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Establishment of Anticancerouspotential of Pyocyanin Pigment Produce by P. Aeruginosa

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ABSTRACT
Two strains of Pseudomonas aeruginosa, SSH-3 and HSS-6 wereisolated from slaughter house effluent sample and clinical sample, respectively. P. aeruginosa produce a major toxin i.e, Pyocyanin, which exerts damaging effects on mammalian cells. Pyocyanin is a phenazine derivative, which displays redox activity and associated with anticancer activities. The pyocyanin pigment was extracted from fermentation broth of both the isolates by chloroform extraction method andsubsequently purified by silica column chromatography and thin layer chromatography. The purified pigment from P. aeruginosa SSH-3 and HSS-6 and standard pyocyanin has Rf value of 0.833. Pyocyanin as a pigment was confirmed by UV-Vis spectrophotometric analysis with absorption maxima at 270-280nm, FTIR and NMR. The growth inhibitory effect of pyocyanin was determined by MTT assay. The anticancerous activity of purified pyocyanin extracted from P. aeruginosa MH038270 has been established against Hep2C cell lineand this pigment was found toinhibit the proliferation of Hep2C cell line.

KEYWORDS: Pseudomonas aeruginosa, redox-active, purification, characterization, anticancerous agent.

I. INTRODUCTION

Cancer is a major disease, which occurred due to uncontrolled growth of cells [1]. Cervix carcinoma is one of the most frequent malignancies. This is the most common cause of death because of cancer in women and also most frequent cause of death worldwide[2]. According to World Health Organization, around 5, 70,000 new cases have been reported, representing 6.6% of all female cancers in 2018. The methods available for the treatment of cancer are surgery, chemotherapy and radiation therapy. Many researchers studied to find out an effective solution for this serious health issue. The therapeutic methods have some side effects including the effect of cytotoxic agent on non-cancerous cells [3].Natural metabolites derived from different organisms have proven to be promising sources of anticancerous agent [4]. In recent years, biological compounds and synthetic molecules isolated from fungus, bacteria and plants have demonstrated medicinal effect on various types of cancer cell lines.

Pyocyanin is produced by opportunistic pathogen P. aeruginosa, well known to play role in it'svirulence [5]. Phenazine-1- carboxylic acid, the first phenazine formed in shikimic acid pathway, is converted to pyocyanin in two steps. These two steps are catalyzed by enzymes PhzM and PhzS. It has been already reported that the physical presence of PhzS is necessary for the activation of PhzM, suggesting that a protein-protein type of interaction is occurred in synthesis of pyocyanin [6]. Phenazines are associated with antitumor activities [7].Pyocyanin has natural ability of modulating redox cycles and causes oxidative stress which plays an important role in its detrimental effects on host cells.Pyocyanin generate reactive oxygen species (ROS) and the cells that are respiring actively such as cancer cells, appear to be more susceptible to ROS. Normal cells (non-malignant) preserve redox homoeostasis by tightly controlling the equilibrium between generation and elimination of ROS, maintaining basal ROS at low levels.

Pyocyanin is a zwitterion having lowmolecular weight of about 210 Dalton, it readily diffuses through cell membranes and permeates it, where it generates ROS and leads to oxidative stress [8, 9]. After entering into cells, NAD(P)H reduced pyocyanin nonenzymatically and subsequently pyocyanin reduces molecular oxygen to superoxide [10], and hydrogen peroxide (H_2O_2) [11]. Pyocyanin can inhibit fungal [12, 13, 14],bacterial growth [15] and mammalian cells [16, 17]. Additionally in eukaryotic cells, pyocyanin also interfere with activities of topoisomerase I and II [18]. The development of pyocyanin as anticancer agent is very important in biotherapy. Therefore, this work was focused to study the effect of purified pyocyanin on cancer cell line.

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II. MATERIALS AND METHODS

EXTRACTION OF PYOCYANIN PIGMENT

The inoculated media of both the culture (SSH-3 and HSS-6) produce vigorous blue greenpigmentation within the optimized incubation period. P. aeruginosa also produce other extracellular pigments such as; pyoverdin, pyorubin and pyomelanin which are responsible for this color change elaborated by pseudomonads [19].The biomass of P. aeruginosa was separated by centrifugation at 10,000 rpm for 15 minafter incubation periodand supernatantwas filtered through 0.45µm pore size membrane filter. Solvent (chloroform) extraction method was used for separation of pigment[20].The blue color solution present in solvent layer was separated from aequous layer by using seperating funnel. The blue colored solution turned to reddish pink by the addition of 0.1NHCl.

