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Research Article

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Identification of Bioactive Compounds, Characterization, Optimization and Cytotoxic Study of Pyocyanin against Colon Cancer Cell Line (HT-29)

Sivasankara Narayani S[1*], Saranya P[1], Lokesh P[2], Ravindran J[3]

[1]*Department of Microbiology, Ayya Nadar Janaki Ammal College, Tamil Nadu, India*
[2] *School of Biosciences and Technology, Vellore Institute of Technology,Vellore, Tamilnadu, India*
[3]*Department of Preclinical, University of Kuala Lumpur, Ipoh, Malaysia*

ABSTRACT

The aim of the study is to examine the cytotoxic effect of pyocyanin against the colon cancer cell line (HT-29). The organism was stained and found to be gram negative. The culture was further subjected to various biochemical characterization tests and it was confirmed as Pseudomonas sp. Pyocyanin extracted from pseudomonas sp. was analyzed using standard techniques. Out of seventeen isolates, seven isolates responded positive for starch and catalase test. Ten isolates were positive for MR-VP and hydrogen sulphide test whereas all isolates exhibited negative result for IAA production. Only one culture, showed positive for triple sugar iron agar test and for antibiotic sensitivity test. The culture is also subjected to optimization studies by analysing the effect of pH, media, light and dark conditions and solvent selection on pyocyanin production. Pyocyanin is also characterized using techniques such as UV-visible spectroscopy, FTIR and GC-MS. The sample was also tested for in vitro cytotoxicity against colon cancer cells and it showed that it destroys 50% of HT-29 cells at the concentration of 45.29 µg/ml. From the results of the study, it can be concluded that pyocyanin possesses potent cytotoxicity against colon cancer cells.

Keywords: Cytotoxicity; Pyocyanin; Colon cancer; Characterization; Optimization

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INTRODUCTION

Colorectal Cancer (CRC) is a common cancer with rising incidence and increased mortality rate all over the world. It includes malignant growth from the mucosa of the colon and rectum [1]. The pigment pyocyanin produced by *Pseudomonas. sp* has a high cytotoxic effect against Panc-1 cells and induces apoptosis/necrosis in these cells [2]. Many types of pigments are produced by *P. aerugionosa*. They are pyoverdin, pyorubin (red), pyocyanin and pyomelanin (brown) [3]. The colours provide the attractive look to the industrial products such as food, textiles, and pharmaceutical products.

Pigments from natural sources have obtained increased attention since past because of minimal toxicity and less side effects when compared with synthetic pigments. Many artificial synthetic colourants have been widely used in foodstuff, dye, and pharmaceutical industries that may cause hazardous effects. Natural pigments are also used as an alternative for synthetic pigments. Natural pigments are obtained from plants and microorganisms. Microbial pigments have many advantages of artificial and inorganic colours. The microbial pigment production is more

efficient and beneficial than the synthetic pigment production. Microbes can be grown easily in the culture media and they are independent from weather conditions [4].

The most characteristic feature of *Pseudomonas aeruginosa* is the production of soluble pyocyanin pigment: a water soluble blue green phenazine compound. From the beginning, pyocyanin had been used as a reversible dye with a redox potential similar to that of menaquinone. Pyocyanin has various pharmacological effects on prokaryotic cells. Its biological activity might be attributed to similarity in the chemical structure of isoalloxazine, flavoproteins, flavin mononucleotide and flavin adenine dinucleotide compounds [5]. In addition, the bioprocess and downstream processing of pyocyanin for aquaculture applications have been reported [3]. The phenazine-based pyocyanin pigment has a particular interest for its capability to generate reactive oxygen species (ROS). Tumour cells are susceptible to reactive oxygen species produced by pyocyanin since it interferes with topoisomerase I and II activities in eukaryotic cells [6]. Pyocyanin also has got application in biosensors as a redox compound for carrying out electron transfer between enzyme molecules and the electrode material. Therefore, the biosensors based on pyocyanin were also expected to have application in different fields such as agricultural, medicine and environment [7].

Pyocyanin can be used as electron shuttle in microbial fuel cells by enabling bacterial electron transfer towards the microbial fuel cells (MFC) anode [8]. It was observed that the addition of pyocyanin to MFC-containing *Brevibacillus sp.* PTH1 doubled the rate of electron transfer [9]. In addition, pyocyanin could conjugate to organic compounds and form new complexes which are used in organic light emitting devices (OLED). These devices were gaining importance due to their low voltage requirements, wide colour range, and light weight [10]. In this study, the pyocyanin was extracted from *Pseudomonas sp.* and then scrutinized using typical techniques and to examine the cytotoxicity against the colon cancer cell line (HT-29).

MATERIALS AND METHODS

Identification of Bacteria

The collected bacterial strain from the ANJAC Bio fertilizer centre was confirmed by morphological and biochemical characterization.

Extraction of Pyocyanin

Pseudomonas sp., was grown in the culture until pigment was produced. The culture broth was then centrifuged at 10000 rpm for 20 mins and then supernatant was collected. This supernatant was used for extraction. The extraction procedure was described by Ingledew and Campbell, with some modification. The chloroform at 1:2 ratio was added to the supernatant then it formed the two-separating phase, blue chloroform phase and aqueous phase. It was separated by using separating funnel. After separation the blue chloroform was concentrated with 0.1 N HCL under continuous stirring until the whole blue pigment was converted into acidic form (Red) and then the acidified layer pH was neutralized to 7 by using 0.4 M borate NaOH buffer. The absorbance of Pyocyanin was determined by measuring colorimetrically at 520 nm [11].

$$\text{Concentration of pyocyanin pigment } (\mu g/ml) = O.D_{520} \times 17.072$$

The concentrated pyocyanin pigment was evaporated at room temperature, then the needle crystals are formed.

