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Molecular Detection and Expression of Azurin: An Anti-Cancer Protein from Local Isolates of *Pseudomonas* species

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Abstract

Azurin was recognized as anticancer drug in 2000, it plays an important role in inducing apoptosis and cytotoxicity. Azurin may incorporate with cytosol and nuclear material after penetrating the tumor cells and stabilize p53 (a tumor suppresser protein) that facilitate to increase its level inside the cell. A 447 bp PCR amplified fragment of Azurin from *Pseudomonas aeruginosa* was cloned in pTG19-T cloning vector. The DNA sequence predicts a pre-protein with a signal peptide of 19 amino acids followed by 128-aminoacid mature azurin protein. azurin gene was cloned in pET22b vector. Recombinant rpET22b (+)-Azu vector was cloned in BL21-Codon Plus (DE3) cells. Optimized, high expression of azurin gene was obtained at 0.5 mM concentration of IPTG after 6 hours at 37°C. Sequencing shows 99.8% sequence homology with reported sequence of azurin. One silent mutation i.e., CCA to CCG is detected at 17th amino acid. However, resulting amino acid (proline) remains the same.

Keywords: Azurin; *Pseudomonas aeruginosa*; Tumor suppresser protein

Introduction

Cancer is one of the most principle cause of death in the world. Several different, potent and effective drugs against cancer are developed due to quick evolution in the field of medicine and pharmacology. Bacterial proteins are important and modern type of anticancer drugs. There are many other bacterial products discovered until now, among which anticancer activity has been shown such as Azurin and ETA (exotoxin A) produced by *Pseudomonas aeruginosa*, **Enterococcal** anti-proliferative peptide (Entap) from *Enterococcus sp*., diphtheria toxin from *Corynebacterium diphtheria* and an analog of Pep27 peptide (Pep27anal2) from *Streptococcus pneumonia.* Clinical trials and development are going on for these products and they may prove to be of great pharmacological use in treating cancers [1]. Azurin was recognized as a new and interesting anticancer drug in 2000 by Zaborina et al. [2]. He reported that azurin plays an important role in inducing apoptosis and cytotoxicity in murine macrophage cell line J774. As J774 is a transformed cell line which is originated from cell sarcoma, a question arises there whether this azurin can induce cytotoxicity in cancer cell lines in humans. Later on the role of azurin in activating apoptosis and significant cytotoxicity was revealed in cancer cell lines of humans as breast cancer (Michigan Cancer Foundation-7, MCF-7), melanoma (UISO-Mel-2) and osteosarcoma (U2OS) cells [3,4]. Azurin the single copper protein is a simple redox protein that is derived from different bacterial strains of *Pseudomonas* and *Alcaligenes* and facilitates denitrification metabolism. X-ray crystallographic 3D structures of this protein derived from bacteria has been established and the structure around the copper site has been developed [5]. A special domain of azurin "Azu 50-77 or p28" consisted of 28 amino acids was recognized to be the most preferable entry domain. In tumor cells, it functions as a dominant protein transduction domain (PTD) [6]. Studies revealed that the entrance mechanism of azurin along with its procured peptides neither depend on the clathrins nor on membrane bounded glycosaminoglycans. But there are significant chances of involvement of N-glycosylated proteins in recognition as well as cholesterol reduction from membrane. The protein has key role in regulating the cellular mechanisms including cell growth, genomic stability and cell death. All of these mechanisms may be controlled by the same protein. However, this protein is an important transcription factor and may function as a sequence-specific transcription regulator for many pro-apoptotic genes, namely *bax* (BCL2 associated X) and cyclin-dependent kinase inhibitor 1 *gene (p21)*. These genes produce Bax protein associated with apoptosis and protein 21 associated with growth arrest as well as hindrance to the cell cycle [7]. In-spite of the fact that the process of apoptosis initiated by p53 is not completely deduced but p53 play an important role in azurin mediated-apoptosis. It is interesting to know that azurin a bacterial protein exhibits its ability to complex with several targets inside or outside the mammalian cells. Azurin inhibits ephrin/EphB2 signaling, interferes in upstream signalling and inhibits the cancer cell growth [8].

Materials and Methods

Samples of *P. aeruginosa* were collected from local diagnostic Laboratory. Total DNA was isolated from bacterial cell, using classical method of Sambrook and Russels.