PURIFICATION OF PYOCYANIN

The extracted pigment from both the cultures was purified bycolumn chromatography, using silica gel (mesh size) (C18 1g/6mL) as stationary phase and a solution of methanol and chloroform of 1:1 ratio was used as mobile phase [15]. The pyocyanin pigment previously stored in sterile container at 4°C was re-dissolved in 5mL of chloroform and adsorbed onto silica gel.The crude pigment adsorbed on silica column was equilibrated with washing solution (1% methanol in chloroform). The adsorbed pigment was eluted with mobile phase and three bands of light blue, dark blue and yellow in color were appeared. Further, to check the purity of eluted fraction, thin layer chromatography (TLC) has been done by using methanol and chloroform (1:1) as mobile phase and silica as stationary phase. The R_f value of purified pigment was checked and compared with standard pyocyanin.

CHARACTERIZATION OF PYOCYANIN

The purified pigment in fractions of both P. aeruginosa SSH-3 and HSS-6 were subjected to UV-Visible spectrophotometery. Ultraviolet and visible absorption spectra of purified pigment dissolved in mobile phase was recorded over a range of 200-800 nm. Further characterization of purified compound was also done by FTIR and Proton NMR and compared with standard pyocyanin. These analyses showed the presence of relevant bonds and functional groups in their respective structure.

DETERMINATION OF ANTICANCER ACTIVITY OF PURIFIED PYOCYANIN FROM P. aeruginosa MH038270

Cytotoxic study of purified pyocyanin from P. aeruginosa MH038270 (HSS-6) was determined in Hep2C cell linein vitro. Cells were grown in Dulbecco's modified Eagles medium (HiMedia) supplemented with 10% FBS (Fetal Bovine Serum). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C.

The viability of the cells was determined by the ability of cells to cleave MTT 3-(4,5-dimethylthiazol2-yl)-2,5- diphenyl tetrazolium bromide.This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol2-yl)-2,5-diphenyltetrazolium Bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters into the mitochondria, where it undergoes reduction by succinate dehydrogenase to an insoluble colored (dark purple) product named formazan.

Briefly, purified pyocyanin was pre-solubilized in methanol to give a stock solution of 20mg/mL. The confluent monolayers of Hep2C cells were grown in 96 wells microtitre plate for 24h. Cells were incubated with various concentrations of the sterile purified pyocyanin (0.5µg/mL, 1.5µg/mL, 2.5µg/mL, 3.5µg/mL,4.5µg/mL, 5.5µg/mL, 6.5µg/mL, 7.5µg/mL and 10µg/mL) in triplicate at 37°C in a CO_2 environment for 48h. The control was performed using methanol alone instead of pigment. At the expiration of the 48h incubation period, 100µL of medium were removed from each well and 20µL of the MTT solution (5mg/mL in distilled water) was loaded to each well. The plates were incubated for 1h at 37 °C. After 1h treatment period, media of wells was discarded and 100µL of DMSO was added to each well to solubilise the crystals of formazan. The reading of the plates was taken in ELISA reader (MULTISCAN EX, Thermo Electron Corporation, China) and the optical density was determined at 570nm. The viability of cell after treatment was also examined microscopically.

III. RESULTS AND DISCUSSION

EXTRACTION AND PURIFICATION OF PYOCYANIN

The pigment produced by P. aeruginosain optimized mediumwas extracted by chloroform extraction method. Pyocyanin is soluble in chloroform producing blue color [15]. It has been reported that the bright green colour of broth

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inoculated with P. aeruginosa culture developed when water-soluble blue pyocyanin combines with pyoverdin[21].A change in color from blue to deep pink or red observed after the addition of 0.2N HCl,confirmed only the presence of pyocyanin pigment [22, 23].The pyocyanin pigment is dark-red in acidic condition because of the basic property of one of the nitrogen atoms and blue in alkaline reaction [24].

The extracted crude pyocyanin was purified by column chromatography using silica gel. Pure pyocyanin was eluted early as blue band with elution buffer (1:1 methanol and chloroform). Similar results during the course of purification of pyocyanin were obtained by Sudhakar, (2013) [23].According to Devnath et al, 2017,they also found a single blue colour band during purification which indicated that no other pigment was present in the extracted solution [25].

The TLC of the purified compound obtained from column chromatography was performed against standard pyocyanin. A spot of blue color of pigment (Fig.1) with retention factor (Rf) value 0.833 observed. The results are comparable with the previous finding where Rf value of 0.83 was reported for purified pyocyanin [26]. Baron and rowe, 1981have obtained RF value for purified pyocyanin of 0.71 by eluting the pigment with chloroform solvent [15].

