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Research Article

SCREENING, CHARACTERIZATION, ANTIMICROBIAL AND CYTOTOXIC POTENTIAL OF PYOCYANIN PRODUCING *PSEUDOMONAS* SPP. ISOLATED FROM THE SPONGE

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Abstract- Pyocyanin is a blue pigment, redox active secondary metabolites produced by sponge associated bacteria. The aim of this study was to investigate the bioactive potential of pyocyanin from *Pseudomonas* sp. a sponge associated bacterium. A total of 23 pigmented bacterial strains were isolated from a sponge *Callyspongia* sp. All the colonies were screened for their antimicrobial activity against human bacterial and fungal pathogens by cross streaking method. For antioxidant activity, DPPH method was adopted, whereas the cytotoxicity property was checked by MTT assay. The pigment production medium was also optimized with different nutritional sources. Out of 23 strains tested, only 3 strains were chosen based on the good activity and finally one strain (PA1) was used for the study. This strain was tentatively identified as Pseudomonas sp., through biochemical characterization. The results revealed that the pigment pyocyanin exhibited high activity than commercial antibiotics. It was found that Klebsiella pneumoniae was inhibited high with the diameter of 20 mm followed by *S. aureus* (22 mm), *Salmonella typhi* (26 mm), *E.coli* (20 mm), and *Bacillus subtilis* (19 mm). It was also found that the pyocyanin inhibited the growth of *Candida krusei* (10 mm), *Aspergillus flavus* (12 mm) and *Candida albicans* (16 mm). The minimum inhibitory concentration was observed between 10 and 20 µg/ml. The antioxidant activity was observed at lower concentration of 0.2 µg/ml. In MTT assay on viability of HepG2 cells, the toxicity was found at 100 µg/ml. The active compound was identified and the results showed that the presence of alkali and most of the functional group was OH-C=N and CH_3.This confirms the presence of pyocyanin. Thus, this pigment pyocyanin may be a potential drug candidate for future studies.

Keywords- *Pyocyanin, antimicrobial activity, cytotoxicity, Minimum inhibitory concentration (MIC), MTT assay*

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Introduction

The marine microbial active metabolites are the new source of most antimicrobials used for the treatment of infections and having wide range of biological and pharmacological applications [1]. Due to the overuse of antibiotics, bacteria was developed antibiotic resistance and gene mutation, making existing drugs shown the less effective for the human health. This is forced the researchers to find a new replacement compound for treating infections with eco- friendly. From that the marine sponges are the reservoir of dense and distinctive microbial communities. They comprise with 40% of microbial load in their body canal system [2]. These organisms play a significant role in their interactions, including with nitrification, metabolic activity, UV protection, and secondary metabolite production. However, the precise nature of the interaction between sponges and microbes is unknown [2-3]. The marine microbial communities have the robust potent activity than that of terrestrial microbes, they are totally different from one other, because of their environmental conditions. The marine microorganisms living in the sea should be able to survive with low nutrition, high salinity and high pressure. That is why they are largely gram negative rod shaped bacteria and their membrane evolved to adopt the aquatic environment. These characters are highlighted, and differences between the marine and terrestrial microbes [4]. However, marine bacteria attract the researchers because they are potentially produces compounds with biological properties. The number of bacterial species produces the pigments are vital to cellular physiology and survival [5]. Many of these have antibacterial, anticancer and immunomodulatory activities. The marine *Steptomyces* sp., *Bacillus* sp., Vibrio sp., and *Pseudomonas* sp., also produces the pigments and they have a biological function with different nature [6]. Thus, this study mainly focuses on the *Pseudomonas* sp. The *Pseudomonas* sp., is a gram negative rod shaped bacterium which has shown the broad antagonistic activity against both bacterial and fungal pathogens by the production of secondary metabolites, the metabolites are appeared as pigmentation, it may be varied by the presence of colour and has different biological activity, for example the yellow pigment known as pyoverdine and the bluish known as pyocyanin, these are called as phenazine compounds [7-8]. The pyocyanin is different from the pyoverdine because the pyocyanin is redox active metabolites, it is derived from the phenazine -1- carboxylic compound, the PCA and Chrismic acid which is sequentially modified and creates the phenazines derivatives then it was converted as PCA to pyocyanin [8]. Pyocyanin is a water soluble molecule, weak acidic, aromatic agent in nature. The structure contains nitrogen backbone, phenolic groups and involved in a variety of biological activities including gene expression, maintain fitness to bacterial cell and biofilm formation, and able to regulate the signal transduction pathway, ROS, modifying the structure of protein and leads to the cytotoxic effects of cancer cells [9-10]. It can able to survive an extreme environment and easily diffuses the membrane to inhibit the bacterial, fungal and mammalian cells. Based on the uniqueness the present study was focused on the pyocyanin and their biological activity.

