

Pyocyanin induced in vitro oxidative damage and its toxicity level in human, fish and insect cell lines for its selective biological applications

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Abstract Pyocyanin is a redox active phenazine pigment produced by *Pseudomonas aeruginosa*, with broad antibiotic activity having pharmacological, aquaculture, agriculture and industrial applications. In the present work cytotoxicity induced by pyocyanin is demonstrated in a human embryonic lung epithelial cell line (L-132), a rainbow trout gonad cell line (RTG-2) and a *Spodoptera frugiperda* pupal ovarian cell line (Sf9). For toxicity evaluation, cellular morphology, mitochondrial function (XTT), membrane leakage of lactate dehydrogenase, neutral red uptake, affinity of electrostatic binding of protein with sulforhodamine B dyes, glucose metabolism, and reactive oxygen species, were assessed. Results showed that higher pyocyanin concentration is required for eliciting cytotoxicity in L-132, RTG-2 and Sf9. The microscopic studies demonstrated that the cell lines exposed to pyocyanin at higher concentrations alone showed morphological changes such as clumping and

necrosis. Among the three cell lines L-132 showed the highest response to pyocyanin than the others. In short, pyocyanin application at concentrations ranging from 5 to 10 mg l⁻¹ were not having any pathological effect in eukaryotic systems and can be used as drug of choice in aquaculture against vibrios in lieu of conventional antibiotics and as biocontrol agent against fungal and bacterial pathogens in agriculture. This is besides its industrial and pharmacological applications.

Keywords Pyocyanin · Toxicity · IC₅₀ · Vibriosis · Antibacterial · Antifungal

Introduction

Pyocyanin (5-methyl-1-hydroxyphenazine) is a biologically active blue coloured phenazine pigment produced by the bacterium, *Pseudomonas aeruginosa*. It is a redox active natural compound which has various pharmacological effects on eukaryotic and prokaryotic cells. Pyocyanin is a nitric oxide (NO) antagonist in various pharmacological preparations (Warren et al. 1990; Vukomanovic et al. 1997) and it has been used as a model for flavins in electrochemical and spectroscopic studies (Morrison et al. 1978). It also finds application in medicine, food and environment and as mediator in biosensors (Ohfuji et al. 2004). Pyocyanin can be used as electron shuttle in

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microbial fuel cells enabling bacterial electron transfers (Pham et al. 2008; Rabaey and Verstraete 2005).

Besides its pharmacological and industrial applications, pyocyanin has broad antibiotic activity against various genera and species of bacteria (Arunkumar et al. 1997; Vijayan et al. 2006; Priyaja 2012), fungi (Costa and Cusmano 1975; Kerr et al. 1999) and protozoa (Dive 1973). It has also been demonstrated that environmental isolates of *Pseudomonas* can control pathogenic vibrios (Vijayan et al. 2006; Preetha et al. 2010; Priyaja et al. 2014) and improve larval survival in shrimp hatchery systems (Pai et al. 2010). Pyocyanin has been identified as the key molecule produced by *Pseudomonas* that inhibits growth of pathogenic vibrios in aquaculture systems (Preetha et al. 2010; Priyaja et al. 2014).

Despite their wide applications, toxicity of pyocyanin on eukaryotic systems is controversial. Pyocyanin induced toxicity from clinical isolates of *P. aeruginosa* in cell lines and primary cell cultures has been well documented (Muller 2006). However, the in vitro toxicity studies and the IC₅₀ value of pyocyanin from environmental isolates in various biological systems including human cell lines have yet to be accomplished. Antagonistic *Pseudomonas* species are gaining increasing attention as biological control agents for pathogenic fungi and bacteria in agriculture (Anjaiah et al. 2003; Bano and Musarrat 2003, Rangarajan et al. 2003; Kumar et al. 2005), vibrios in aquaculture (Chythanya et al. 2002; Rattanachaiy et al. 2011) and also as augmenters in bioremediation programs (De Meyer et al. 1999; Chaerun et al. 2004; Hasanuzzaman et al. 2004; Tang et al. 2007). Considering the above applications and to facilitate its safe usage toxicity of purified pyocyanin from an environmental isolate of *P. aeruginosa* (MCCB 118) has been tested on human, fish and insect cell lines chosen based on its possible exposure to handlers (humans) and the very sensitive aquatic (fish) and terrestrial (insect) systems as representatives.

Materials and methods

All chemicals used for the experiments, unless specifically stated otherwise, were purchased from Sigma- Aldrich, USA.

Cell lines used for the study, and their growth conditions

The insect cell line, Sf9, originated from *Spodoptera frugiperda* pupal ovarian tissue, the fish cell line, RTG-2, originated from rainbow trout (*Oncorhynchus mykiss*) gonadal tissue and the human embryonic lung epithelial cell line, L-132, were used for the study. These cell lines were purchased from National Centre for Cell Science (Pune, India). The insect cell line, Sf9 was maintained at 28 °C in Grace's insect medium, the fish cell line, RTG-2 at 25 °C in minimum essential medium (MEM) and the human embryonic lung epithelial cell line, L-132 at 37 °C in Dulbecco's modification of minimum essential medium (DMEM). All the media were supplemented with 10 % fetal bovine serum and antibiotic mixture containing 100 µg ml⁻¹ streptomycin and 100 IU ml⁻¹ penicillin.

Bacterial isolate used as the source of pyocyanin and its molecular characterization

Pyocyanin used for the study was extracted from *P. aeruginosa* strain MCCB118. The isolate was collected from marine sediment onboard FORV Sagar Sampada (Fisheries and Oceanographic Research Vessel, Govt. of India) cruise number 233, from the Arabian Sea at a depth of 500 m (7°00'19"N, 77°20'30"E). It was maintained in ZoBell's marine agar slants (2216E Hi Media Laboratories, Mumbai, India) and deposited at the National Centre for Aquatic Animal Health, Cochin University of Science and Technology (Kerala, India). For molecular characterization, 1.5 kb fragment of the 16S rRNA gene was amplified from genomic DNA and sequenced to determine the uniqueness of the *Pseudomonas* isolate. Total genomic DNA was extracted following the method of Lee et al. (2003) with slight modification. Briefly, aliquots of 2 ml overnight culture grown in medium containing 0.5 % peptone, 0.1 % yeast extract were centrifuged and the resultant pellet re-suspended in 1 ml lysis buffer (0.05 mM Tris-HCl, 0.05 mM EDTA, 0.1 M NaCl, 2 % sodium dodecyl sulfate (SDS), 0.2 % polyvinylpyrrolidone (PVP), 0.1 % β-mercaptoethanol). This was followed by the addition of 20 µg ml⁻¹ proteinase K and incubated at 37 °C for 1 h and then at 55 °C for 2 h. DNA was extracted by standard phenol-chloroform method