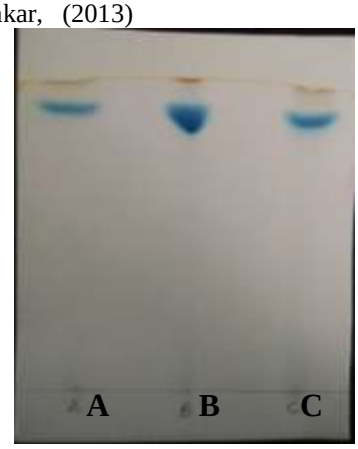

Fig.1. Thin layer chromatography of Pigment; A- Pigment from SSH-3, B- Standard pyocyanin (Sigma aldrich) and C- Pigment from HSS-6

CHARACTERIZATION OF PYOCYANIN PIGMENT

UV-VIS SPECTROPHOTOMETRY

UV-Vis spectrophotometry has been routinely used to determine the absorption maxima of compound.The absorption spectrum of purified compound and standard pyocyanin dissolved in equal ratio of methanol and chloroform was monitored from 200-800nm using UV-Vis spectrophotometry. The result showed the λmaxof purified pyocyanin from P. aeruginosa SSH-3, HSS-6 and standard pyocyanin at 270-280nm (Fig.2).These results were in accordance with the finding of Karpagam et al, (2013) in which maximum absorbance obtained at 278nm [27]. In other study, the maximum absorbance peak of extracted purified pigment dissolved in 0.1N HCl ontained at a range of 270-275nm [25]. These peaks indicated the presence of pyocyanin compound in solution.

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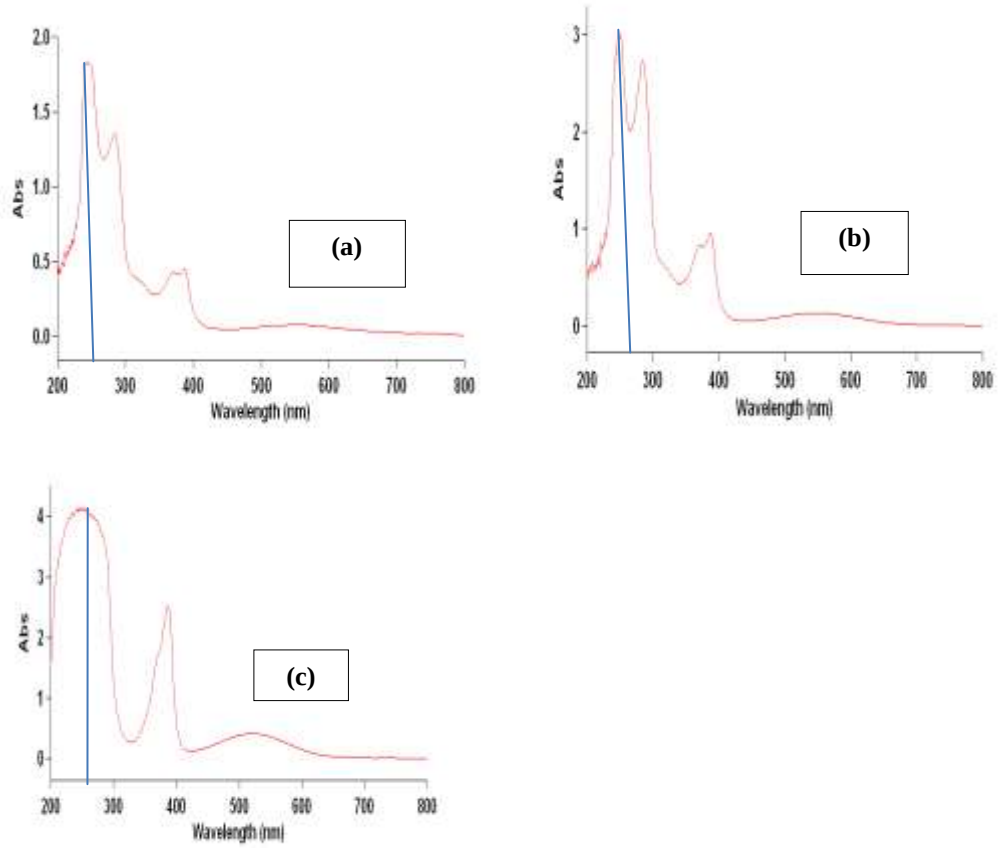

Fig.2. UV-Vis absorption spectrum of (a) P. aeruginosa SSH-3 pyocyanin, (b) P. aeruginosa HSS-6 pyocyanin and (c) Standard pyocyanin

NMR SPECTROSCOPY ANALYSIS

NMR spectroscopy analysis has been done to determine the purity of a sample as well as molecular structure of compound in solution. Proton (^{1}H) NMR technique usually used for structural determination of molecules. Pyocyanin showed the two characteristic peaks. The pyocyanin from P. aeruginosa SSH-3, HSS-6 and standard pyocyanin, exhibited peaks atδ 7.4-7.2 ppm,δ 7.3-7.2 ppm andδ 7.8-7.7ppm respectively, which indicate the presence of N-atom attached to aromatic ring. The peak atδ 4-3.3 ppm showed the presence of methyl group attached to aromatic nitrogen atom (Fig.3). It has been also reported in previous finding that Proton NMR and 13C NMR peak of pyocyanin clearly indicated the presence of methyl groups linked to the aromatic nitrogen atom [28]. These results clearly demonstrate that the produced pyocyanin produced by P. aeruginosa was purified to homogeneity and their structures are in accordance with previous reports [27, 29, 30, 31].