Optimization

Effect of pH on pyocyanin production: The nutrient medium was prepared and initial pH of medium could that support the production was determined by adjusting the pH of the medium to various values such as (3, 4, 5, 6,7,8,9 and 10). After sterilization the culture was inoculated and incubated at 37^{o}C for 3-4 days. After incubation colour

change was observed and then extraction was carried out. The absorbance solution was measured calorimetrically at 520 nm. Concentration of pyocyanin pigment was calculated [12].

Effect of media on pyocyanin production: The nutrient medium and King's B medium was prepared. After sterilization the culture was inoculated and incubated at 37^{o}C for 3-4 days. After incubation colour change was observed and then extraction was carried out. The absorbance solution was measured calorimetrically at 520 nm. Concentration of pyocyanin pigment was calculated.

Selection of solvent for pigment production: The extraction of culture broth was standardized using solvents such as ethanol, methanol, acetone, chloroform, petroleum ether, diethyl ether, ethyl acetate etc.,

Effect of light and dark condition for pyocyanin production: The nutrient medium and King's B medium was prepared. After sterilization the culture was inoculated and incubated at 37^{o}C for 3-4 days in dark and light condition. After incubation colour change was observed.

Characterization pigments: The pigment was characterized through UV spectrophotometric, FT-IR (Fourier Transform-Infrared spectroscopy) and GC-MS (Gas chromatography-Mass spectrometry).

UV-Visible spectroscopy: The initial characterization of pyocyanin was carried out by using UV-visible spectroscopy, absorbance solution was scanned from 250 nm to 800 nm wavelengths. The chloroform was used as a blank [13].

FT-IR: FT-IR analysis is a technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. The technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to IR rays absorbs infrared energy at frequencies, which are characteristic to that molecule. During FT-IR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the IR rays at different frequencies is translated in to an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analysed and matched with known signatures of identified materials.

GC-MS analysis: The GC-MS analysis of the sample was carried out on Agilent chromatography GC (Model 7820A series) fitted with detector VL-MSD (Model 5977E). The carrier gas Helium was flow at constant 2 ml/min; the GC oven temperature started at 100^{o}C for 1 min then increased at 10°C/min to 270^{o}C held for 30 mins. 1.0 µl of the sample was automatically injected into the column (DB-5) with the injector temperature at 270°C. The injections were performed in split less mode [14].

Effect of Pyocyanin Against Colon Cancer Cells (HT-29)

MTT assay: Pyocyanin sample was tested for *in vitro* cytotoxicity, using HT-29 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1 × 10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24-48 hour at 37ºC. The wells were washed with sterile PBS and treated with various concentrations of the PS sample in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37ºC in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using graphPad Prism 6.0 software (USA) [15].

RESULTS

Collection of Organisms

The organism was fetched from the Biofertilizer production centre, Department of Microbiology, ANJAC, Sivakasi.

Identification of Organism

The organism was once again identified and confirmed using staining and biochemical test.

Gram staining

The organism was gram stained and confirmed to be a gram negative rod (Figure 1).

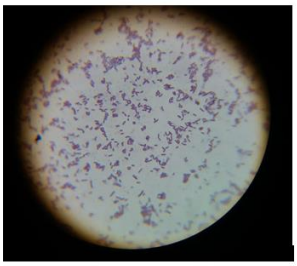

Figure 1: Gramstaining of the organism

Motility

The organism was found to be motile while seen underneath microscope.

Colony Morphology

Large, opaque, flat colonies with the irregular margins and green colour were detected in the nutrient agar plates.

Odour

Different fruity odour was emanated from the culture.

Biochemical Characterization

Biochemical characterization results were depicted (Figure 2 and Table 1). The culture used in this study was able to produce indole which exposed a cherry red colour, the MR VP broth also remains yellow, in which not as much amount of acid is produced from the fermentation of glucose by our culture. In VP test, acetyl methyl carbinol is not produced from the glucose fermentation so the broth remains yellow [15].

It also have the capability to utilise the citrate and it was confirmed by turning of green colour to blue colour. There was no characteristic change detected in methyl red test and voges proskauer test. It is also evidenced that as catalase positive it could produce the bubbles in hydrogen peroxide [16].In the starch agar, a zone is molded around the culture where it has streaked which proves that our culture was able to hydrolyse the starch. In skim milk agar also a zone is formed around the culture streaked which demonstrates that our culture could hydrolyse casein and also our culture could degrade the gelatin (Figure 2 and Table 1).

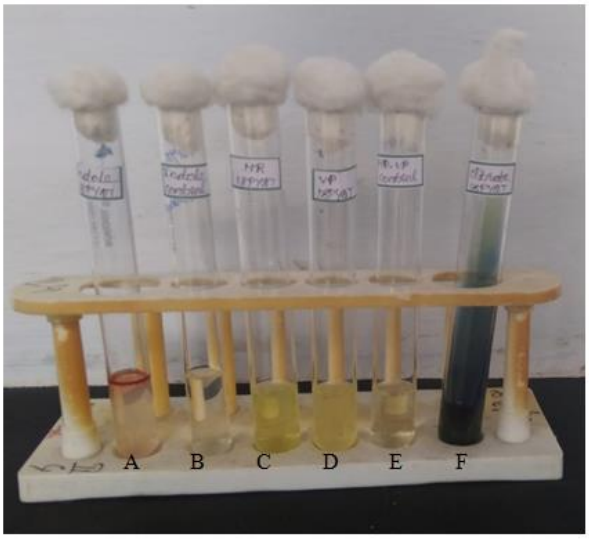

Figure 2: Biochemical characterization of organism Sample: A – Indole, B – indole control, C – Methyl red, D – Voges Proskauer, E – MRVP Control, F – Citrate