Material and Methods

Pathogens used in this Study

Clinical pathogens including bacteria and fungi namely *Bacillus subtilis*, *Salmonella Typhi*, *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Candida albicans*, *Candida krusei*, and *Aspergillus flavus* were used in this study. All the bacterial pathogens were kindly provided by Dr.T.Ramamurthy, Dy.

Director (Senior), National Institute of Cholera and Enteric Disease, Kolkata.

Isolation of sponge associated bacteria

The marine sponge sample was collected from the Tuticorin coast, Tamil Nadu, India, transferred to a sterile polythene bags and transported to the laboratory at 4ºC for the isolation of sponge associated microorganisms. The sample was brought into room temperature upon arrival to the laboratory and washed with sterile water to remove the debris from the sample, then the sponges were chopped into tiny pieces and one gram of sample was transferred into 99 ml of sterile distilled water, shaken well for 30 minutes, finally the homogenized sample was serially diluted up to 10-9. Then the diluted to spread plated on the nutrient agar plates and incubated for 3 days at room temperature. After incubation, the morphologically distinct colonies were selected and obtain a pure culture. The pure and pigmented isolates were identified through microscopic and biochemical characterization followed by the growth response in different ranges of pH, and salt concentration [1].

Primary screening for the isolates:

All the pure isolates were primarily screened by cross streaking method to find out the antimicrobial activity of the selected human bacterial pathogens. In this study, *Bacillus subtilis*, *E. coli*, *Salmonella Typhi*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were used as test pathogens. The pigment producing isolate was streaked perpendicularly across the pathogens in nutrient agar (NA) plates and incubated at 37ºC for 24h. The highly active isolates were selected for further study [1].

Identification of Sponge associated bacteria:

All the isolates which exhibited antimicrobial activity were identified through microscopic and biochemical analysis described in the Bergeys Manual of determinative bacteriology 2nd Edition, 2001-2012.

Fermentation for pigment production:

Based on the screening the selected isolate was cultured for pyocyanin production. The nutrient broth (NB) was supplemented with glycerol (1 ml), $MgCl_2$ (0.14g), asparagine (1%) for the production of pigment. A single colony from the nutrient agar plate was inoculated into the 10 ml of nutrient broth and incubated at room temperature for 24h. After incubation, the bacterial culture was transferred into the 500 ml Erlenmeyer flask containing 200 ml of sterile nutrient broth and incubated for 72h at room temperature. After incubation, the culture was filtered through Whattman filter paper no: 1, then the cells was harvested at 10,000 RPM for 20 minutes and the cell free supernatant was separated [16].

Pyocyanin extraction

The solvent extraction was used for the first step in the separation process. The cell free supernatant was extracted with chloroform and shaken vigorously for 30 minutes. Two distinct layers were separated where one was the pigment and remaining material of culture. The extraction was repeated twice, then the pigmented chloroform layer was collected and sequentially extracted with 2 ml of 0.2 N HCl[11].