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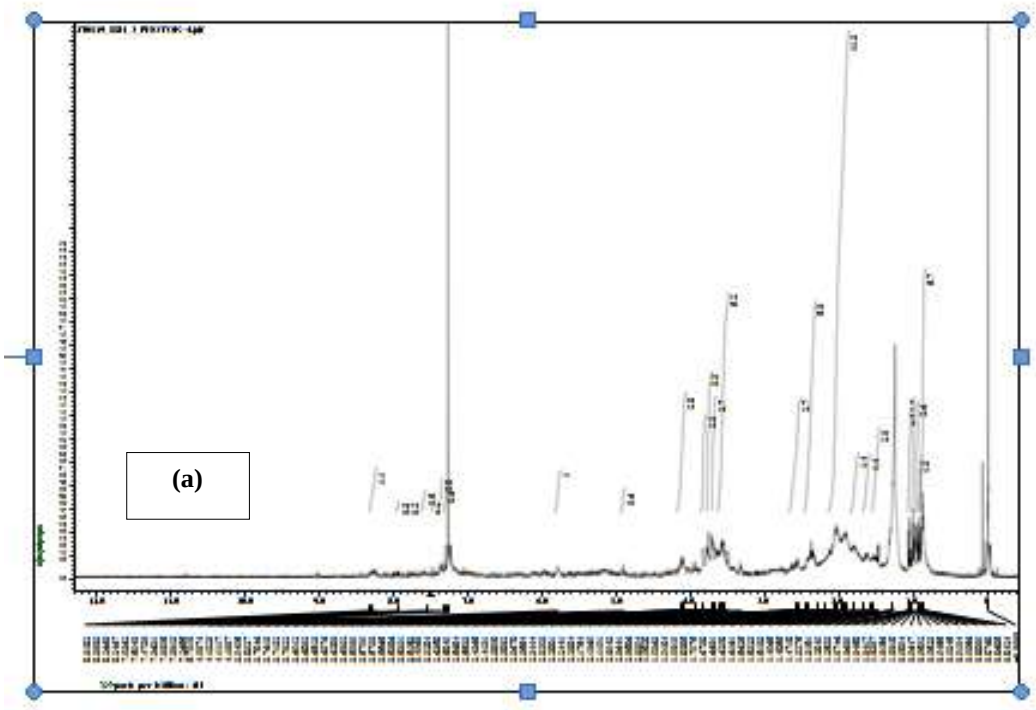