Primer designing and PCR amplification

The Azurin gene sequence was available at NCBI database under the name of *Pseudomonas aeruginosa* azurin (azu) gene, PA01 chromosome (NCBI Reference Sequence: NC_002516.2). Primers were designed for this Azurin gene manually by adding restriction sites of *Nde*I and *Bam*HI at 5' and 3' ends respectively. Primer properties were checked on Oligo Calc and Thermo Fischer Scientific Tm Calculator. The sequence of primers and restriction sites are given in Table 1.

Polymerase chain reaction was carried out in 50 µl reaction mixture.

Primers	Sequence	No	T_m	GC %
AZU-f	5'-TTCCATATGCTACGTAAACTCGCTGCGGTA-3'	30 nt	68.9°C	50%
AZU-r	5'-GGATCCTCACTTCAGGGTCAGGGTGC-3'	26 nt	67.6°C	61.54%

Table 1: Names, sequences and properties of primers specific for Azurin gene.

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30 cycle PCR was done with initial denaturation for 1 minute at 96°C, denaturation at 96°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 1 minute and final extension for 7 minutes at 72°C. The PCR product was extracted by Vivantis Quick Gel Extraction kit.

Cloning of Azurin (447 bp) in pTG19-T cloning vector

The PCR product eluted was ligated with TA cloning vector pTG19-T (25ng/ µl) and left for overnight incubation between 16-22°C. The ligation mixture was used to transform competent cells by using heat shock method. White colonies were selected and inoculated in 10 ml LB broth and kept overnight at 37°C in shaking incubator for plasmid preparation.

Plasmid preparation of Azu/pTG19-T transformants

The recombinant pTG19-T plasmids were extracted by Thermo Scintific GeneJet Plasmid Miniprep Kit [9,10]. The recombinant plasmid (Azu/pTG19-T) was digested with *Eco*RI and *Hin*dIII restriction enzymes to confirm the presence of Azurin (447bp) insert. For subcloning, the recombinant plasmid DNA (Azu/pTG19-T) and the expression vector [pET 22b (+)] both were restricted with same enzyme (*Nde*I and *Bam*HI).

Expression vector contains sites for both *Nde*I and *Bam*HI. First the plasmid was incubated overnight at 37°C with single enzyme *Nde*I. After confirmation, second enzyme *Bam*HI was added and left for 3 hours at 37°C. The digestion mixture was loaded on agarose gel electrophoresis to obtain the digested Azurin gene insert. The gel slice containing the digested Azurin insert was cut out with sharp sterile blade. The 447 bp gene insert digested with *Nde*I and *Bam*HI was eluted by using Vivantis Quick Gel Extraction kit.

The ligation mixture of Azurin gene and pET22b (+) digested with *Nde*I and *Bam*HI was prepared. The mixture was kept overnight at 16-22°C. rpET22b/Azu Plasmid was transformed to DH5 alpha and Bl21 codon plus cells. The recombinant plasmid was confirmed by restriction digestion and sequencing. The sequencing was done using the dideoxy chain termination method on Beckman Coulter CEQTM 8000 DNA sequence. Sequence alignment was performed using the DDBJ (DNA Data bank Japan) sequence analysis software and Clustal W.

Expression of Azurin in *E. coli* BL21 expression system

The *E. coli* strain BL21 DE3 codon plus cells harboring rpET22b/Azu plasmid were grown overnight in shaker at 37°C by inoculating a single colony into 10 ml LB broth containing ampicillin under sterile conditions. Non recombinant or plain pET22b (+) plasmid harboring cells were also cultured as a negative control.

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The *E. coli* strain BL21 DE3 codon plus cells harboring rpET22b/Azu plasmid were grown overnight in shaker at 37°C by inoculating a single colony into 10 ml LB broth containing ampicillin under sterile conditions. Non recombinant or plain pET22b (+) plasmid harboring cells were also cultured as a negative control.