Table 1: Results of biochemical characterization of culture used in this study

S.no	Biochemical test	Characterization	Inference
1.	Indole	Cherry red ring	Positive
2.	Methyl red	No characteristic colour change	Negative
3.	Voges Proskauer	No characteristic colour change	Negative
4.	Citrate utilization	Blue colour change	Positive
5.	Catalase	Bubbles are observed	Positive
6.	Oxidase	Purple colour in the oxidase disc is observed	Positive
7.	Starch hydrolysis	Zone was formed around the organism streaked in starch agar	Positive
8.	Casein hydrolysis	Zone was formed around the organism in skim milk agar	Positive
9.	gel Liquification	Degrades the gelatine	Positive

Identification

From the above consequences, the organism has been recognized as *Pseudomonas sp.*

Pigment Extraction

The full-fledged culture of *Pseudomonas* was reserved in a centrifuge tubes and then they are exposed to centrifugation then supernatant was engaged, after that the pigment was unglued into two phases i.e., chloroform phase and aqueous phase (Figure 3). In the chloroform phase, the blue pigment was separated in which, water is insoluble and it is concentrated with 0.2 N HCL and then it turned into red colour and then the pH was neutralized by adding 0.4 M borate NaOH buffer until the colour transforms into blue (Figure 4). Then the pyocyanin was collected by vanishing of chloroform phase. Final product of pyocyanin was bluish in colour. Thus, the blue pigment was extracted [17].

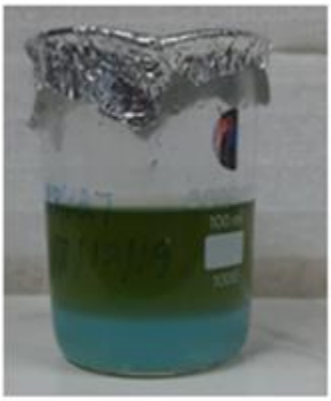

Figure 3: Phenol chloroform added supernatant

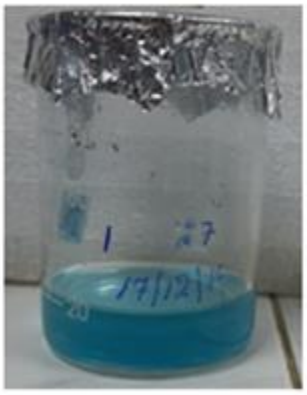

Figure 4: Bluish extract of pyocyanin

Total Pyocyanin Content

The total pyocyanin content was extracted and found to be 13.09 ± 0.39 µg/ml.

Selection Media for Pigment Production

The two diverse media (Nutrient broth and King's B broth) were designated for growth and pigment production. Among the two diverse media, Nutrient broth produced 13.39 µg/ml and King's B broth produced 0.56 µg/ml (Table 2). From this result, it was observed that nutrient broth showed the enlarged amount of pigment production and showed additional growth rate at 520 nm when related to the King's B media [18,19]. Hence, nutrient broth can be preferred for pyocyanin production. After 3-4 days incubation, the culture broth colour was altered into green colour thus pigment production was detected and extraction process was carried out (Figures 5-7 and Table 2).

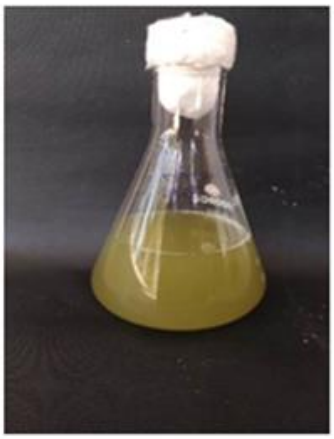

Figure 5: *Pseudomonas* in nutrient broth

Figure 6: *Pseudomonas* in kings B broth

Table 2: Pyocyanin yield in nutrient broth *Vs.* pyocyanin in Kings B broth

Trial	Pyocyanin in nutrient broth (µg/ml)	Pyocyanin in Kings B broth (µg/ml)
1	3.19	0.56
2	3.89	0.5
3	3.09	0.59

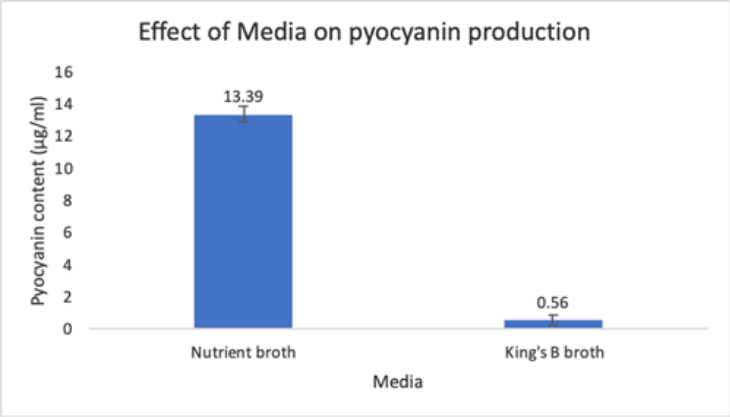

Figure 7: Pyocyanin in nutrient broth vs. Pyocyanin in Kings B broth

From this media optimization step, it has been concluded that nutrient broth can produce more pyocyanin pigment when compared to king's B medium [20].