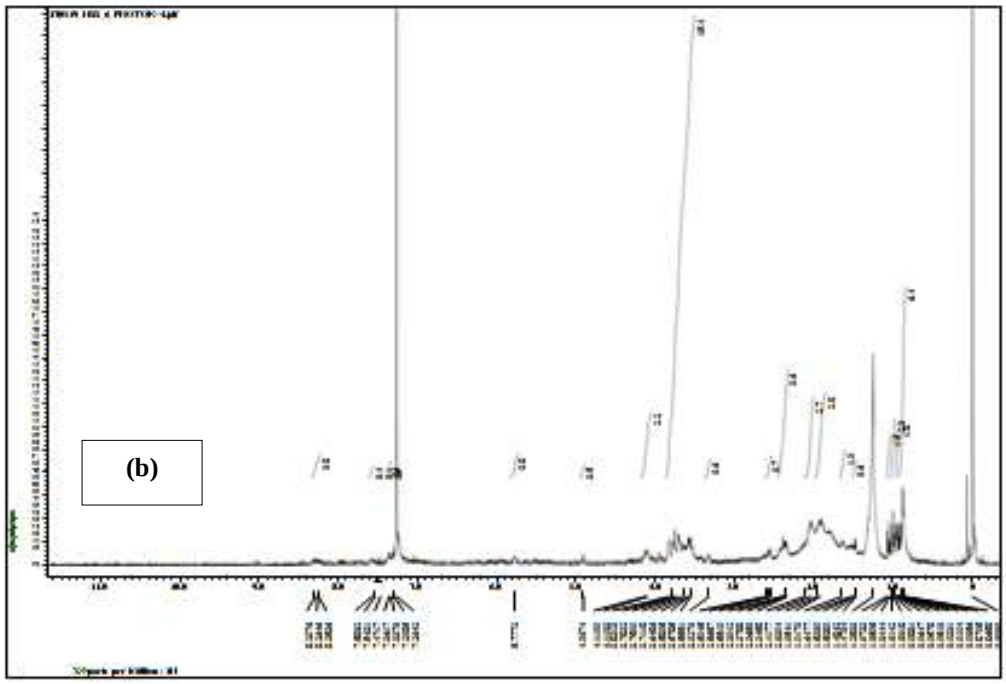

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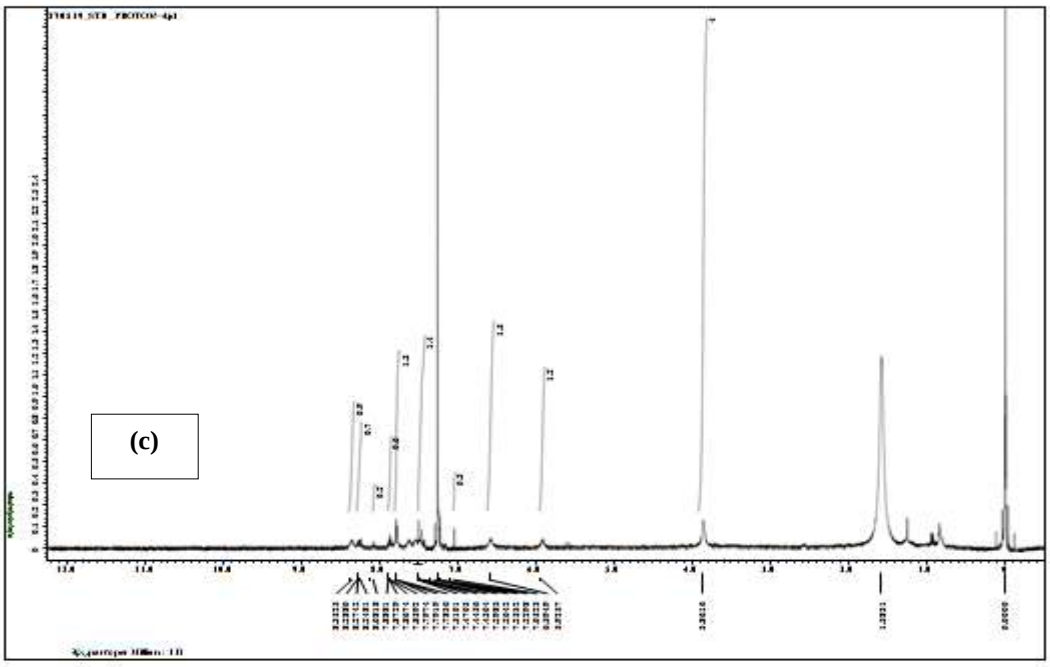

Fig.3. NMR spectra of the purified pyocyanin (a) obtained from P. aeruginosa SSH-3, (b) obtained from P. aeruginosa HSS-6 and (c) NMR spectrum of Standard pyocyanin

FTIR STUDIES

Further, the characterization of purified compound of different selected isolates has been done by analyzing their infrared spectra. FTIR spectroscopy technique is used to detect different functional group. The FTIR spectra showed broad stretch of –OH group between 3600-3300 cm^{-1}. The compound showed absorption from 1600-1300 cm^{-1} due to vibration of C-C stretching in the aromatic ring. In the pyocyanin of P. aeruginosaSSH-3 and HSS-6, a hump appeared at 1648.9 and 1650.4 cm^{-1},respectively, which was due to the C=N bond. The peak obtained in the range 1300-1100 cm^{-1} corresponds to C-O stretching. The presence of –CH3 stretching was also confirmedbetween 2900-2700 cm^{-1}(Fig.4). The comparison of the spectra revealed that purified compound was pyocyanin because most of the groups present in the structure of standard pyocyanin were present in purified products. The FTIR analysis of purified pyocyanin obtained from P. aeruginosa SSH-3 and HSS-6was same as that of FTIR of standard pyocyanin pigment. These FTIR analysis characteristics is in corborration of previous studies characterization[25, 31].

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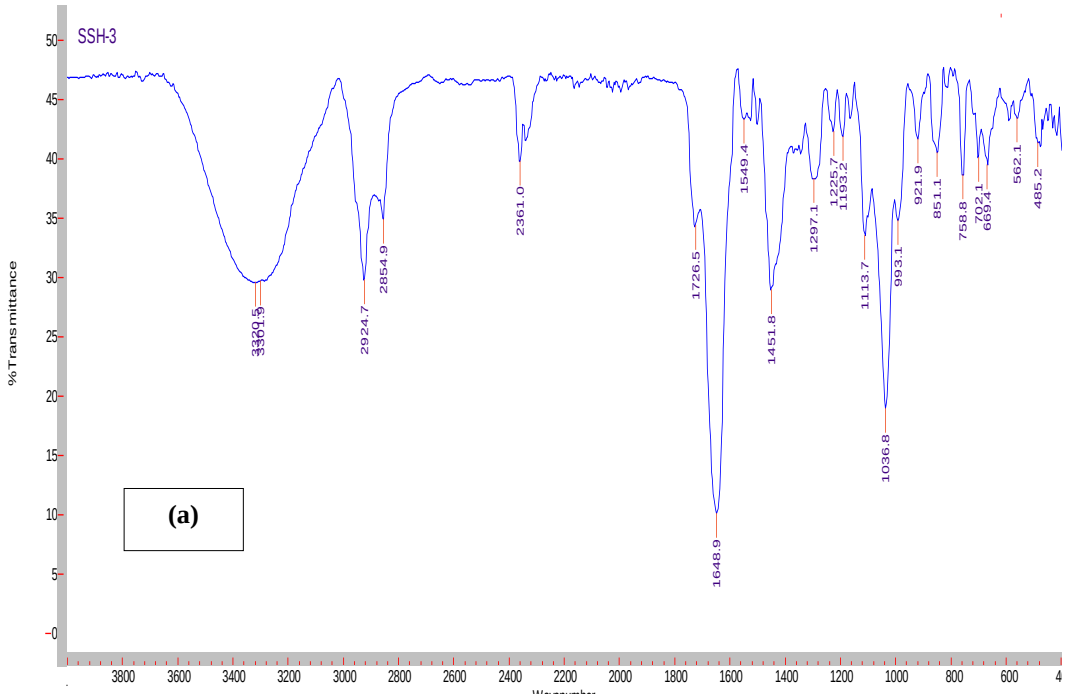