Antimicrobial activity of pyocyanin

The bioactivity of pyocyanin was analyzed by disk diffusion method with Muller Hinton agar (MHA) plates. Nalidixic acid (NA 30), Rifambicin (RIF 5), Co-trimaxazol (COT-25) antibiotics were purchased from Hi-media were used as control. Overnight grown test pathogens were plated as lawn culture and left it for 60 seconds. After that extracted pyocyanin was impregnated in the disks at different concentration (10-50 µg/ml) and placed onto the plates and incubated the plates at 37ºC for 48 h. After incubation the inhibitory activity was observed and recorded.

Minimum Inhibitory Concentration (MIC)

The pyocyanin was screened further for MIC. The MIC was performed in 96-well microtitre plates. The extracted PCN was taken at different concentrations, ranged between 10-100 µg/ml. The selected bacterial pathogens were grown on the nutrient broth (NB) and fungal pathogens in sabouraud dextrose broth (SDB) respectively. The cells were seeded into the plate; NB and SDB maintained as a control, the PCN was taken and seeded into the each wells, and incubated the plates at 37ºC for 48 h. After incubation, the plates were read for the absorbance at 595 nm [21].

Antioxidant Activity of PCN

The radical scavenging activity was measured by DPPH method. 0.2ml of DPPH in methanol was mixed with 100 µl of two fold increasing concentrations (12.5 to 500 µg/ml) and 50 mM Tris HCl buffer (pH 7.4). The mixture was shaken vigorously and left it for room temperature for 30 minutes in the dark. The ascorbic acid was maintained as control and read the absorbance at 517 nm [17]. The scavenging activity was calculated as the % of inhibition, according to the following formula.

Scavenging activity= (A0-A1) /A0×100

Where the A0 is the absorbance of blank, A1 is absorbance of extract or ascorbic acid. Antioxidant activity was expressed as inhibitory concentration (IC50).

Cytotoxicity study

The cytotoxic property of pyocyanin was carried out by MTT assay against HepG2-cell lines. HepG2 cells were grown in RPMI-1640 medium which was supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml streptomycin solution. The 100 µl of cells were seeded into the 96 well micro-titer plates (5×103 cells/well), incubated the plates at 37ºC+5% CO_2 for 24h. After incubation, the growth media was removed and added fresh media with the different concentration of pyocyanin ranged from 10-100 µg/ml then incubated the plates at 37ºC for 24 h. After treatment the media were carefully removed from the wells and washed with the DMEM media, then 200 µl of MTT (5mg/ml) was added to the each well and again incubated in darkness at 37ºC for 6 h. After incubation, 100 µl of DMSO was added into each wells, left it for 45s and read the absorbance at 570 nm [26] and IC_{50} value was calculated, using the formula:

$$IC_{50} = \frac{Mean\ OD\ of\ untreated\ cells\ (control) - Mean\ OD\ of\ treated\ cells}{Mean\ OD\ of\ untreated\ cells\ (control)} \times 100$$

Characterization with thin layer chromatography

The extracted pigment was collected and the purity was characterized by thin layer chromatography. The sample was spotted by using capillary tubes on to the silica coated plate, allowed to dry and then the plates were developed with the solvent mobile phase by using chloroform:methanol solvent system in 1:1 ratio for 30 minutes. The developed plates were observed through UV- transilluminator and the retention factor was calculated by the formula:

Rf value = Distance travelled by the solute / Distance travelled by the solvent

Characterization by HPLC

The purity of the compound was checked with analytical cartridge Shimadzu (C-18 column) on a water pump 515 pump on a methanol water elution system.

FTIR

The FTIR spectrum of the pyocyanin was conducted via the IR-470 model. The functional group of IR spectra was determined with the help of FTIR correlation charts. The FTIR % transmittance was recorded varies the number (cm^{-1}). The wave number region was 3500-500 (cm^{-1}). The pyocyanin was prepared of one compression drop between two KBr disks.