Effect of pH on Growth

When compared to the acidic and alkaline pH, neutral pH was suitable for the growth of organism. The pyocyanin yield was found maximum at the pH of 7 which was 13.45 ± 0.58 µg/ml (Figure 8 and Table 3). Thus, the pH also plays a vital role in the growth of the microorganism.

Table 3: pH Vs. Pyocyanin in nutrient broth

pH	Pyocyanin (µg/ml)
5	2.08
6	11.07
7	13.45
8	3.619
9	7.56

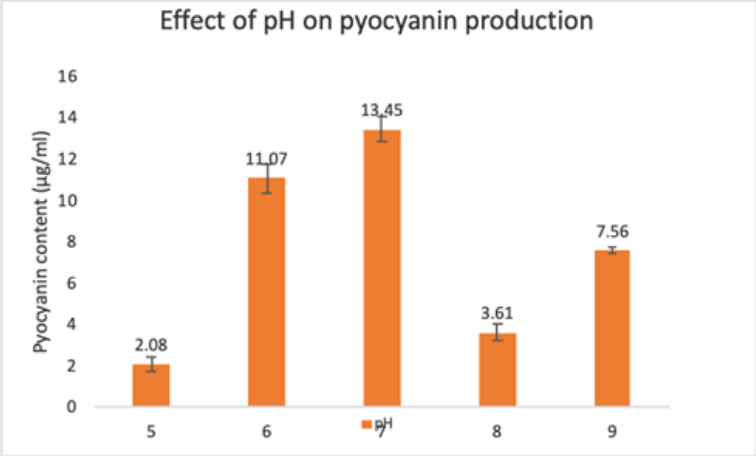

Figure 8: pH Vs. Pyocyanin in Nutrient broth

Effect of Light on the Growth

The growth of the bacteria also depends up on the light. When the culture is kept under dark condition the growth rate of culture was much faster than in light condition. Thus, light plays a vigorous role for the progression and pigment production of the organism [21].

Selection of Suitable Solvent for Pigment Production

Among the solvents tested such as ethanol, acetone, methanol, chloroform and ethyl acetate, the chloroform was an ideal solvent. Chloroform was used for extracting the maximum amount of water insoluble membrane bound pigment.

From the optimization studies, it was resolved that more amount of pigment was produced when using nutrient broth with pH 7. Moreover, dark condition is favourable for maximum production and chloroform was a best solvent for extracting more pigments [22].

Characterization of Pyocyanin

Visible absorption spectrometry of pyocyanin: The UV-Visible Spectra of pyocyanin pigment in crude form exhibited the peak at 761.50 nm, which indicates the presence of pyocyanin (Figure 9 and Table 4).

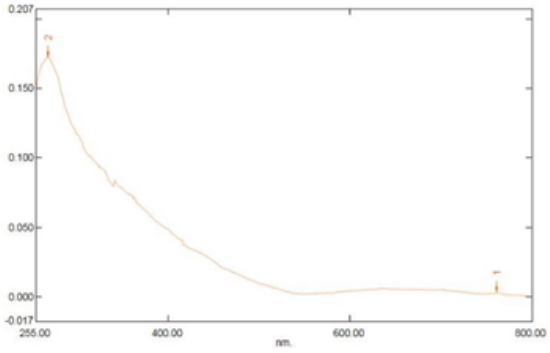

Figure 9: UV-Visible spectroscopy of pyocyanin

Table 4: UV-Visible absorption spectrometry

S.no	Wavelength (nm)	Absorption
1	268	0.003
2	761.5	0.173

FT-IR Analysis of Pyocyanin

The pyocyanin crude form was processed for recognizing the functional group through Fourier Transform Infrared Spectroscopy (FTIR). The results showed prominent bands of absorbance for pyocyanin at around the peak area of 442 cm^{-1} to 2967.28 cm^{-1} (Figure 10 and Table 5). The peaks denote presence of alkyl halide, alkene, aromatic, ester and nitro compounds [23].

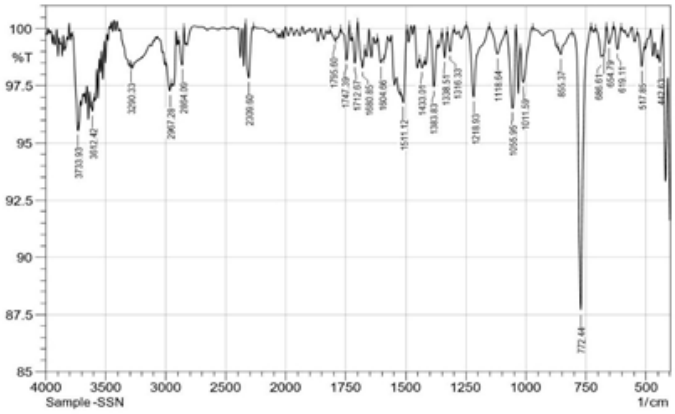

Figure 10: FTIR spectrum of pyocyanin

Table 5: Functional groups of the pycocyanin revealed by FTIR

S.no	Peak value (cm^{-1})	Bond	Class of compounds
1.	442.63	C-I stretch	Alkyl and Aryl Halides
2.	517.85	C-I stretch	Alkyl and Aryl Halides
3.	619.11	C-Cl stretch	Alkyl and Aryl Halides
4.	654.79	C-Cl stretch	Alkyl and Aryl Halides
5.	686.61	C-Cl stretch	Alkyl and Aryl Halides
6.	772.44	C-Cl stretch	Alkyl and Aryl Halides
7.	855.37	C-Cl stretch	Alkyl and Aryl Halides
8.	1011.59	C-F stretch	Alkyl and Aryl Halides
9.	1055.95	C-F stretch	Alkyl and Aryl Halides
10.	1118.64	C-F stretch	Alkyl and Aryl Halides
11.	1218.93	C-F stretch	Alkyl and Aryl Halides
12.	1316.33	NO_2 stretch	Nitro Compounds
13.	1338.51	NO_2 stretch	Nitro Compounds
14.	1383.83	NO_2 stretch	Nitro Compounds
15.	1433.01	NO_2 stretch	Nitro Compounds
16.	1511.12	NO_2 stretch	Nitro Compounds
17.	1604.66	C=C stretch	Aromatic Compounds
18.	1680.85	C=C stretch	Alkenes
19.	1712.67	C=O stretch	Aldehydes
20.	1747.39	C=O stretch	Esters
21.	1795.6	C=O stretch	Acid Chlorides