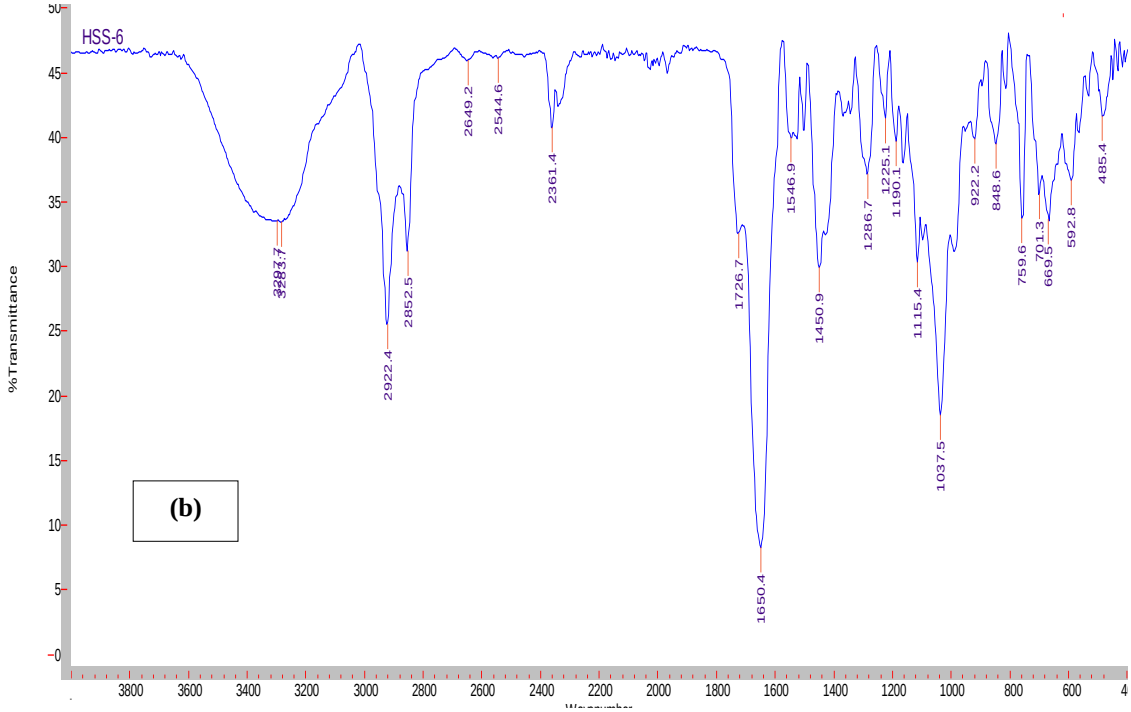

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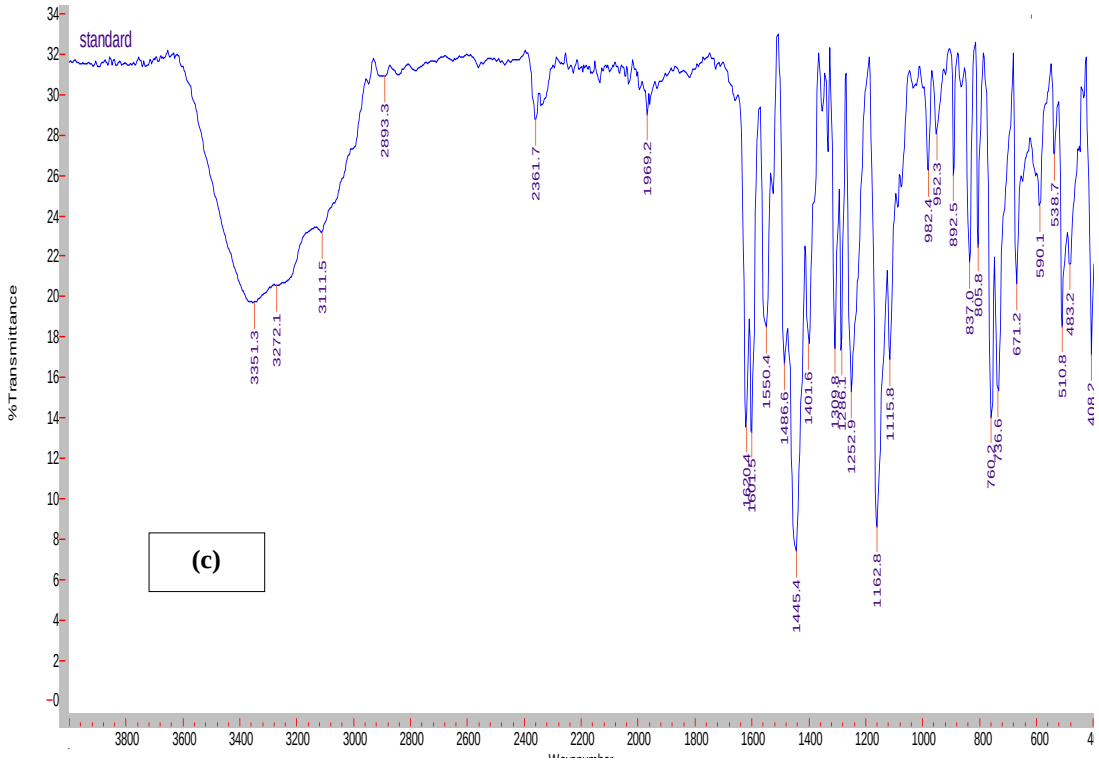

Fig.4. FTIR spectra of the purified pyocyanin (a) obtained from P. aeruginosa SSH-3, (b) obtained from P. aeruginosa and (c) FTIR spectra of standard pyocyanin

DETERMINATION OF ANTICANCEROUS ACTIVITY

The purified pyocyanin obtained from P. aeruginosa HSS-6 had cytotoxicity with IC_{50}of1.4µg/mL.A significant decrease in viability of cells was achieved and