Induction with isopropyl-β-D-thiogalactoside (IPTG)

Overnight culture of BL21 DE3 codon plus cells harboring rpET22b/Azu plasmid was inoculated in flasks containing 10 ml LB broth and ampicillin. The flasks were incubated in shaker at 37°C and 150-170 rpm until the OD_{600} reached to 0.6-0.7. One of these flasks was taken as an un-induced control while one set of flasks was induced with different concentrations of IPTG (0.1 mM, 0.2 mM, 0.3 mM and 0.5 mM) and the other set with different concentrations of Lactose (5 mM, 10 mM, 15 mM and 20 mM). After induction, these flasks were again incubated under similar conditions for the intervals of 2, 4, 6 and 8 hours, collecting the samples (about 1 ml) in 1.5 ml eppendorf at each interval. The samples collected at various intervals were centrifuged at 12000 rpm, 37°C for 1 minute. The supernatant was discarded, and the cell pellet was suspended in 100 µl of 50 mM Tris (pH 8.0). Then, 100 µl of 1X SDS gel loading buffer was added and the samples were boiled in a water bath for 3 minutes and centrifuged for 1 minute. The samples were cooled and stored at -20°C until use. The prepared samples were analyzed on 15% sodium dodecyl sulphate polyacrylamide gel electrophoresis to check the relative expression of the target protein [11].

Effect of different incubation time on protein expression

The time of incubation had vivid effect on the expression and production of protein. The induced cultures could grow for 2, 4, 6 and 8 hours, collecting samples at each interval to screen the optimal expression of protein of interest. 1 ml of each induced culture was withdrawn after 2, 4, 6 and 8 hours and cells were harvested by centrifugation for recovering the pellet which was resuspended in 50 mM Tris-Cl buffer pH 8.

Results

PCR amplification of Azurin gene from *Pseudomonas aeruginosa*

Azurin gene was ampilified by PCR by using DNA extracted from *Pseudomonas aeruginosa*, 10 pmoles of forward (AZU-f) and reverse (AZU-r) primers (Table 1) 10 IU of *Taq* DNA *polymerase* (Fermentas) 1 X PCR buffer, 1.5 mM concentration of $MgCI_2$, 2.5 mM DNTPS with total volume of 50uI (Figures 1 and 2).

Ligation of Azurin (447 bp) in pTG19-T cloning vector

The PCR product eluted was ligated with TA cloning vector pTG19-T (25ng/ µl) and left for overnight incubation between 16-22°C. The concentrations of 30 µl reaction mixture are given in Table 2. The recombinant plasmids (Azu/pTG19-T) were subjected to restriction analysis to confirm the presence of Azurin insert (447 bp) (Figure 3). The restriction enzymes selected were *Eco*RI and *Hin*dIII as the TA vector pTG19-T contains the sites of both enzymes (Figure 4). The recombinant plasmid confirmed as positive through previous restriction analysis was digested with *Nde*I and *Bam*HI to obtain the Azurin fragment to be cloned. The gene was engineered to contain *Nde*I and *Bam*HI sites (Figure 5).

Effect of IPTG/Lactose concentration on protein expression

The cultures were grown until the O.D reached 0.6 and induced with different concentrations of lactose (5 mM 10 mM, 15 mM and 20 mM) and IPTG (0.1 mM, 0.2 mM, 0.3 mM and 0.5 mM) and allowed to incubate in shaker at 37°C for 6 hours. All the prepared control and induced cultures were analyzed on 12% SDS-PAGE. The protein of interest can be seen expressed and produced in the lanes 3, 4, 7, 8 and 9. The lanes 3 and 4 represent protein production upon induction with 15 mM and 20 mM lactose respectively. The lanes 7, 8 and 9 represent protein production upon induction with 0.2 mM, 0.3 mM and 0.5 mM IPTG respectively (Figure 6).

These results clearly showed that recombinant protein was expressed when induced with IPTG/lactose and not produced when left un-induced and 0.3 mM and 0.5 mM IPTG was optimized for the use in further protein production and analysis (Figures 7 and 8).

Figure 1: Sequence of Azurin gene (taken from NCBI database) with designed primers. Restriction sites in primers are shown in green. Start and stop codons are indicated in red.
*Forward Primer contains *Nde*I Restriction site (CATATG)
**Reverse Primer contains *Bam*HI Restriction site (GGATCC

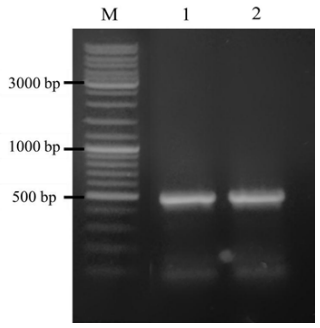

Figure 2: 1% Agarose gel electrophoresis showing PCR amplification of Azurin gene. Lane M: Gene Ruler DNA ladder mix (Thermo Scientific #SM0331). Lane 1 and 2: PCR product of 447 base pair fragment.