22.	2864.09	C-H stretch	Aldehydes
23.	2967.28	C-H stretch	Alkanes

GC-MS Analysis

gas Chromatography Mass spectrum (GC-MS) analysis showed the presence of Phenol,2,4 bis(1,1 dimethylethyl),n-Hexadecanoic acid, n-Hexadecanoic acid,9-Octadecenoic acid, Oleic Acid, 2-Heptadecenal,Oleic Acid,Cyclopentadecanone,2-hydroxy-Butyl 9-tetradecenoate, Cyclopentadecanone, 2-hydroxy-2,3-Dihydroxypropyl elaidate,2,3-Dihydroxypropyl elaidate, Erucic acid, Difluoro(methylamino)phosphines..., 1 Butane, 2-phenyl-3-(trimethylsil...,Cyclotetradecane, Hexahydropyridine, and 1-methyl-4-[4..., Tris (tertbutyldimethylsilyloxy) (Figure 11 and Table 6) [24].

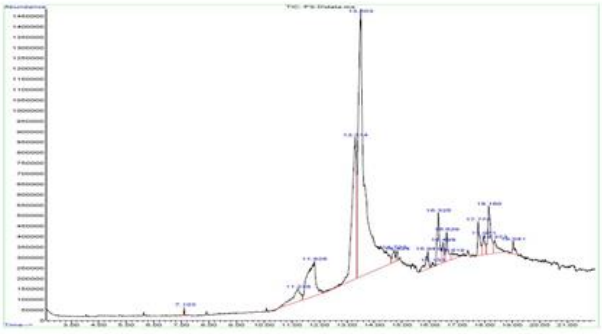

Figure 11: GC MS analysis of pyocyanin

Table 6: Compound identification in GC MS

S. No.	R. T. (mins)	Name of the compound
1.	7.108	Phenol,2,4 bis(1,1 dimethylethyl)
2.	11.24	n-Hexadecanoic acid
3.	11.826	n-Hexadecanoic acid
4.	13.31	9-Octadecenoic acid
5.	13.5	Oleic Acid
6.	14.719	2-Heptadecenal
7.	14.823	Oleic Acid

8.	15.939	Cyclopentadecanone,2-hydroxy-
9.	16.138	Butyl 9-tetradecenoate
10.	16.327	Cyclopentadecanone, 2-hydroxy-
11.	16.497	2,3-Dihydroxypropyl elaidate
12.	16.629	2,3-Dihydroxypropyl elaidate
13.	16.818	Erucic acid
14.	17.773	Difluoro(methylamino)phosphines...
15.	17.972	1 Butane, 2-phenyl-3-(trimethylsil.
16.	18.161	Cyclotetradecane
17.	18.369	Hexahydropyridine, 1-methyl-4-[4...
18.	19.04	Tris(tert-butyldimethylsilyloxy)...

As pyocyanin comprises of Hexadecanoic acid it can be used in cosmetics as the colouring substances. Oleic acid presence was also indicated by GCMS which is used in soaps so it can also be used for soap preparation. The Erucic acid which is existing in paint is also present in our compound, so we can formulate paints and they can also be used as a bio fuel. Thus, pyocyanin can be used for variety of purposes based on the existence of compounds.

Effect of Pyocyanin Against Colon Cancer Cells (HT-29 cells)

The pyocyanin sample was tested for *in vitro* cytotoxicity, by using HT-29 cells **(**colon cancer cells) and it was found to be that the pyocyanin demolished the proliferation of HT-29 cells, thus pyocyanin possess cytotoxic activity. Related to the control cells, less amount of growth was detected on the pyocyanin treated cells. Percentage of cell viability was decreased when the concentration has been enlarged (Figures 12 and 13). IC50 Rate of our compound is is 45.29 μg/ml. IC50 rate was calculated by graph pad prism software (Table 7) [25].

Table 7: Log vs. normalised response variable slope

log(inhibitor) vs. normalized response-Variable slope		
Best-fit values		
LogIC50		1.656
HillSlope		-0.9224
IC50		45.29

95% CI (profile likelihood)		
LogIC50		1.596 to 1.715
HillSlope		-1.021 to -0.8342
IC50		39.47 to 51.86
goodness of Fit		
Degrees of Freedom		28
R squared		0.9781
Sum of Squares		744.5
Sy.x		5.157
Number of points		
# of X values	3	30
# Y values analyzed	3	30

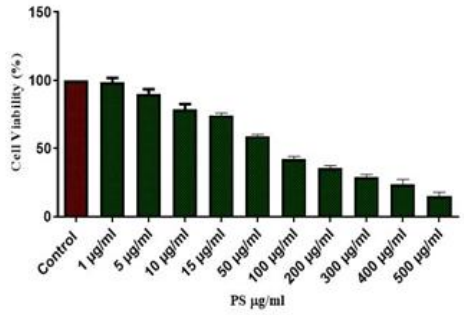

Figure 12: Cytotoxic effect of pyocyanin against HT-29 cells (colon cancer cells). The cell number (% of control) was determined by MTT assay. Each value represents the mean ± standard deviation of three determinations