sensitivity of the Hep2C cell line to pyocyanin was dependent on dose. The maximuminhibition (91.9±1.46%)was achieved after 48h with 10µg/mL concentration of pyocyanin (Fig.5).The prime reason behind anticancer effect of pyocyanin was its capability to generate reactive oxygen species (ROS) and these ROS interferes with the topoisomerase I and II activities in eukaryotic cells (tumor cells) [33].

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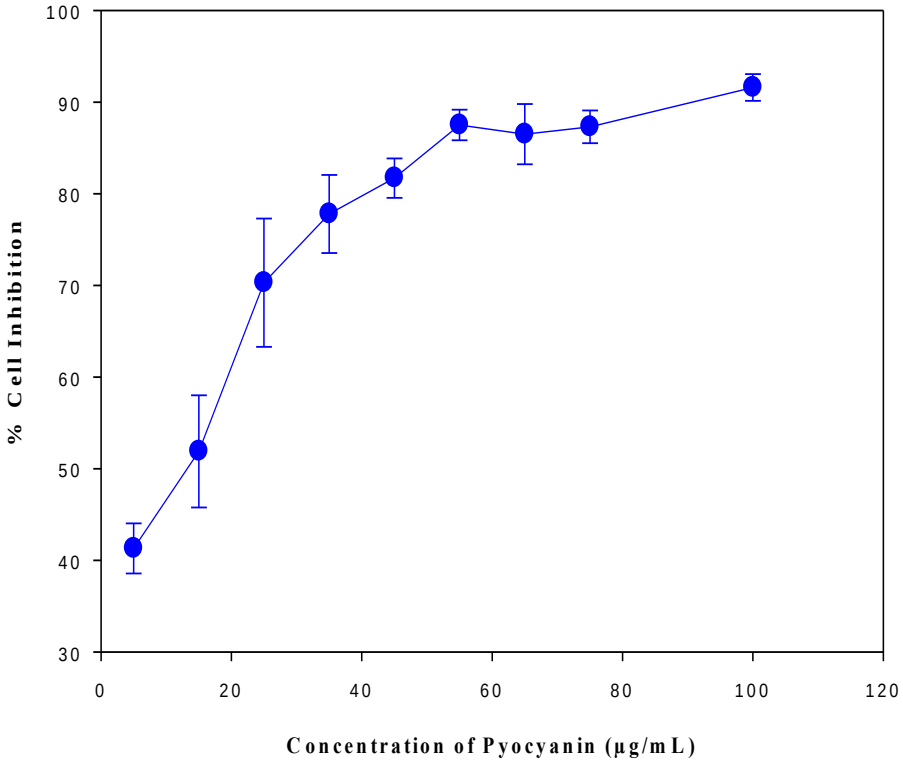