Results

Isolation of sponge associated bacteria

A total of 23 bacterial isolates were obtained from sponge *Callyspongia* sp. Among these 23 marine strains, 3 showed the pigment production on nutrient agar plates [Fig-1]. The strains were designated as PA1, PA2, PA3 and were primarily screened against human bacterial pathogens namely *Bacillus subtilis*, *E. coli*, *Salmonella Typhi*, *Klebsiella pneumoniae* by cross streaking method.

The results revealed that PA1 exhibited good inhibitory activity against *Bacillus subtilis*, *Klebsiella pneumoniae*, *E.coli* and *Salmonella Typhi* (Table 1 &Fig.2). The prominent blue-green pigment producing PA1 was identified as *Pseudomonas* sp., through by microscopic and biochemical analysis (Table 2).

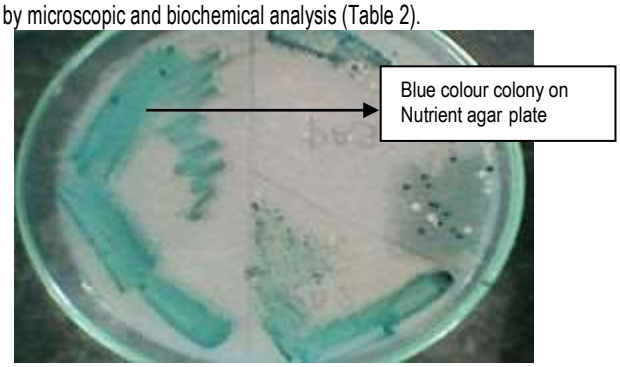

Fig-1 The blue pigment producing a strain on nutrient agar plate.

Fig-2 Cross streaking of PA1 against (i) *E.coli* (ii) *Salmonella Typhi* and (iii) *Klebsiella pneumoniae*

Table-1 Primary screening of three potential isolates against bacterial pathogens

Cross Streak Method				
Bacterial Strains	*Bacillus subtilis*	*E. coli*	*Salmonella* Typhi	*Klebsiella pneumoniae*
PA1	+	+++	++	+++
PA2	-	+	+	++
PA3	+	+	-	++

(+ positive activity, ++ good activity, +++ high activity, - no activity).

Antimicrobial activity

The pyocyanin was subjected to antibacterial and antifungal activity against human bacterial and fungal pathogens in the disk diffusion assay. It was found that the high activity was observed against *Klebsiella pneumoniae*, *S.aureus*, the zone of inhibition was 22 mm and 20 mm respectively. The moderate activity was found against *Bacillus subtilis* (19 mm), *Salmonella Typhi* (26 mm), *E.coli* (20 mm). Also, the antifungal activity was found against C. albicans and *Aspergillus flavus*. The zone of inhibition was observed 16 mm and 12 mm respectively. However, the results of commercially used antibiotics showed only minimum activity against human pathogens but the pyocyanin had the potent inhibitory activity against both fungal and bacterial pathogens at 20 µg/ml than in commercially available antibiotics (Table 3 and Fig 3 & 4).

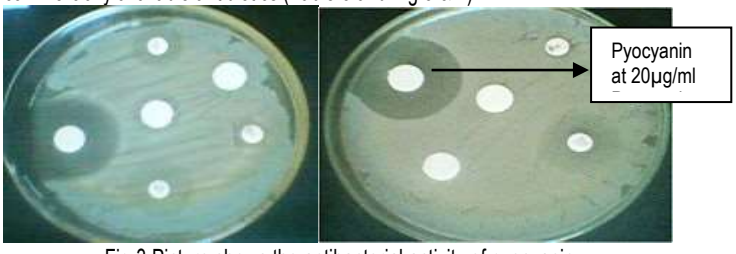

Fig-3 Picture shows the antibacterial activity of pyocyanin.