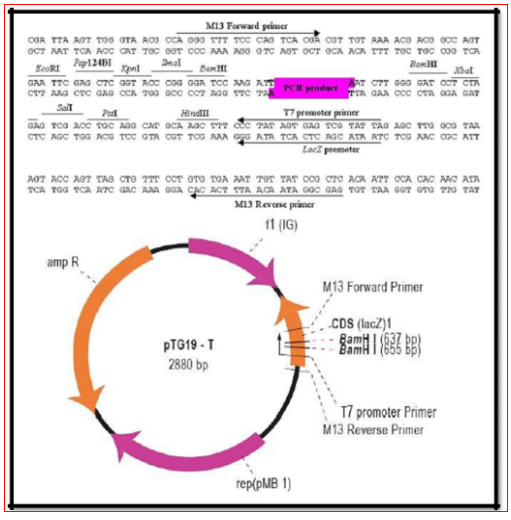

Figure 3: Map and multiple cloning site of pTG19-T.

Components	Volume
Water	8 µl
5X Ligase Buffer	6 µl
T4 DNA Ligase	1 µl
Vector pTG19-T	3 µl
Eluted DNA	12 µl
Total volume	30 µl

Table 2: Components of Azu/pTG19-T ligation mixture.

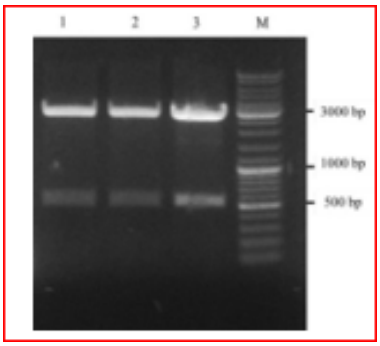

Figure 4: 1% Agarose gel showing recombinant Azu/pTG19-T digested with *Eco*R1 & *Hind* III. Lane M represents Gene Ruler DNA ladder mix (Thermo Scientific #SM0331). Lane 1-3 showing Azurin insert was digested out.

Effect of different incubation time on protein expression

Protein expression was clearly visible after 4, 6 and 8 hours in the lanes 4, 5, 7, 8 and 9, 10 respectively (Figure 5). The lane 1 indicated marker (lysozyme 14.3 kDa). Lane 6 is a negative control (positive vector without IPTG induction). The effect of incubation time post induction upon the growth of bacteria is shown in Figure 5. It was found that the growth increases as the incubation time is increased.

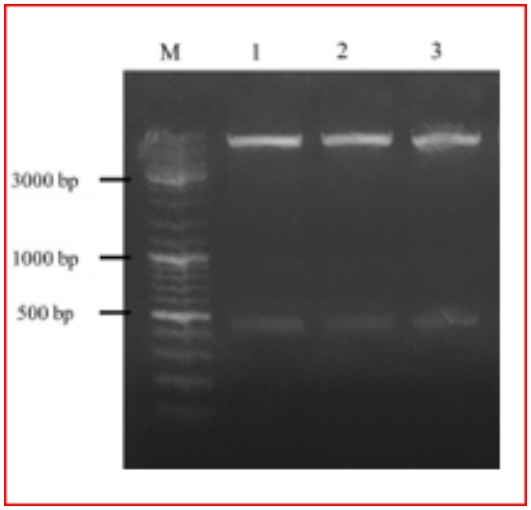

Figure 5: 1% Agarose gel showing recombinant Azu/pET22b digested with Nde1 & BamHI. Lane M represents Gene Ruler DNA ladder mix (Thermo Scientific #SM0331). Lane 1-3 showing that Azurin insert was digested out.