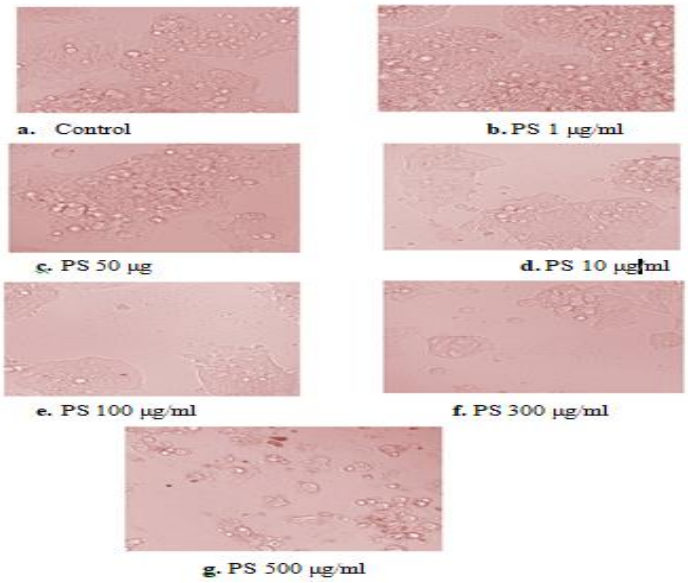

Figure 13: Control cells and Pyocyanin treated HT-29 cell in different concentrations

DISCUSSION

In the current study, the culture bought from the ANJAC Biofertilizer centre, Department of Microbiology was examined. The organism was gram stained and found to be as gram negative rod. The organism were found to be motile while seen under microscope. Large, opaque, flat colonies with the irregular margins, with green colour were observed in the nutrient agar plates. Different fruity odour was emitted by the culture. The culture was confirmed as *Pseudomonas sp.* using biochemical characterization which includes Indole production test, citrate utilisation test, catalase, oxidase test, starch hydrolyse test, casein hydrolyse test, MR VP Test and gel liquification test.

In the contemporary study, culture was able to produce indole which showed the cherry red colour, the MR VP broth also remains yellow, in which only the less amount of acid is produced from the fermentation of glucose in the culture and also in VP test acetyl methyl carbinol is not produced from the glucose fermentation so the broth remains yellow. It also have the ability to utilise the citrate and it was confirmed by turning of green colour to blue colour. There is no characteristic change was observed in methyl red test and voges proskauer and it also proved that as catalase positive as it could produce the bubbles in hydrogen peroxide, in the starch agar also a zone is formed around the culture where it has streaked which says that our culture has not only have the ability to hydrolyse the starch but also in skim milk agar also zone formation around the culture streaked was observed which proves that our culture could hydrolyse caesin and our culture could degrade the gelatin. Similarly, Ningthoujam [26] isolated bacterial strain, DN1, degrading p-nitrophenol (PNP) from garden soil by selective enrichment in M63 medium. Repeated sub culturing in Nutrient Agar (NA) plates, NA slants and Basal Salts Medium (BSM) containing PNP (BSM+PNP) was done to isolate pure colonies. The organism is gram negative, aerobic, catalase positive, oxidase positive and rod shaped with mostly single arrangement. It showed bluish green pigmentation on various specialized media such as *Pseudomonas* P medium, *Pseudomonas* F medium, Modified F medium, *Pseudomonas* Isolation Agar (PIA) and Hi Fluoro *Pseudomonas* Agar. DN1 gave positive results with motility, citrate utilization, urease, Nitrate

Reduction (NR) and gelatin liquefaction tests but negative results were observed with Methyl Red (MR), Voges Proskauer (VP) and indole tests. It hydrolyses casein and lipase positive but starch hydrolysis is negative.

Acid production from carbohydrates also was also tested (glucose and lactose) and showed negative result. It can grow at 42°C but not at 4°C and tolerates <5% NaCl concentration. Optimum pH for PNP degradation was found to be 7.0. Among several media tested such as M9, M63 and BSM, BSM was found to be the optimum medium for biodegradation of PNP. DN1 could degrade upto 100 mg L^{-1} PNP using the xenobiotic as sole carbon or carbon and nitrogen sources. On the basis of gross morphological, micromorphological, physiological and biochemical tests DN1 was definitively identified as *Pseudomonas aeruginosa* strain DN1. Based on literature evidences, this is the first report of a *Pseudomonas aeruginosa* strain that is able to degrade p-nitrophenol (PNP).

Similarly, Shivangi Singh [13] isolated seventeen isolates from the rhizosphere of different vegetable crops and all the seventeen isolates are suspected as *Pseudomonas sp*. All isolates were grown on nutrient agar medium (NA) and incubated at 28 ± 2 ºC to study various morphological characters. After 24 hours of incubation growth of culture was observed**.** Some of the isolates showed light green, circular, shining, slimy, irregular in morphology and some were yellowish. The biochemical characterization of culture was done. Catalase test, starch hydrolysis, MR-VP test, IAA Production, TSIA test, hydrogen sulphide and antibiotic sensitivity test was conducted. Out of seventeen isolates seven isolates showed positive for catalase, and starch hydrolysis test whereas negative for MR-VP and hydrogen sulphide. Ten isolates showed positive for MR-VP and hydrogen sulphide whereas negative for catalase and starch hydrolysis. All isolates showed negative result for IAA production. Out of seventeen, only one culture showed positive for triple sugar iron agar test for Antibiotic Sensitivity test.