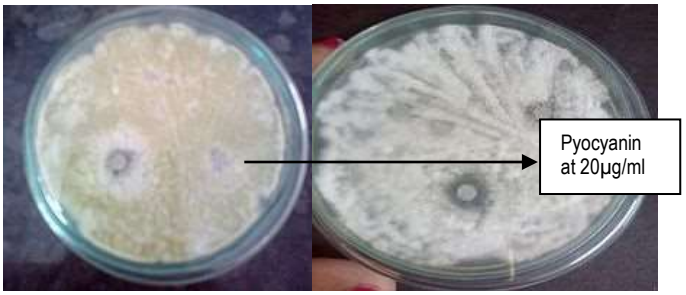

Fig-4 Picture shows the antifungal activity of pyocyanin

MIC

The pyocyanin was further determined by MIC, it was carried out in microtitre plate. The pyocyanin was subjected for their ability to be active against bacterial and fungal pathogens and the results revealed that the minimum inhibitiory activity was observed in 10 µg/ml of concentration against all tested pathogens. The inhibition was also found in 20 and 30 µg/ml of concentration against pathogens. The result revealed that the growth was inhibited at lowest concentration mostly against *Bacillus subtilis*, *E. coli*, *S. aureus*, S. Typhi, *Klebsiella pneumoniae* and also fungal pathogens namely C. albicans and *Aspergillus flavus* (Table 4).

Antioxidant

In the present study, the antioxidant activity of PCN was analyzed compared with the ascorbic acid. The scavenging activity was higher than the ascorbic acid (Fig 5). The results showed that the good antioxidant activities were obtained from the pyocyanin at 0.2 µg/ml of concentration. The antioxidant activity was observed even at lower concentration than that of ascorbic acid.

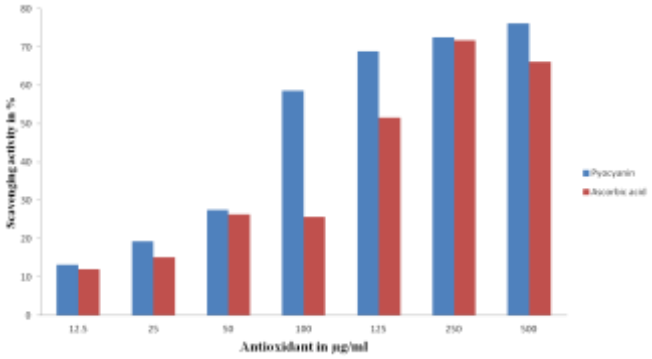

Fig-5 The DPPH scavenging activity of pyocyanin

Effect of pyocyanin on viability of cells in MTT assay

The cytotoxic property was determined by MTT assay. Initially, cell line showed that 90% of viability at low concentration and when concentration increased the viability was observed up to 80% of viability. Finally, the toxicity was found at 100 µg/ml for 24 h on HepG2 cells (Fig 6). The results revealed that the pyocyanin had the inhibitory property at the highest concentration of cancer cells.

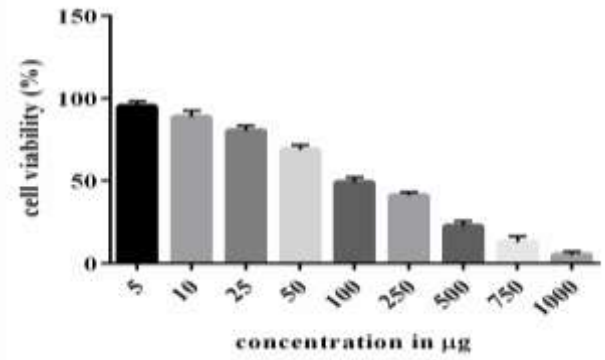

Fig-6 The cytotoxicity of pyocyanin on HepG2 cancer cell lines

Extraction and purification of pyocyanin

The mass culture of PA1 was performed in order to isolate and characterize secondary metabolites.