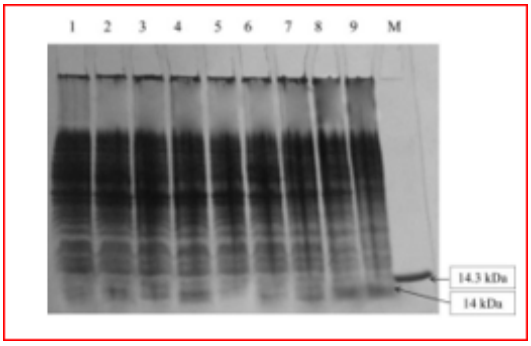

Figure 6: SDS-PAGE analysis of the pellet fraction from the small scale production of Azurin in *E. coli* at 37°C for 6 hours (Effect of IPTG/ Lactose concentrations upon protein expression). Lane 1: with 5 mM lactose; Lane 2: with 10 mM lactose; Lane 3: with 15 mM lactose; Lane 4: with 20 mM lactose; Lane 5: negative control (uninduced); Lane 6: with 0.1 mM IPTG; Lane 7: with 0.2 mM IPTG; Lane 8: with 0.3 mM IPTG; Lane 9: with 0.5 mM IPTG.

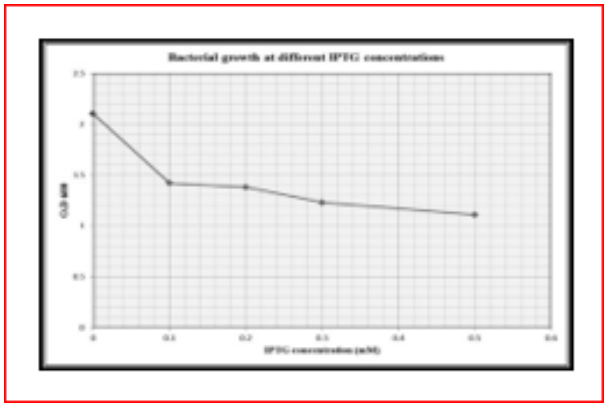

Figure 7: Graph showing effect of different concentrations of IPTG on bacterial growth.

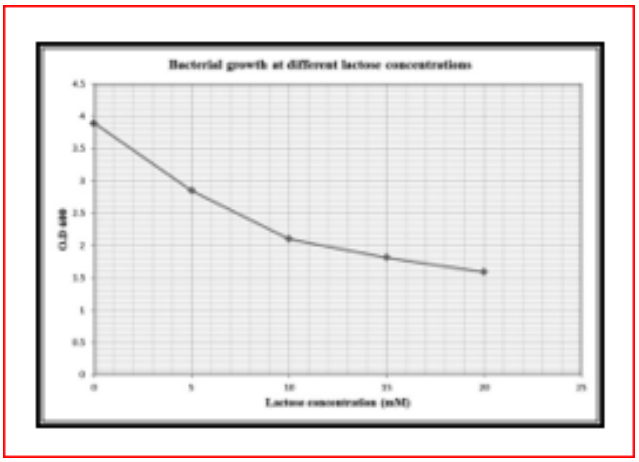

Figure 8: Graph showing effect of different concentrations of lactose on bacterial growth. The effect of different concentrations of IPTG/ lactose on bacterial growth after 6 hours is shown in Figure 5 and 6. It was found that bacterial growth decreases as the concentration of IPTG/ lactose increases.

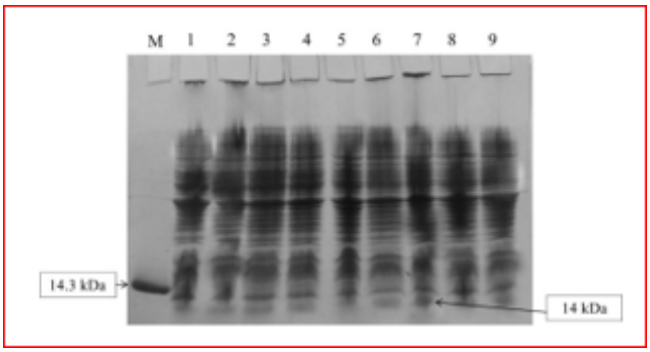

Figure 9: SDS-PAGE analysis of the pellet fraction from the small scale production of Azurin in *E. coli* at 37°C for 2, 4, 6 & 8 hours (Effect of incubation time upon protein expression).. Lane 1: Induction with 0.3 mM IPTG for 2 hrs; Lane 2: Induction with 0.5 mM IPTG for 2 hrs; Lane 3: Induction with 0.3 mM IPTG for 4 hrs; Lane 4: Induction with 0.5 mM IPTG for 4 hrs; Lane 5: Negative control (uninduced); Lane 6: Induction with 0.3 mM IPTG for 6 hrs; Lane 7: Induction with 0.5 mM IPTG for 6 hrs; Lane 8: Induction with 0.3 mM IPTG for 8 hrs; Lane 9: Induction with 0.5 mM IPTG for 8 hrs.