All isolates were sensitive against antibiotics such as Ciprofloxacin, gentamicin and Ceftriaxone. Formation of zone of inhibition was observed around the antibiotic used. The largest zone of inhibition was observed in ciprofloxacin whereas smallest zone of inhibition was observed in ceftriaxone. On the basis of morphological and biochemical characterization seven isolates of *Pseudomonas sp*. were selected for the further evaluation of their growth promoting traits in brinjal. In the study they have used the isolates to inoculate the brinjal plant by seedling dip method and analyse various parameters such as root length, shoot length, fresh root and shoot weight and dry root and shoot weight. All the seven isolates significantly showed plant growth promoting activity by increasing root and shoot length, root fresh and dry weight and shoot fresh and dry weight as compared to the control but among the treatments one particular isolate showed the highest root length (18.52 g ± 0.31), shoot length (45.03 g ± 0.65), root fresh weight (3.50 g ± 0.36), root dry weight (1.58 g ± 0.11), shoot fresh weight (45.73 ± 0.34) and shoot dry weight (8.64 g ± 0.28) followed by another culture whereas minimum root length (11.73 g ± 0.34), shoot length (36.06 ± 0.67), root fresh weight (1.71 g ± 0.12), root dry weight (0.78 g ± 0.03), shoot fresh weight (35.73 g ± 0.34) and shoot dry weight (5.69 g ± 0.04) was observed in same genus of different culture.

Correspondingly, Savitha De Britto [23] studied seven bacterial strains isolated from agricultural waste soil and screened for pyocyanin production. Among the seven isolated bacterial culture KU-BIO2 produce more amount of pyocyanin. This culture was identified as *P. aeruginosa* by using 16S rRNA sequencing. The sequence of particular culture showed high similarity with various other isolates of *P. aeruginosa.* It was already reported for the beneficial application such as bioremediation or oil degradation and production of biosurfactant.

Likewise, Abdul-Hussein [5] examined the *Pseudomonas aeruginosa* culture isolated from the soil sample of Basra city located in India. Correspondingly, Sudhakar [25] isolated *P. aeruginosa* SU1 from soil sample collected from Sathyabama University. Likewise, Moayedi [17] used total of 10 isolates. The specimens were isolated from wound and one isolated from urinary tract infection. The cultures were donated by the lab of Shaheed Motahari Burns Hospital, Tehran, Iran. In addition to that, 10 *P. aeruginosa* strains isolated from ten agricultural and oil contaminated samples (taken from regions with the lowest risk of hospital specimen containment). Similarly, Shivangi Singh [15] collected the soil samples from the rhizosphere (approximately 15 cm deep) of different vegetable crops (brinjal, tomato, potato, pea and ladyfinger) from five locations like Chatha, R.S Pura, Vijaypur, Ramgarh and Supwal of Jammu region. The samples were mixed and air dried for further isolation of bacteria.

Likewise, Savitha De Britto [7] collected the soil samples from the corn, soybean, sweet potato, watermelon seeds and groundnut and isolated organism.

Correspondingly, Feghali and Nawas (2018) used *P. aeruginosa* isolates in his study. He collected from the clinic, courteously provided by the clinical microbiology laboratory of the Lebanese American University Medical Center-Risk Hospital (LAUMC-RH).

In the present study, pyocyanin extracted from *Pseudomonas sp.* was 13.09 ± 0.39 µg/ml. Correspondingly, Naziya [13] worked on the pyocyanin pigment. In their study the maximum amount of pigment obtained was 3.548 ± 2.2 µg/ml.

In the present study, nutrient broth yielded more amount of pyocyanin of about 13.39 µg/ml than King's B medium which is 0.56 µg/ml. Likewise, Savitha DeBritto [27] used the King's A broth amended with soya as a nutrient to get maximum pyocyanin followed by the nutrient broth amended by sweet potato. The maximum production in nutrient broth was observed in the sweet potato broth with a concentration of 1.702 µg ml^{-1} and the minimum production was observed in the soya bean broth with a concentration 0.1702 µg ml^{-1}. The maximum production in King's A broth was observed in the soya bean broth with a concentration 2.560 µg ml^{-1}. The minimum production was observed in the watermelon broth with a concentration 0.5106 µg ml^{-1}.

Correspondingly, Abdul-Hussein [21] used different media for enhancement of pigment production for *Pseudomonas* (green). They found out that mineral salt medium and peptone water were much better support medium for producing of blue-green pigment. They also used mineral mannitol, including broth and broth malt with cooked meat extract. It showed less improvement at same temperature and period of incubation. The other bacterial pigment was cultured on nutrient agar (NA) and peptone glycerol casein (PGC) gave a good recorded result. In their findings also nutrient agar supplement induce *Pseudomonas* to produce more amount of pyocyanin.

Likewise, Barakat [19] isolated *P. aeruginosa* which produce blue green pigment, using King's broth medium. Pigment production was indicated by change in colour of King's broth to a blue green. The appearance of pigment starts after 24 h of incubation and increased with time until 72 h. Pyocyanin quantification assay is based on the pyocyanin's measuring colour at 520 nm in acidic phase and using 0.2 M HCl used as a blank. Absorbance was multiplied by 17.072 the extinction coefficient and the yield were expressed in micrograms (µg) of pyocyanin produced per milliliters (ml) of solution (µg/ml). Pyocyanin concentration obtained after 72 h of incubation was 26 µg/ml. In the study they got maximum amount of pyocyanin on basal King's B medium (25.5 µg/ml) when compared to nutrient broth (5.28 µg/ml) tryptone water (9.55 µg/ml) and peptone water (11.74 µg/ml) broth. Extracted pyocyanin showed inhibition of bioactivity at 20.8 mm against gram $^{-}$ve and 18.3 mm against gram $^{+}$ve and fungal strains that recorded using King B medium.