Table-2 Biochemical characterization and growth at different pH and NaCl concentrations by the isolate PA1

Characteristics	Isolate PA1
Grams staining	Negative rod
Oxidase	Positive
Catalase	Positive
Motility	Positive
MR	Negative
Vp	Negative
Citrate utilization	Positive
Glucose Fermentation	Acid
H2S production/Motility	Negative/motile
Gelatin hydrolysis	Positive
Casein hydrolysis	Positive

Growth response at Different pH

pH – 5	pH- 6	pH- 7	pH-8	pH-9
_	+	++	++	+

Growth response at Different Nacl % concentration

0.5	1	1.5	2	2.5	3	3.5	4	4.5	5	5.5	6	6.5	7	7.5	8
-	-	-	+	+	+	+	+	+	+	++	++	++	++	+++	+++

Table-3 Antimicrobial activity of pyocyanin and different antibiotics

Human Pathogens	Nalidixic acid (NA 30)	Rifampicin (RIF 5)	Co- Trimoxazol (COT 25)	Pyocyanin -20
Bacillus subtilis	14 mm	14mm	17mm	19 mm
E. coli	14mm	16mm	15mm	20mm
Salmonella Typhi	-	-	-	26mm
Klebsiella pneumoniae	16mm	13mm	16mm	20mm
Staphylococcus aureus	14mm	12mm	15mm	22mm
Candida albicans	-	-	-	16mm
Candida krusei	-	-	-	10mm
Aspergillus flavus	-	-	-	12mm

Table-4 The Minimum inhibitory activity profile of pyocyanin against pathogens [- no visible growth, ++ mild growth, +++ strongest visible growth]

SN	Concentration of the pyocyanin pigment (µg/ml)	MIC of the pyocyanin pigments for pathogens							
		B. subtilis	*E. coli*	*K. pneumoniae*	*S.* Typhi	*S. aureus*	*C. albicans*	*C. Krusei*	*A. flavus*
1	10	-	-	-	-	-	-	-	-
2	20	++	-	-	-	-	-	++	+++
3	30	+++	-	-	-	-	-	++	-
4	40	+++	+++	++	++	++	+++	+++	+++
5	50	+++	+++	+++	+++	+++	+++	+++	+++
6	60	+++	+++	+++	+++	+++	+++	+++	+++
7	70	+++	+++	+++	+++	+++	+++	+++	+++
8	80	+++	+++	+++	+++	+++	+++	+++	+++
9	90	+++	+++	+++	+++	+++	+++	+++	+++
10	100	+++	+++	+++	+++	+++	+++	+++	+++

The PA1 strain grown in a fermentation medium possessed a very strong pigment production on modified nutrient broth. The culture was extracted with the chloroform and subsequently with 0.2 N HCl. In chloroform extraction the blue layer was formed and collected. Again HCl was added and the colour turned to pink, which indicates the presence of pyocyanin in the mixture. Then, in TLC, the separated metabolite was partially identified based on the Rf value. The result showed the blue colour in the TLC plates and Rf value was found as 0.71 (Fig.7).

The results of HPLC revealed that the pyocyanin was observed at 14.725 retention time and 90% area was observed in the chromatographic result which was confirmed the presence of pyocyanin in the mixture (Fig.8). In FTIR spectra results showed that the compound in different strains was observed the band between 3500-3000 cm-1 which indicates the presence of amide group (OH) and the appearance of bands 3000 is an indication of C-H stretch for aromatic compound. These were observed in 1561-1403.30 cm^{-1} due to vibrations of carbon – carbon stretching in the aromatic ring. Absorption used between 1561-1272 cm^{-1} indicating the alkali C-O at 1040.54 cm^{-1} (Fig.9), most of the functional groups present was pyocyanin (Table 5).