Discussion

Pseudomonas aeruginosa is aerobic, rod-shaped and gram negative bacterium that is responsible to cause diseases in plants, animals and humans [12]. It is a source of anti-cancer proteins like Azurin and Exotoxin A [1]. Azurin, has anti-cancer abilities. To check the expression of azurin under the control of strong promoter (T7 promoter), Azurin from *Pseudomonas aeruginosa* was cloned in plasmid expression vector pET22b (+). *E. coli* BL21 DE3 is used as host. The expression vector (pET22b) utilizes strong promoter of T7 bacteriophage. The promoter is strongly recognized by T7 RNA polymerase which transcribes the mRNA more efficiently as compared to the polymerase provided by the host *E. coli* [13]. As an expression system, BL21-CodonPlus (C+) were used, due to codon bias problems with the heterologous gene. BL21 DE3 Codon+ cells have extra copies of tRNA genes that allow more codon usage as compared to regular strains. Also, these cells may prove to be cost effective and time saving alternative to solve the problems like re-cloning and site directed

mutagenesis [14]. Azurin gene (447 bp) encodes for 149 amino acids with 19 amino acid signal peptides, at N-terminus. The signal peptide, also referred to as localization sequence (usually 5-30 amino acids long) is present at the N-terminal of synthesized protein that helps to localize the protein towards its secretory pathway [15]. Azurin was expressed in Bl21 codon plus cells both the concentration of inducer and time of incubation have effect on the expression and production of azurin. The concentration of IPTG and incubation time was optimum at 0.5 mM and 6 hours respectively. IPTG is optimized instead of lactose because IPTG is not broken down or used by the cells because it is not part of cellular metabolic pathways. Hence IPTG is more useful inducer of lack operon as compared to lactose because the concentration of IPTG remains constant. As azurin is a stable protein and it can withstand long incubation time so good expression was observed both at 6 and 8 hours. Together with the incubation time, the temperature at which cells are grown is very important. 37°C incubation temperature gave robust, fast and nice growth of culture. It is very crucial that the induction must be carried out at an Optical density at 600 nm (OD600) of 0.7 because the cells at this OD have reached their exponential growth phase and great majority of cells are healthy and alive which makes them perfect for protein expression. The nucleotide sequence of azurin gene was determined through sequencing with M13 forward and reverse primers. Azurin shows 99.8% homology with reported sequence of azurin. There is only one silent mutation at 17^{th} amino acid i.e., CCA to CCG (Figure 6). However, the resulting amino acid (proline) remains the same. The predicted amino acid sequence of the mature azurin protein is in completely matched with the known amino acid sequence of azurin. Several research studies report that not all strains of *Pseudomonas aeruginosa* contain the gene encoding for Azurin protein. This may be due to the reasons that some strains might have undergone deletions or mutations in that gene region and thus are devoid of azurin protein. These physiological variations result in varying ability of *P. aeruginosa* for azurin production [16]. Reported that out of 95 samples of *Pseudomonas aeruginosa*, only 10 samples were found to be azurin producing strains. In most bacteria, PCR is effective method of determining the presence or absence of azurin [17].

Conclusion

Azurin gene is successfully cloned in BL21 codon plus cells by using different growth conditions and induction time; it is possible to acquire more information about the expression of azurin in BL21 codon plus cells. More studies can be done using different expression of azurin in *E. coli* for enhanced azurin synthesis different strains of *P. aeruginosa* were grown at $CuSO_4$ and KNO_3 in anaerobic conditions and purified by gel filtration and ion exchange chromatography.

Author Contributions

Farheen Aslam, Adeen Farooq, Hina Qaisar conceived and designed the experiments; Adeen Farooq, Hina Qaisar performed the experiments; and Saima Iftikhar Bajwa and Farheen Aslam and Faiza Saleem supervised and analyze the data; Shagufta Naz contributed reagents/materials/analysis tools; Farheen Aslam and Adeen Farooq and Faiza Saleem wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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