following Sambrook and Russell (2001). The gene coding 16S rRNA was amplified by PCR using the universal primers NPIF (5′GAG TTT GAT CCT GGC TCA3′) and NPIR (5′ACG GCT ACC TTG TTA CGA CTT3′) (Reddy et al. 2000) in a 25 µl reaction mixture containing 1× Thermopol buffer (New England Biolabs (NEB), Ipswich, USA), 200 µM dNTPs, 2.5 mM MgCl₂, 10 pmol each primer, 75 ng DNA template, 1 U Taq polymerase (NEB, USA). Thermal cycled (Eppendorf, Germany) included 35 cycles of 94 °C for 20 s, 58° C for 30 s, and 72 °C for 2 min followed by a final extension at 72 °C for 10 min. The 1.5 kb DNA product was gel purified using a Wizard SV PCR purification kit (Promega, Madison, WI, USA) and was sequenced in ABI 3700 sequencer at M/s Microsynth AG (Balgach, Switzerland). The sequence obtained was matched with the GenBank database using the BLAST algorithm (Altschul et al. 1990) and submitted under the accession number EF062511.

Extraction and purification of pyocyanin from the environmental isolate of *P. aeruginosa*

The *P. aeruginosa* MCCB 118 was inoculated into 1,000 ml ZoBell's broth (0.5 % peptone, 0.1 % yeast extract and 0.01 % ferric phosphate) prepared in seawater with a salinity 10 g l⁻¹ and incubated at 30 °C in a shaking incubator at 150 rpm. After 24 h, the blue coloured broth (due to the presence of pyocyanin) was transferred into 50 ml Oakridge tubes (Tarsons, India) and centrifuged at 10,000g for 20 min at 4 °C. The supernatant was transferred into a separating funnel (Magnum, Gurgaon, India) and mixed with chloroform at 1:1.5 ratio (supernatant:chloroform). This mixture was shaken well and kept undisturbed for 5–10 min for the pyocyanin fraction in the broth to get extracted to the chloroform layer. The blue colored chloroform layer with pyocyanin formed below the aqueous layer in the separating funnel was collected in a conical flask protected from light, to prevent oxidation. This chloroform fraction was then transferred into a 500 ml vacuum rotary flask and concentrated in a vacuum rotary evaporator at 40 °C (Büchi, Flawil, Switzerland). The vacuum concentrated pyocyanin fraction was purified using silica gel column, having 3 cm diameter and 60 cm length (Magnum, India). The column was packed with silica having a mesh size 100–200 and equilibrated

using chloroform–methanol solvent system in the ratio 1:1 and the concentrated pyocyanin fraction was loaded on to the column. Chloroform–methanol solvent system in the ratio 1:1 was used as the mobile phase to separate pyocyanin. The column run blue coloured pyocyanin fraction was collected and further concentrated in vacuum rotary evaporator at 40 °C (Büchi, Switzerland). The purified and concentrated pyocyanin was taken in a pre-weighed amber coloured bottle and passed through a jet of nitrogen gas to remove solvents and to dry the product. Weight of the dried pyocyanin was determined gravimetrically. This was then dissolved in 500 µl dimethyl sulfoxide (DMSO) and used for all assays.

Confirmation of purity of pyocyanin by HPLC analysis

A portion of purified pyocyanin along with an authentic pyocyanin standard, obtained from M/S Color Your Enzyme, Ontario, Canada were analysed by high pressure liquid chromatography (HPLC) according to a method described by Fernández and Pizarro (1997). HPLC analysis was performed on a Dionex, model ultimate 3000 (Germany) gradient elution system attached to a C₁₈ column (250 × 4.6 mm) and a detector monitoring at 280 nm controlled by the software programme Chromleon (version 6.80). A gradient method was used for eluting samples employing solvent systems A and B. Solvent A was water–trifluoroacetic acid (100:0.04, v/v) and solvent B was acetonitrile–water–trifluoroacetic acid (90:10:0.04, v/v/v). Elution was carried out as follows: solvent A was maintained for 15 min and then changed to 90 % A and 10 % B. This mixture was used for 10 min. Subsequently a linear gradient with a ratio of 70 % A and 30 % B was applied for another 15 min. These conditions were maintained for further 5 min (until 45 min from onset). Finally, solvent composition was changed with a ratio 64 % A and 36 % B and maintained until the end.

Cytotoxicity assays

Combined colorimetric assays for the quantification of the membrane integrity, mitochondrial metabolism, lysosomal integrity and activity, total protein synthesis rate of cells, glucose metabolism and free radical accumulation were performed in each cell line

sequentially by 24 h exposure to pyocyanin in triplicate. Accordingly, six different parameters were evaluated to assess the toxicity. The leakage of intracellular lactate dehydrogenase (LDH), reduction of 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT), uptake of neutral red (NR), and affinity of electrostatic binding of protein with sulforhodamine B dyes (SRB) were assessed with a commercial kit (Cytotox-PAN I, Xenometrix, Heford and Worcester, UK). Glucose metabolism and accumulation of free radicals were evaluated with GOD-PAP end-point glucose assay kit (Biolab Diagnostics, Mumbai, India) and hydrogen peroxide assay kit (BioVison, Milpitas, CA, USA) respectively.

Exposure to pyocyanin and sequential assay procedure for six parameters

Cell cultures in 96 well plates were developed from each cell line by adding 0.2 ml cell suspension in growth medium containing approximately 5×10^5 cells ml^{-1} and incubating for 12 h at appropriate temperature. Different concentrations of purified pyocyanin prepared in growth medium were added to the wells to attain final strength ranging from 6.25 to 200 $\mu\text{g ml}^{-1}$ for XTT (mitochondrial activity), neutral red up take (plasma membrane damage) and SRB (protein synthesis), 0–200 $\mu\text{g ml}^{-1}$ for LDH and H_2O_2 , and 25–200 $\mu\text{g ml}^{-1}$ for glucose consumption in triplicate for each concentration. Cells without pyocyanin were kept as negative control and a solvent control using DMSO.

