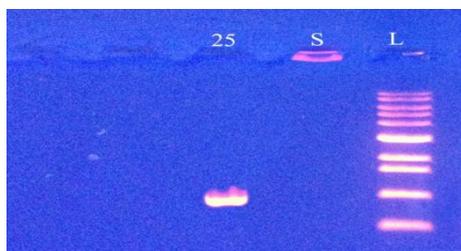


Figure (5): Enzymatic activity detection by Topogen kit. Where L: ladder, S: substrate of KDNA and 25: tube number where enzyme found.

3.2.5 Determination of molecular weight of DNAgyrase:

The molecular weight (MW) was determined by gel filtration depending on the size of the separate molecules with their charge^[8]. Sepharose 6B for gel filtration was used to estimation the molecular weight of DNA gyrase that purified from clinical *pseudomonas aeruginosa* in the presence of five standard proteins, Urease (409 K Dalton), α -hemolysin (102 K Daltons), Alkaline phosphatase (80 K Daltons), Pepsin (34 K Daltons) and Casein (31 K Daltons). Blue Dextran 2000 was applied first to column to detect the void volume (V_0). DNA gyrase and each of standard proteins were applied to column and eluted separately. The elution volume (V_e) of each of standard protein was recorded, and then V_e/V_0 was estimated for each one. The molecular weight of DNA gyrase was determined by blotting the relationship between V_e/V_0 and log of MW of standard proteins as showed in figure (6). By standard curve, the molecular weight of α -hemolysin is determined and it is nearly (109K Daltons).

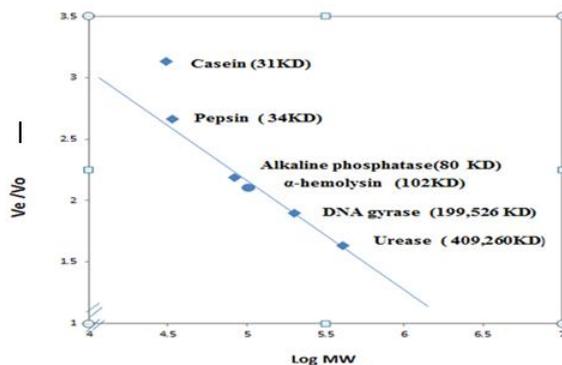


Figure (6): Standardization of the DNA gyrase molecular weight based on the elution volume to the void volume ratio (V_e/V_o ratio) by gel-filtration chromatography.

3.2.6 Inhibition activity studies of compound (Q3) on purified DNA gyrase:

In silico and *in vitro* studies showed that compound (Q3) has clear impact on DNA gyrase of clinical *P.aeruginosa*, the inhibition sensitivity of purified DNA gyrase was investigated with compound (Q3). Since DNA gyrase is nuclear enzyme that play important role in DNA transcription, replication, recombination and chromosome segregation in prokaryotic cells^[14]. DNA gyrase is promoting and catalyzing the relaxation of knotted DNA, also DNA gyrase has distinct potential to catalyze the decatenation of intact double strand DNA to enable enzyme to separate DNA molecule during mitosis^[15], *P.aeruginosa* DNA gyrase activity was determined by using decatenations reaction.

By utilizes the kinetoplast DNA (2.5 KB) from *Crithidia fasciculata*, since it forms a large network of interlocked (catenated) circles^[16]. DNA gyrase promoting decatenates the circles from the network, Separations occurred and relaxed DNA monomers (gyrase assays) were performed at 50 volts and band clearly appeared on agarose gel. While the catenated circles are unable to enter an agarose gel and obtained in wells^[11]. The purified enzyme was incubated in standard reaction with various concentrations of compound (Q3) ranging (250, 500, 750, 1000, 2000 and 3000 $\mu\text{g/ml}$) with (200 ng) of KDNA (substrate) for 30 minutes^[11]. After loading the samples on agarose gel (0.9%) and power running at 50 volts for 60 minutes, purified DNA gyrase enzyme at concentration succeeded to decatenation of the substrate (catenated DNA) and separated it into relaxed DNA monomers that able to passed through agarose gel.^[11] (Figure 7).

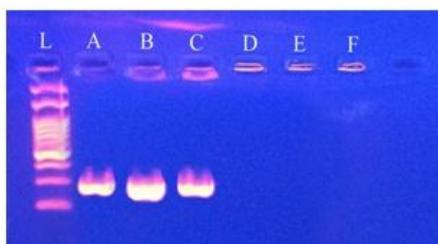


Figure (7): Inhibition studies of prepared compound (Q3) against purified DNA gyrase.

The results of inhibition studies showed that compound (Q3) showed no inhibition activity against purified enzyme at concentration (250, 500 and 750 $\mu\text{g/ml}$) (A, B and C) respectively, as the DNA gyrase proceeded catalyzing decatenation and succeed to separate catenated DNA to relaxed DNA monomer that able to enter agarose gel when power run at 50 volts. Meanwhile compound (Q3) showed full inhibition activity against purified DNA gyrase at concentration (1000, 2000 and 3000 $\mu\text{g/ml}$) (D, E, and F) respectively. Where compound (Q3) showed necessary concentration to inhibit full activity of purified DNA gyrase of *P.aeruginosa* is (1000 $\mu\text{g/ml}$) (D), and that agree with Robert^[13]. *In silico* studies showed that compound (Q3) has highest functional score against DNA gyrase, also compound (Q3) has functional group 2 OH and aromatic ring with fitted orientation and lower molecular weight (278 Daltons), all those enable compound (Q3) make covalent bonding (hydrogen bond) with side chain of tyrosine amino acid of active site at 122 position of sequence subunit A of DNA gyrase^[17]. Blocking the active site of DNA gyrase was banned interaction with substrate KDNA during incubation. Therefore, KDNA obtained in wells unable to enter agarose gel with its big size (2,5kb)^[11].

VI. CONCLUSION

The Purification of DNA gyrase from *P.aeruginosa* have been studied. However, salicyl aldehyde (Q3) showed full inhibition against purified DNA gyrase on agarose gel at (1000 $\mu\text{g/ml}$) concentration its mean approximately 4-fold higher than MIC for this species (256 $\mu\text{g/ml}$) during *in vitro* studies, and the molecular weight of purified DNA gyrase was found to be (199, 560 KDalton).

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