Fig.5. Anticancer effect of pyocyanin on Hep2C cell

Hassani and his colleagues (2012) also studied the cytotoxic effect of pyocyanin from mutant and wild type strains of P. aeruginosa on RD cells [32]. Zhao and his colleagues (2014) studied the proliferation of HepG2 cells in the presence of 10µg/ml pyocyanin. According to their study, a nine day treatment of pyocyanin led to 67% decline in HepG2 cell numbers compared to the control [33].Moayedi and his colleague (2018) reported the cytotoxic effects of pyocyanin on human pancreatic cancer cell line (Panc-1). According to their study, there was inhibition of 98.69 ±0.23 and 89.88±1.86% of 6mg/ml of pyocyanin extracted from clinical and soil isolates of P. aeruginosa, respectively [34].Other study investigated the anticancerous effect of pyocyanin on proliferation of L132 cell line (human lung epithelial cell line) extracted from P. aeruginosa CGR-3 was found to inhibit 61% of cells at 0.318µg/mL concentration [35]. It has also been reported that pyocyanin has negligible effect on normal cell line (L929) even at higher concentration which make it very useful in food productsas antioxidant and antimicrobial agents [31].The invertedmicroscopic view of Hep2C cancer cells after treatment with pyocyanin of P. aeruginosa HSS-6 has been shown in (Fig.6).

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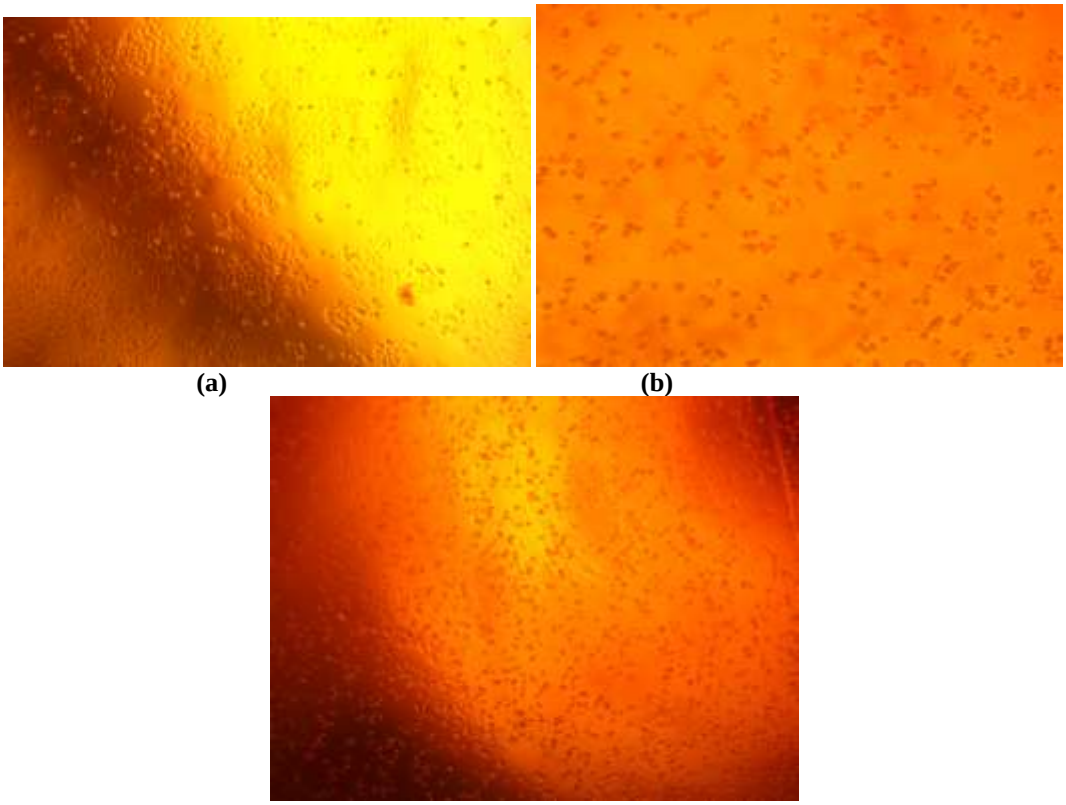

(a) **(b)**

(c)

Fig.6. Inverted microscopic view of cells after treatment with pyocyanin ofP. aeruginosa HSS-6 (a) Control (cells and methanol), (b) Cells after 24h treatment with pyocyanin and (c) Cells after 48h treatment with pyocyanin

IV. CONCLUSION

Pyocyanin is one of major virulence factor produced by P. aeruginosa. Besides its role in virulence, pyocyanin pigment has been also recognized for its various properties. Pyocyanin pigment is a natural product which can be used as antioxidant, antimicrobial and as anticancerous agent.Pyocyanin showed a very high cytotoxic effect on cancerous cell line Hep2Cleads to reduction in viability of these cells. Pyocyanin does not have any inhibitory or cytotoxic effect on normal L929 cells, which indicate its safety of use in human. For the introduction of pyocyanin as a potent anti-tumor biodrug, various invivo studies will be carried out using animal models.

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CONFLICT OF INTEREST

The authors have no financial conflicts of interest to declare.

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