After 24 h incubation the wells were observed under inverted phase contrast microscope (Leica, Wetzlar, Germany) and sequential cytotoxicity assays were performed. Supernatants from each well and controls were collected for LDH, glucose and hydrogen peroxide assays. The remaining growth medium in the wells was removed, the cells were washed with PBS and 200 μl fresh medium along with 50 μl pre-warmed XTT solution were added and incubated for 4 h in dark. The same cells were used for SRB and NRU tests.

Extra cellular lactate dehydrogenase assay (LDH)

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is rapidly released into the cell culture supernatant upon damage of the plasma membrane. In this assay LDH catalyzes the conversion of lactate to

pyruvate through the reduction of NAD^+ to NADH/H^+ and the NADH reduces the tetrazolium salt, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride to form water-soluble formazan dye. Formation of formazan is directly proportional to membrane damage (extracellular LDH activity) (Decker and Lohman-Matthes 1988; Korzeniewski and Callewaert 1983).

Aliquots of 20 μl supernatants from each well including control were mixed with 240 μl NADH and pyruvate from a mixture prepared at the ratio of 16 and 3.4 ml respectively. Immediately after the addition, the reading was measured kinetically at 340 nm for 25 min at 37 °C. The kinetic readings were compared with control and the response of cells against various concentrations of pyocyanin was measured. The extracellular LDH activity was calculated as nanomoles NADH consumed/min/ml as per the manufacturer's protocol (Cytotox-PAN I, Xenometrix, Switzerland).

Extracellular LDH was measured kinetically for 10 min at 37 °C, and calculated the percentage enzyme activity by comparing with that of the growth medium.

XTT assay

The assay is a colorimetric method based on the determination of cell viability utilizing the reaction of a tetrazolium salt (2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide, XTT) with the mitochondria of metabolically active cells. After 4 h of incubation with 50 μl pre-warmed XTT solutions at 37, 28 and 25 °C in human, insect and fish cells, respectively, the formazan formed was measured at 480 nm in a microplate reader (Tecan Infinite Tm, Austria) with reference wave length at 690 nm.

Neutral red uptake assay (NRU)

Neutral red (3-amino-*m*-dimethylamino-2-methylphenazine hydrochloride) uptake assay is a survival/viability test based on the ability of living cells to incorporate the neutral red dye on their lysosomes, where it accumulates in membrane-intact cells. Following the sequential assay procedure, XTT solution from each well was discarded and washed with 300 μl of wash solution. Two hundred μl of neutral red labeling solution diluted (1:200) with growth medium was added and incubated for 3 h at appropriate

Fig. 1 HPLC of pyocyanin: (a) pyocyanin from marine isolate *P. aeruginosa* MCCB117, (b) pyocyanin authentic standard (Color Your Enzymes, Kingston, ON, Canada)

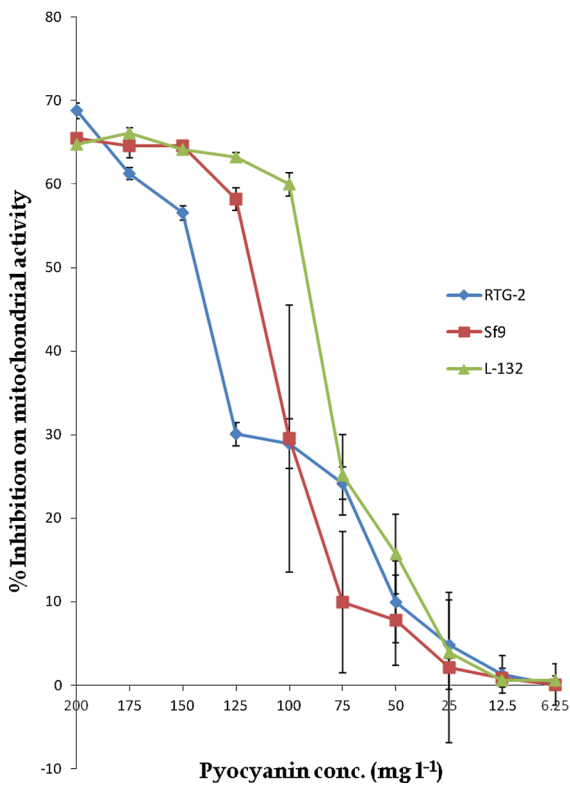
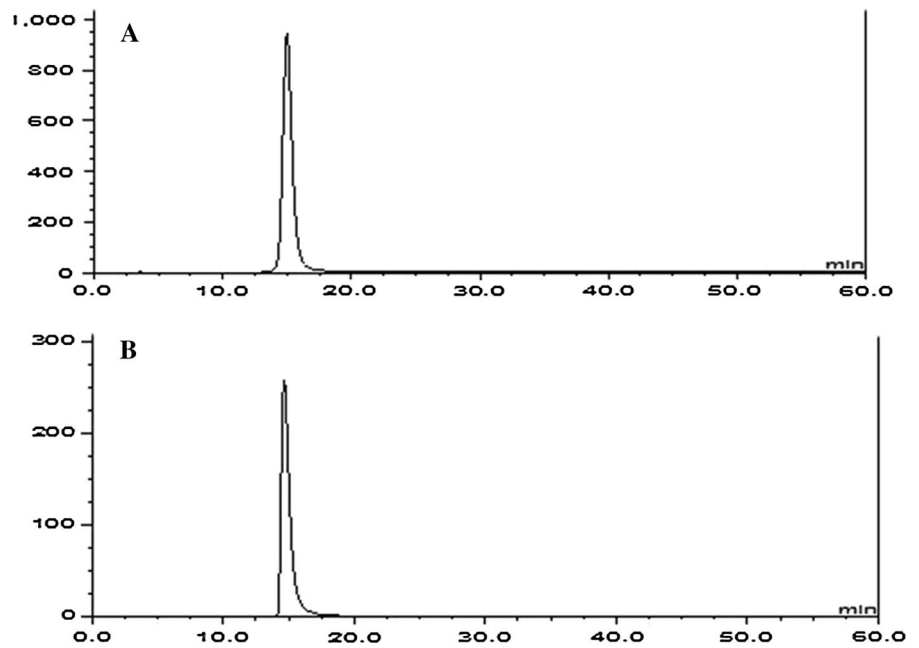