In the present study, when the culture is subjected to the pH 7 culture gives the more pyocyanin as 13.5 µg/ml rather than the other acidic and alkaline pH. Thus, pH plays a vital role for the growth of the organism. Correspondingly, Naziya [23] have the maximum amount of the pigment produced as 3.548 ± 2.2 µg/ml in pH 7 under the dark condition. Likewise, Barakat [6] worked on different pH and temperature. It was experimentally tested for bioactivities. Activities and yield of pyocyanin was increased with the decrease of pH 7-6. They also analysed favourable temperature to yield maximum pigment which was found to be 28ºC. Lower activities were recorded for pH 9 at 40^{o}C. On condition of pH 7 maximum yield was obtained at 75.15 µg/ml. The work revealed that 28ºC was favourable temperature for the maximum production of pyocyanin at the concentration of 76.11 µg/ml.

In the present study, when the culture was kept in dark place the culture grows fast rather than culture kept in the light condition. Thus, light play a vital role for the growth of the organism.

Likewise, Barakat [17] observed the growth of the microorganisms in the dark room. He got best amount of culture growth in dark condition. Correspondingly, Naziya [18] concluded that the designed selective wavelength lamp model for light experiments is a more suitable technique than the paper wrapping method. The model was also advantageous over paper wrapping method. As once designed, it is easy to handle and also gives maximum pigment yield production in darkness.

In the present study, UV-Visible spectra of pycocyanin was identified by UV-Visible Spectroscopy. The UV-Visible Spectra of reaction mixture showed the peak at 761.50 nm, which indicates the presence of pyocyanin. Likewise, Mahmood [13] analysed the maximum absorbance using UV spectroscopy. The absorbance of his extracted solution showed maximum peak at 512 nm. This peak indicates the presence of pyocyanin compound. Correspondingly, Abdul-Hussein [15] also extracted bluish colour of pyocyanin which converted to red with HCl, and spectrophotometric analysis of pyocyanin showed a peak at 520 nm. Likewise, Savitha De Britto [6] checked the absorbance peaks in five different nutrient supplement broths. They were 299 and 260 nm in the corn, 256 nm in the soya bean, 222 nm in the sweet potato, 229 nm in the watermelon seed and 235 nm in the groundnut broths respectively.

In our present study, the pyocyanin was analysed for identifying the functional group through FTIR. The results showed prominent bands of absorbance at around the peak area is 442 cm^{-1} to 2967.28 cm^{-1}. The peaks denote the presence of alkyl halide, alkene, aromatic, ester and nitro compounds. Similarly, Savitha De Britto [8] compared standard pyocyanin FTIR data with extracted pyocyanin. Standard FTIR data revealed the presence of an Alkenyl C=C stretch at 1604, a C=O-H stretch Aldehyde at 1405 and a C-C-C stretch, with a medium stretch of 1169. Corresponding to the standards, the test samples exhibited similar molecular functional groups at 1604, 1405 and 1169. The standard pyocyanin peak was eluted at 1.05 mins with an m/z value of 211.05 and production of 197.04. Similarly, the extracted samples peaks were eluted at 1.08 and 1.07 for sweet potato and soya bean respectively.

In our study, GC-MS analysis showed the presence of Phenol, 2,4 bis (1,1 dimethylethyl), n-Hexadecanoic acid, n-Hexadecanoic acid, 9-Octadecenoic acid, Oleic Acid, 2-Heptadecenal, Oleic Acid, Cyclopentadecanone, 2-hydroxy-Butyl 9-tetradecenoate, Cyclopentadecanone, 2-hydroxy-2,3-Dihydroxypropyl elaidate, 2,3-Dihydroxypropyl elaidate, Erucic acid, Difluoro(methylamino)phosphines..., 1 Butane, 2-phenyl-3-(trimethylsil..., Cyclotetradecane, Hexahydropyridine, 1-methyl-4-[4..., Tris(tertbutyldimethylsilyloxy) compounds. Correspondingly Abdul-Hussein [9] demonstrated that, GC-mass spectral analysis of pyocyanin extracted from *Pseudomonas aeruginosa*. The study revealed the chemical structure of pigment as hemi pyocyanine. Similarly, Barakat [4] also identified by mass spectrum library which showed the presence of 1-hydroxy-N-methyl phenazine with intense molecular ion peak at 196 m/z.

In our cytotoxic study, the pyocyanin sample was tested for *in vitro* cytotoxicity, against colon cancer cells (HT-29) and it is found to be that the pyocyanin destroys the 50 percentage of the HT-29 cells at the concentration of 45.29 µg/ml. From this study, it showed thus pyocyanin possess the cytotoxic activity against colon cancer cells.

Correspondingly, Vipin [27] explained that the Pyocyanin exhibited toxicity against glioblastoma cells**.** Pyocyanin exhibited 33.41%, 36.89% and 66.34% cytotoxicity at 50 µg/ml, 100 µg/ml and 200 µg/ml concentrations respectively. No significant difference was observed in cytotoxicity between 50 µg/ml and 100 µg/ml concentrations of pyocyanin.

CONCLUSION

The results of the study showed that pyocyanin possess potent cytotoxic activity against colon cancer cells (HT-29). Understanding the whole mechanistic action of pyocyanin might prove its efficacy on treating all types of cancer. In addition to this, it can also be beneficial in formulating soaps, paints, food colorants and biofuel which can be used in day-todays life.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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