Table-5 FT-IR profile analysis of pyocyanin functional groups

Functional group	FTIR spectra
C-H	2925.35
C-N Nitro group	1561.87
C-H stretch	1403.30
Alkalyl C-O	1040.54
C-H	848.82

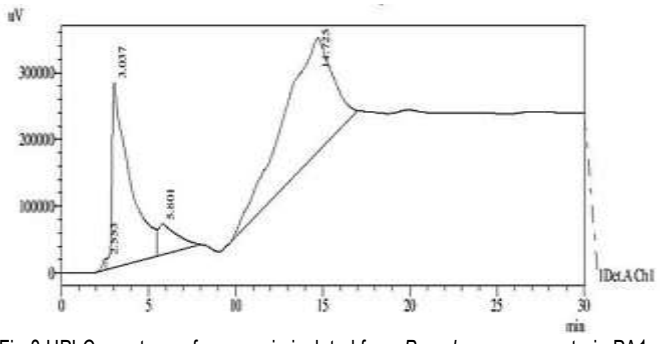

Fig-8 HPLC spectrum of pyocyanin isolated from *Pseudomonas* sp., strain PA1

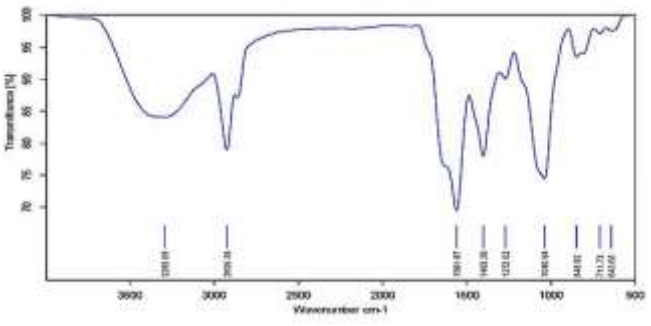

Fig-9 FT-IR of Pyocyanin isolated from *Pseudomonas* sp., strain PA1

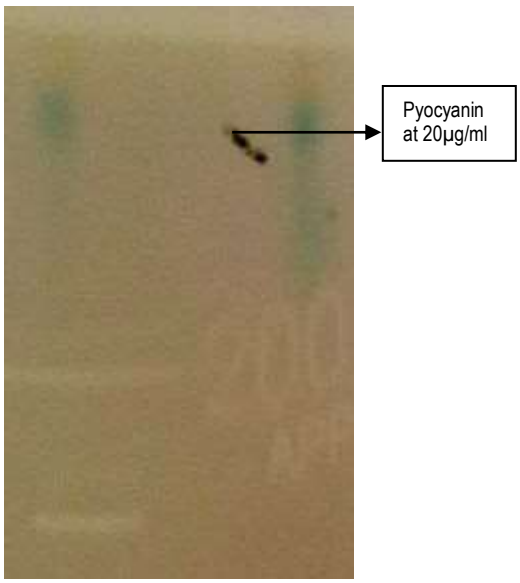

Fig-7 Separation of pyocyanin in Thin Layer Chromatography

Discussion

The marine sponges harbour a rich diversity of microorganism in their body system and 40% of microbial abundance was observed in the sponge cells. There are reports suggests that the symbiotic bacteria produced a variety of metabolites in the marine ecosystem which is beneficial for human health. They are potent and having biological and pharmacological property [1-3]. Symbiotic marine bacteria which can produce the pigments was found to able to inhibit other organisms for their survival. Interestingly, this type of pigmentation having the tremendous range of biological activity. Based on this, the researches looking for the pigmented bacteria as a biological control, but the research still not completed. In this study the pigmented bacteria and their biological property was mainly studied. The marine sponges were collected from the Tuticorin coastal area, Tamil Nadu, India. Based on the morphological features the collected sponge was identified as *Callyspongia* sp. There are many reports revealed that this kind of sponges are very common in Indian coastal area including Tuticorin coast [11]. A total of 23 strains was isolated from the *Callyspongia* sp., and they were shared by *Vibrio* sp., *Salmonella* sp., *E. coli* and *Pseudomonas* sp. Out of these, only 3 bacteria produced pigment on nutrient agar plates. The antagonistic activity of the pigmented bacteria was checked by the cross streaking method and the results revealed that the strain PA1 showed the highest inhibition activity against *E. coli*, *S. Typhi* and *Klebsiella pneumoniae* when compared with other strains. Hence, the PA1 was taken for further study. The potent PA1 was identified as *Pseudomonas* based on microscopic and biochemical studies. The blue pigment known as PCN is a well-known secondary metabolite extracted from the *Pseudomonas* sp., it can easily diffuse the membrane and it has an ability to inhibit the growth of different microorganism and to exhibit antimicrobial and anticancer activity. This is in agreement with the results of Basis *et al*., (2006) and Saha *et al*., (2008) [12-13]. Mainly it causes depletion of oxygen supply to the cells so that the growth was completely inhibited when grown on agar plates containing blue pigment of most Gram positive and Gram negative organisms [13]. In this study, the pigment was collected by the fermentation process with various carbon and nitrogen sources. After that the pigment was extracted with chloroform and 0.2N HCl to obtain the pinkish red colour, it indicated the presence of pyocyanin. Then the Pyocyanin was confirmed by thin layer chromatography and the Rf value was 0.75. This study is endorsed by the works carried out elsewhere [14-16]. HPLC results revealed that the major peak was observed at 14.92 retention time which was confirmed the purity of active compound. Then the functional characters were categorized based on FTIR result, it showed the