Fig. 2 Pyocyanin induced inhibition of mitochondrial dehydrogenase activity in the different cell lines tested

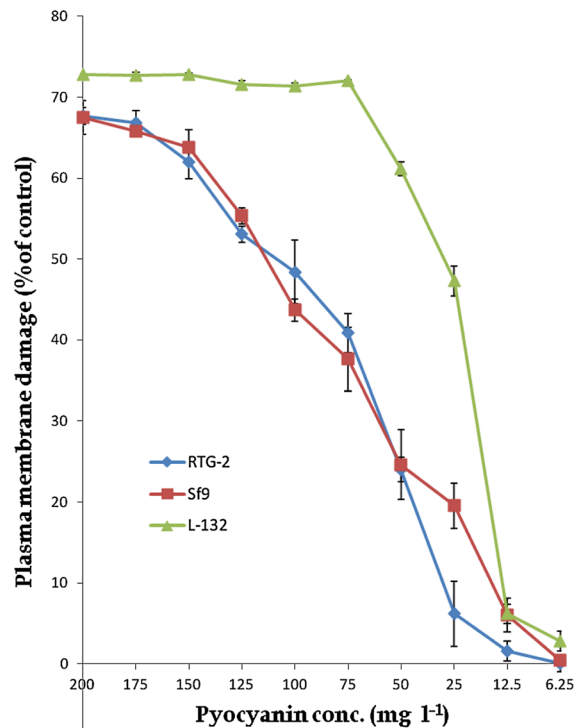


Fig. 3 Pyocyanin induced oxidative damage of plasma membrane of the different cell lines tested

temperature depending on the cell line (25, 28 and 37 °C for RTG-2, Sf9 and L-132 cell lines, respectively). The labeling solution was removed and 100 µl of fixing solution was added to each well and removed after 1 min, added 200 µl of solubilization solution was added and incubated for 15 min at room temperature (RT). After incubation and gentle mixing using a multichannel pipette the absorbance was measured at 540 nm using a micro plate reader (Tecan Infinite™, Männedorf, Switzerland) with reference wavelength at 690 nm and the percentage was calculated.

Sulforhodamine B assay (SRB)

Sulforhodamine B is a bright pink aminoxanthene dye with two sulfonic groups that binds to basic amino acid residues under mild acidic conditions, and dissociate under basic conditions. The solubilization solution from neutral red uptake assay was removed, retaining the cells attached to the wells. The wells were washed with 300 µl wash solution and 250 µl of fixing solution was added, the plate was incubated for 1 h at 4 °C. The cells were washed and 50 µl of labeling solution was added and incubated for 15 min at RT. The wells were washed 2 times with 400 µl of rinsing solution and air dried. After drying 200 µl of solubilization solution was added and incubated for 1 h at RT and the absorbance was read at 540 nm with reference wavelength at 690 nm. Percentage inhibition of various cells at each concentration of pyocyanin was calculated based on the formula, percentage inhibition/activity of cells = $[100 - (\text{average absorbance of cells at a particular concentration of the compound} / \text{average absorbance of control cells without the compound}) \times 100]$ (Vichai and Kirtikara 2006).

Assay for glucose metabolism

Glucose assay allows to determine the physiological state of cultured cells by measuring glucose consumption. An aliquot of 3 µl exposed cell supernatant from each well and control wells were used for the glucose assay. Supernatants were mixed with 300 µl working buffer solution prepared by mixing the enzyme reagent (0.125 mM amino-4-antipyrine, 30,000 U l⁻¹ glucose oxidase, 10,000 U l⁻¹ peroxidase and 100 mM phosphate buffer) with 100 ml assay buffer (16 mM sodium phenolate) and incubated for 15 min at 37 °C. Absorbance of test and standard (100 mg% glucose) were

measured at 500 nm in a microplate reader (Tecan Infinite™, Austria) against blank and control. Inhibition in glucose consumption compared to control cells was calculated.

Determination of pyocyanin-generated hydrogen peroxide

Under aerobic conditions in vitro, pyocyanin results in the formation of the reactive oxygen species, superoxides, and hydrogen peroxide (H₂O₂). In this assay, an aliquot of 50 µl supernatant from each well was mixed with 50 µl hydrogen peroxide assay mixture containing 46 µl assay buffer, 2 µl OxiRed™ probe solution, 2 µl horse radish peroxidase (HRP) (BioV-ison, USA) and incubated at RT for 10 min. Absorbance was measured at 570 nm.

Statistical analysis

The results were the mean values of 3–6 replicates ± standard deviation. Data were analyzed

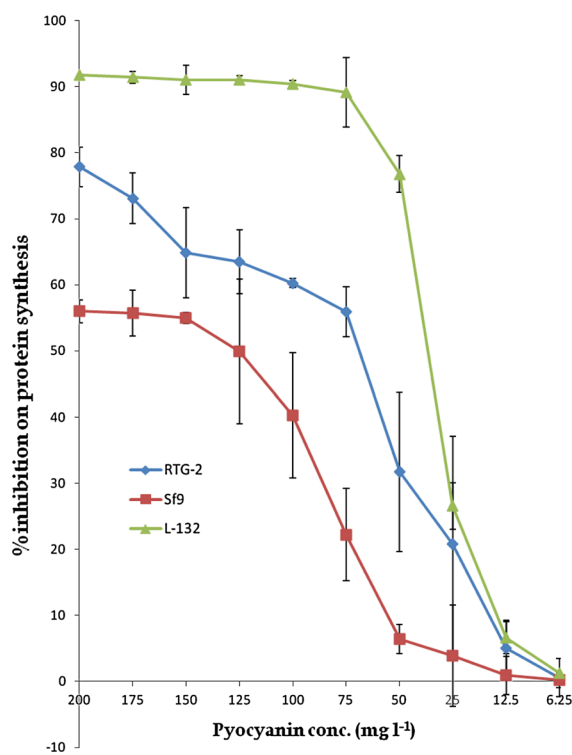


Fig. 4 Pyocyanin induced inhibition of protein synthesis in the different cell lines tested

statistically by probit analysis using SPSS software (SPSS 17.0, SPSS Inc., Chicago, IL, USA) to determine IC_{50} values with 95 % confidence.

Results

Identification of the bacterial isolate

Nucleotide sequence of the isolate, MCCB 118, determined with a 1.5 kb region of the 16S rRNA gene was 99 % identical to *P. aeruginosa* in GenBank BLAST search and the same was submitted with a GenBank accession no. EF062512.

Identification and confirmation of purity of pyocyanin

The HPLC retention time (RT value) of the purified compound was 15 min, identical to that of the pyocyanin standard (Fig. 1).

Cytotoxicity of pyocyanin on various cell lines

In Sf9 cells, IC_{50} values of pyocyanin for inhibition of mitochondrial dehydrogenase was $106.39 \pm 13.92 \text{ mg l}^{-1}$ (Fig. 2), to cause plasma membrane damage $107.77 \pm 28.14 \text{ mg l}^{-1}$ (Fig. 3) and protein synthesis inhibition $133.67 \pm 23.6 \text{ mg l}^{-1}$ (Fig. 4). At 25 mg l^{-1} , pyocyanin caused 3.9 % inhibition in mitochondrial activity, 19.6 % plasma membrane damage and 2.1 % inhibition of protein synthesis. At lower concentration (6.25 mg l^{-1}) the toxicity was undetectable. Whereas at 200 mg l^{-1} these value were 65.5, 67.5 and 56 % respectively. Pyocyanin at higher concentrations (175 and 200 mg l^{-1}) caused morphological changes such as clumping and necrosis of Sf9 cells (Fig. 5).

In RTG-2 cells, IC_{50} values of pyocyanin were 146.19 ± 28.78 , 109.83 ± 11.28 and $76.64 \pm 9.99 \text{ mg l}^{-1}$ in inhibition of mitochondrial dehydrogenase activity, to cause plasma membrane damage and in inhibition of protein synthesis, respectively (Figs. 2, 3, 4). Pyocyanin-induced toxicity in RTG-2

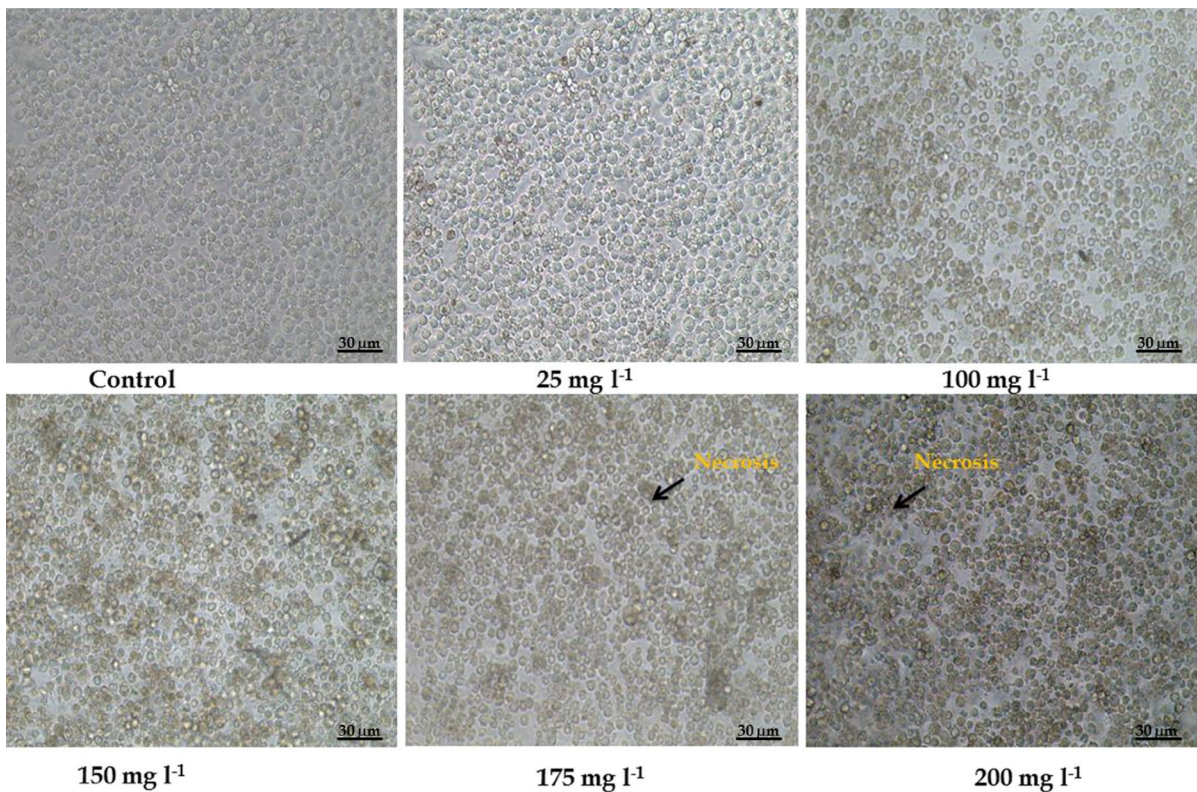


Fig. 5 Pyocyanin induced toxicity in insect cell line (Sf9). Images were taken at $\times 20$ magnification

at 25 mg l^{-1} of pyocyanin was 4.8 % inhibition of mitochondrial activity, 6.2 % plasma membrane damage, and 20.8 % inhibition on protein synthesis. At lower concentration (6.25 mg l^{-1}) the toxicity was undetectable. However, toxicity observed at the maximum concentration of pyocyanin tested (200 mg l^{-1}) was 68.8 % inhibition of mitochondrial activity, 67.7 % in plasma membrane damage and 77.9 % inhibition of protein synthesis. Necrosis and cell rounding were observed at higher concentrations above 150 mg l^{-1} (Fig. 6).

L-132 cells were more prone to pyocyanin-induced toxicity compared to the insect (Sf9) and fish (RTG-2) cell lines (Fig. 7). IC_{50} value of pyocyanin on inhibition of mitochondrial dehydrogenase activity was $112.01 \pm 23.73 \text{ mg l}^{-1}$ (Fig. 2). Meanwhile, the IC_{50} value of pyocyanin induced damage of plasma membrane was $21.79 \pm 14.23 \text{ mg l}^{-1}$ and that of on inhibition of protein synthesis was $32.57 \pm 16.52 \text{ mg l}^{-1}$ (Figs. 3, 4). At a concentration of 25 mg l^{-1} pyocyanin, 3.9 % inhibition of mitochondrial activity, 47.3 % plasma membrane damage and 26.6 % inhibition of

protein synthesis were observed in L-132 cells. At lower concentration (6.25 mg l^{-1}) the toxicity was negligible, whereas at 200 mg l^{-1} the values were 64.8, 72.8 and 91.7 %, respectively.