presence of OH water molecule and C=C, C=N. Several studies also suggested that this prove the presence of pyocyanin presence in the compound [17-18]. Interstingly, the compound showed the good inhibition at 20 µg/ml of concentration against *Klebsiella pneumoniae* and S.aureus and the zone of inhibition was 32 and 30 mm whereas commercial Nalidixic acid (NA 30), Rifambicin (RIF 5), Co-trimaxazol (COT-25) had the moderate activity while compare with pyocyanin, so it was considered as a good antimicrobial agent. It was also found that the pyocyanin inhibits the fungal pathogens like *Candida albicans* and *Aspergillus flavus* as observed by the other workers [19-20]. The antifungal activity showed the zone of inhibition against *Candida krusei* (10 mm). *Candida albicans* (16 mm) and *Aspergillus flavus* (12 mm). In another study it was observed that antimicrobial activity was at 50 µg/ml [21]. Our result also confirmed that the antimicrobial property was observed at low concentration. The research is in still progress to identify the clarity and action of the such pigments. In support of the present study, however, in another study it was proved the blue pigment have the 90-95% of inhibition against other pathogens [22]. The MIC of pyocyanin was also observed at the lowest concentration and the growth was not observed at 10 µg/ml on microtitre plate in *E. coli*, S. Typhi, *Klebsiella pneumoniae*, *S. aureus* and fungal pathogens. This clearly showed that lowest concentration inhibits the bacterial growth than that of highest concentration. In support of this on Basli and Aslim in 2008 reported that pyocyanin was able to inhibit the gram positive and gram negative bacteria [23]. Radical scavenging activity was observed at very minute concentrations. It exhibited 80% of activities compare with the ascorbic acid. Numerous previous reports supported the present findings and all the works found the good antioxidant property [24-25]. In addition to this, the compound showed the cytotoxity effects on HepG2 cells at the highest concentration. The results of this study revealed that the cell death induced by pyocyanin and raised the oxidative damage to the cell that leads to DNA damage, senescence and apoptosis. In another study, it was reported that 112 µg/ml of PCN showed the inhibitory activity against cancer cells [26].

Conclusion

Based on this finding we suggested that the pyocyanin is a natural compound which has the ability to inhibit the other organism's growth. Thus, this compound used as biological agent and creates an eco-friendly activity; further new insights about pyocyanin are building up in the field for new research opportunities.

Application of the Research: This study should be a better understanding about sponge associated bacterial metabolites and their bioactive potential against pathogens applicable for both biotechnology and microbiology.

Research Category: Microbiology

Abbreviations: PCN- pyocyanin, MIC- Minimum Inhibitory Concentration,

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***Research Guide or Chairperson of research: Dr K. Sathiyamurthy**
University: Bharathidasan University, Tiruchy, 620024
Research project name or number: PhD Thesis

Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Marine sponge sample was collected from the Tuticorin coast, Tamil Nadu

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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