In addition, determination of plasma membrane damage induced by pyocyanin (contact time 24 h) through extracellular LDH activity (in terms of % reduction in NADH) showed only 16.52, 19.81 and 43.96 % damage at a concentration 25 mg l^{-1} , and 77, 68.42 and 80 % damage at 200 mg l^{-1} in RTG-2, Sf9 and L-132 cells, respectively (Fig. 8). At lower concentration the toxicity observed was negligible. Besides, control cells (RTG-2, Sf9 and L-132) showed 9.32, 1.13 and 14.82 % normal extracellular LDH activity, respectively.

Effect of pyocyanin-induced stress on glucose metabolism

Pyocyanin-induced inhibition on glucose metabolism was observed in the human cell lines with an IC_{50} value of $19.1 \pm 11 \text{ mg l}^{-1}$. In the fish cell line it was

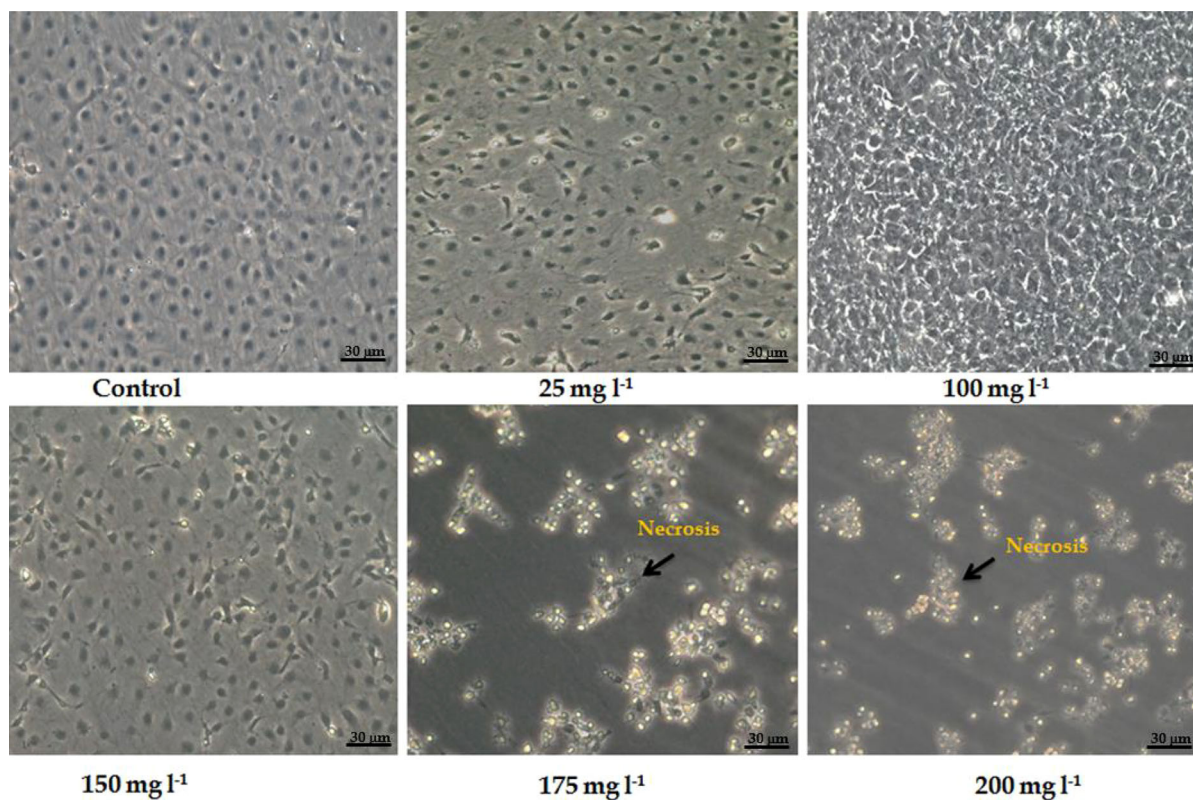


Fig. 6 Pyocyanin induced toxicity in fish cell line (RTG-2). Images were taken at $\times 20$ magnification

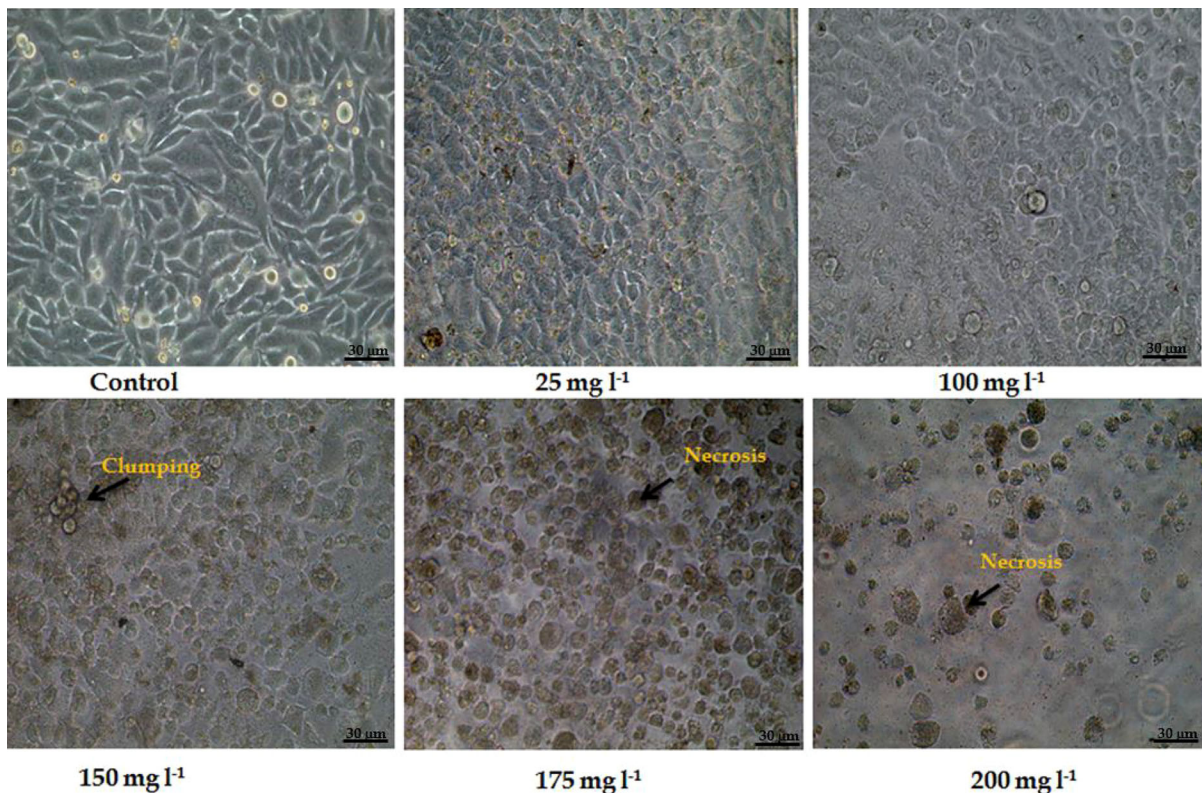


Fig. 7 Pyocyanin induced toxicity in human embryonic lung epithelial cells (L-132). Images were taken at $\times 20$ magnification

$21.1 \pm 5.8 \text{ mg l}^{-1}$ and in Sf9 cells $70.6 \pm 28.7 \text{ mg l}^{-1}$ (Fig. 9). At 200 mg l^{-1} , pyocyanin inhibition of glucose metabolism was 100 % in L-132, 98.2 % in RTG-2 and 70.5 % in Sf9 cells. However, at 25 mg l^{-1} the values were 65.5, 59.4 and 38.4 %, respectively.

Pyocyanin induced hydrogen peroxide production

Exposure of cells (L-132, RTG-2 and Sf9) to pyocyanin resulted in the formation of H_2O_2 in a dose-dependent manner. Base level production of H_2O_2 by untreated cells was $0.026 \mu\text{M}$ for L-132 and Sf9 cells and $0.024 \mu\text{M}$ for RTG-2 cells, respectively. An increase of 46.2, 53.8 and 54.2 %, of H_2O_2 concentration from the base level was observed in Sf9, L-132 and RTG-2 cells in the presence of pyocyanin at a concentration 25 mg l^{-1} with a value of 0.038, 0.04 and $0.037 \mu\text{M}$, respectively. The production of H_2O_2 at 200 mg l^{-1} was 0.06, 0.064 and $0.056 \mu\text{M}$ in Sf9, L-132 and RTG-2 cells, respectively (Fig. 10).

Discussion

The environmental isolates of pyocyanin producing *P. aeruginosa* have been recognized as putative biological control agents on phytopathogenic fungi and bacteria in agriculture (Anjaiah et al. 2003; Bano and Musarrat 2003; Rangarajan et al. 2003), vibrios in aquaculture (Chythanya et al. 2002; Vijayan et al. 2006) and as bioaugmentors in bioremediation (Chae-run et al. 2004; Hasanuzzaman et al. 2004; Tang et al. 2007). Besides its bio-control property, pyocyanin has various industrial and pharmacological applications as well (Warren et al. 1990; Vukomanovic et al. 1997; Jose et al. 2014). However, the pyocyanin-induced toxicity is still a controversial issue as the studies have been mainly performed with clinical isolates focusing on its deleterious effects (Gloyne et al. 2011; Muller et al. 2009). The molecular mechanism and mode of action of pyocyanin in target organism has been studied extensively (Hassan and Fridovich 1980). Britigan et al. (1997) and Hassan and Fridovich (1980)

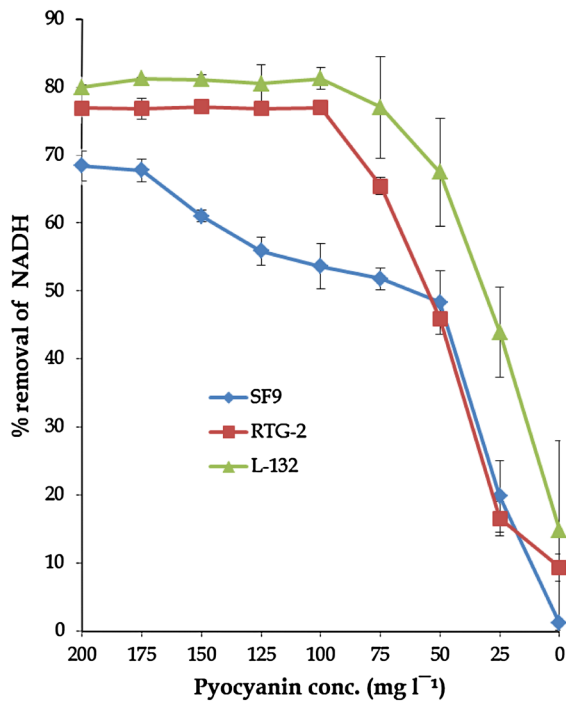


Fig. 8 Extracellular LDH activity in terms of % reduction in NADH of the different cell lines tested

reported that the toxicity is due to its ability to undergo redox cycling in the presence of various reducing agents and molecular oxygen, which leads to the accumulation of toxic superoxide and hydrogen peroxide and, eventually, to oxidative cell injury or death. In this context, determination of the toxicity of pyocyanin from the environmental isolates and its IC₅₀ values in cell lines including that from human gives a much better understanding on the concentration at which it can be applied in various biological systems. This paves the way for safe and environment friendly application of pyocyanin as a drug of choice in aquaculture against vibriosis and bio-control agent against phytopathogenic fungi and bacteria in agriculture besides its manifold industrial application.

Pyocyanin-induced toxicity in human cell lines has been studied extensively. Muller (2006) reported that 1–2 mg l⁻¹ (5–10 μM) pyocyanin from clinical isolates arrested growth of A549 cells and resulting in the development of a morphological phenotype consistent with cellular senescence. On treating the cells with pyocyanin at 2 mg l⁻¹ (10 μM) the senescent phenotype was found stable for 7 days. However, exposure to pyocyanin at 5 mg l⁻¹ (25 μM) resulted in

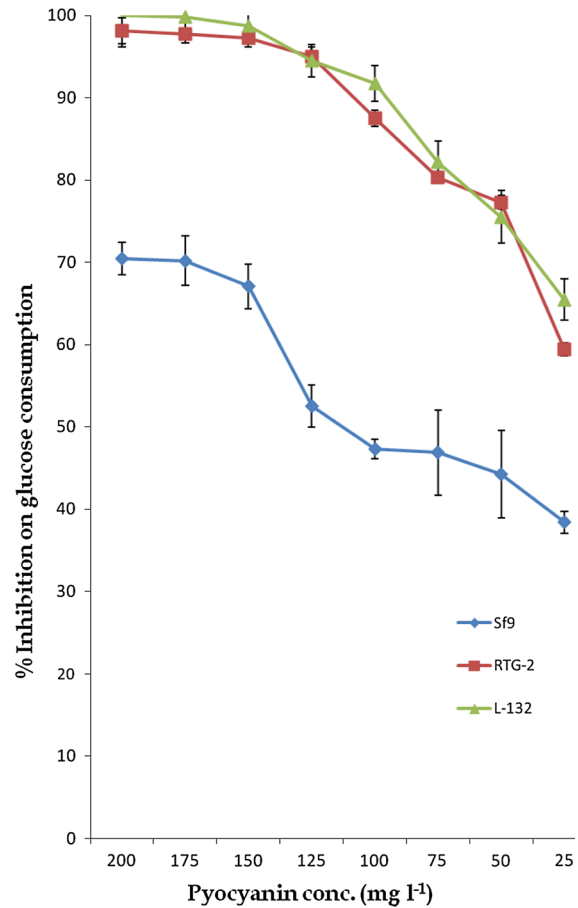


Fig. 9 Pyocyanin induced effect on glucose metabolism in the different cell lines tested

detachment of cells from their substratum, shrinkage, blebbing of cell membranes, and apoptosis leading to cell death (Muller 2006). In our study, pyocyanin at very higher concentrations (175 and 200 mg l⁻¹) only caused significant morphological changes such as clumping, and necrosis as visualized microscopically in all cell lines tested (Sf9, RTG-2 and L-132). Human cell membrane was found to be more susceptible to oxidative damage induced by pyocyanin in comparison with the insect and fish cell lines. The IC₅₀ values on plasma membrane damage for Sf9 cells and RTG-2 cells were almost similar (106.39 ± 13.92 and 109.83 ± 11.28 mg l⁻¹, respectively), whilst for L-132 it was 21.79 ± 14.23 mg l⁻¹. In addition, pyocyanin induced plasma membrane damage could be compared with the extracellular LDH activity which showed percentage activity of 16.52, 19.81 and

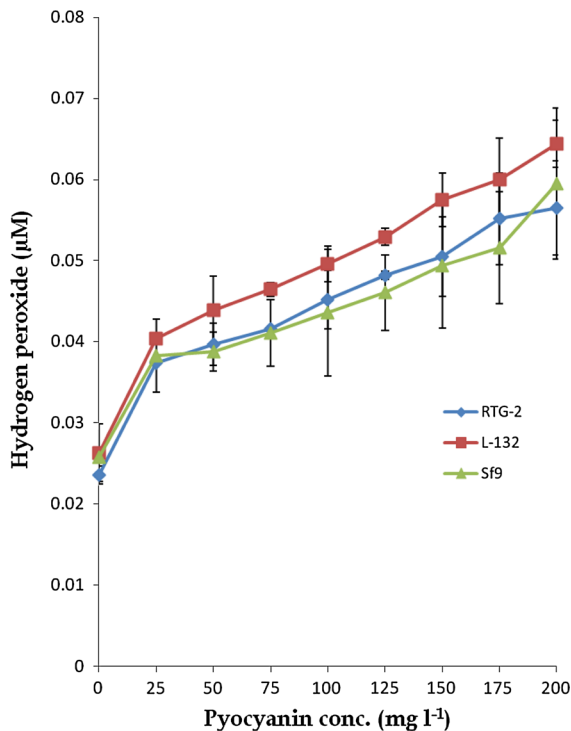


Fig. 10 Pyocyanin induced generation of H₂O₂ in dose dependent manner in the different cell lines tested

43.96 at a concentration of 25 mg l⁻¹ pyocyanin for RTG-2, Sf9 and L-132 cells, respectively.

Measurement of cellular metabolic activity through mitochondrial dehydrogenase system revealed that it required higher pyocyanin concentration for its inhibition. RTG-2 showed highest IC₅₀ value of 146.19 ± 28.78 mg l⁻¹ on mitochondrial dehydrogenase. Whilst, insect cell line (Sf9) and the human cell line (L-132) showed a similar pattern of inhibition having IC₅₀ values of 107.77 ± 28.14 and 112.01 ± 23.73 mg l⁻¹, respectively. Even though at higher pyocyanin concentration Sf9 resisted pyocyanin induced inhibition of protein synthesis with an IC₅₀ value 145.94 ± 25.38 mg l⁻¹ and the human cell line L-132 showed a higher susceptibility with higher damage of the protein synthetic mechanism (IC₅₀ 32.57 ± 16.52 mg l⁻¹) than that of fish cell line (IC₅₀ 76.64 ± 9.99 mg l⁻¹).

In this study, pyocyanin induced toxicity in all cell lines tested could be linked to the oxidative damage due to hydrogen peroxide production in a dose-dependent manner and the lowering in glucose consumption. This supported the findings of Muller

(2006) who pointed out that the toxicity was due to the oxidative stress/injury caused by pyocyanin mediated hydrogen peroxide production in a dose-dependent manner which could be prevented/controlled by using antioxidants. Generally, a millimolar dose of pyocyanin is required to induce toxicity in cells and the data generated in this work point to the fact that this concentration is far beyond the physiological relevance (Imlay 2008).

In conclusion, the study demonstrated the pyocyanin-induced toxicity in various biological systems. Despite the toxicity induced by pyocyanin from clinical isolates, pyocyanin from environmental isolates can be used as a drug of choice against vibriosis in aquaculture and biocontrol agent against fungal and bacterial pathogens in agriculture at 5–10 mg l⁻¹ level which is well below the toxic level in eukaryotic systems. The overall cytotoxicity study with pyocyanin from the environmental isolates revealed that the IC₅₀ values were multifold higher than the required concentration of pyocyanin reported for various biological applications.

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