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Toxicity Evaluation and Antimicrobial Activity of Purified Pyocyanin from *Pseudomonas aeruginosa*

Mohamed N. F. Hamad [1], Diaa A. Marrez [2,*], Sherien M. R. El-Sherbieny [1]

[1] Dairy Department, Faculty of Agriculture, Damietta University, Damietta, Egypt
[2] Food Toxicology and Contaminants Department, National Research Centre, Cairo, Egypt
* Correspondence: diaamm80@hotmail.com;

Scopus Author ID 56288678900

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Abstract: Pigments from microbial sources considered a promising approach in food industry applications as a food colorant and biopreservatives agents. This study aims to evaluate the antimicrobial activity of purified pyocyanin against foodborne pathogens and study the safety of pyocyanin by toxicity determination. Purification and structure elucidation of pyocyanin was carried out using a UV-Vis spectrophotometer, Fourier Transform Infra-Red Spectroscopy (FTIR) and GC-MS analysis. Pyocyanin showed antibacterial activity against 9 species of foodborne pathogenic bacteria with a zone of inhibition from 10.8 to 22.6 mm, and minimum inhibitory concentration (MIC) value ranged between 33.3 to 233.3 µg ml^{-1}. Also, pyocyanin has antifungal activity against 10 mycotoxigenic fungi strains with inhibition zone value ranged from 7.0 to 17.6 mm and MIC value from 58.3 to 250 µg ml^{-1}. No toxicity was observed on shrimp nauplii up to 50 µg ml^{-1} for 12 and 24 h of exposure and up to 100 µg ml^{-1} for 12 h. Also, no toxicity was recorded with pyocyanin using mouse bioassay up to 750 µg ml^{-1}, while 1000 µg ml^{-1} observed toxicity equal to 3.28 MU (mouse unit). Pyocyanin had antimicrobial activity against a wide range of foodborne pathogenic bacteria and mycotoxigenic fungi. Consequently, pyocyanin can be used as a cheap and safe source in the food industry and pharmaceutical applications.

Keywords: Pseudomonas aeruginosa; pyocyanin antimicrobial; toxicity evaluation.

1. Introduction

The most common strategy for controlling food spoilage microorganisms is using chemical food preservatives with high efficiency against both foodborne pathogenic bacteria and mycotoxigenic fungi [1]. However, due to the growing concern about the potential human health risk, environmental contamination and development of foodborne pathogens resistance to these chemical food preservatives, a control strategy depended on food biopreservatives agents has become an alternative approach [2]. The biopreservatives agents have been successfully applied as a food additive to prevent the growth of foodborne pathogens for increasing the shelf life of food [3]. The use of microorganisms like bacteria, fungi, yeast, algae, and actinomycetes extracts as biopreservatives agents have been reported in several studies [4,5].

Pseudomonas aeruginosa produces a wide variety of pigments as secondary metabolites. Four different major pigments of redox-active phenazine compounds that produce by *P. aeruginosa*, including pyocyanin, fluorescein, pyorubrin, and pyomelanin [6]. Pyocyanin is chloroform soluble blue-green pigment produce by an active culture of *P. aeruginosa* [7].

Fluorescein is water-soluble pigment with significant siderophore activity. Pyorubrin is irreversibly reduced to colorless form in reduced oxygen concentration, pyorubrin used as an antibacterial agent, and food colorants [8]. In addition to significant phenazine group, aeruginosin A and B were water-soluble red pigment, which produces by some *P. aeruginosa* with several biological activities [9].

Pyocyanin is an abroad spectrum pigment that has antibacterial activity against a wide range of drug-resistant bacteria and several species of foodborne pathogenic bacteria. Also, pyocyanin exhibited antifungal activity against different species of plant pathogenic fungi and mycotoxigenic fungi [6]. Furthermore, pyocyanin showed antioxidant and anticancer activity against several cancer cell lines [10]. So, the main objectives of this study are isolation, purification, and structure elucidation of pyocyanin from *P. aeruginosa* KT032066 strain. Toxicity evaluation of purified pyocyanin using brine shrimp assay and mouse bioassay. Finally, study the antimicrobial activity of pyocyanin against different strains of foodborne pathogenic bacteria and mycotoxigenic fungi.

2. Materials and Methods

2.1. Bacterial strain.

Pseudomonas aeruginosa KT032066 strain was acquired from Agriculture Microbiology Department, Agriculture and Biology Division, National Research Centre, Cairo, Egypt. *P. aeruginosa* Osh1 strain was cultivated in King's B (KB) g/l: Peptone 20 g, magnesium chloride1.4g, potassium sulfate 10 g, and distilled water 1 liter; pH 7.0 ± 0.2, 25^{o}C) as described by King *et al*. [11].

2.2. Extraction and purification of pyocyanin from P. aeruginosa.

A 24 hour of old culture broth was transferred into a 50 ml centrifuge glass tube and centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was transferred into a 500 ml separating funnel and mixed with chloroform at a 1:1.5 ratio (supernatant: chloroform). This mixture was shaken well and kept undisturbed for 5-10 minutes for the pyocyanin fraction in the broth to get extracted to the chloroform layer.

The blue-colored chloroform layer, along with pyocyanin formed below the aqueous layer in the separating funnel, was collected into a covered conical flask to protect from light to prevent oxidation. This chloroform fraction was then transferred into a 500ml vacuum rotary flask and concentrated in a vacuum rotary evaporator at 40°C.

2.2.1. Silica gel column purification.

The vacuum concentrated pyocyanin fraction was purified by silica gel column; silica gel absorbed crude pigment was loaded on column (60 cm length and 3 cm diameter). The column was packed with silica having a mesh size 100-200 and equilibrated using 1% chloroform-methanol solvent system in the ratio 1:1, and the concentrated pyocyanin fraction was loaded into the column. The purified pyocyanin was eluted with 15% methanol in chloroform. The blue-colored pyocyanin fraction was then collected into a 250 ml conical flask, protected from light [12].

2.2.2. Concentration of the purified pyocyanin.

The column purified pyocyanin fraction 15% methanol in chloroform was further concentrated in a vacuum rotary evaporator at 40°C. The concentrated pyocyanin was taken in

a pre-weighed amber-colored bottle and passed through a jet of nitrogen gas to remove solvents and thoroughly dried. The weight of the dried pyocyanin was taken and subtracted from the weight of an empty bottle to get the dry weight of pyocyanin in milligrams. The concentrated and purified pyocyanin was collected in an amber-colored bottle and stored at -20°C till use.

2.3. Structural elucidation and purity confirmation of pyocyanin.

2.3.1. UV-Vis spectrophotometer.

The purified pyocyanin was subjected to spectroscopic analysis. Ultraviolet and visible absorption spectra of purified pyocyanin dissolved in chloroform and 0.1N HCl were recorded over a range of 200 -700 nm. UV analysis was done using UV spectrophotometer (Shimadzu 1601, Japan). Absorbance maxima determined by UV spectrophotometric analysis [13].

2.3.2. Fourier Transform Infra-Red Spectroscopy (FTIR).

FTIR was employed to explore the functional groups and the chemical bonds in the present compound. The analysis was done using Jasco FTIR Spectrophotometer (FTIR-6100 Jasco, Japan) at National Research Centre. The sample was prepared by homogeneous dispersal of 1 mg of pyocyanin extract in pellets of potassium bromide (Merck, USA). IR absorption spectra were obtained using a built-in plotter. IR spectra were collected over the range of 400-4000 cm^{-1} with a resolution of 4 cm^{-1}. The spectrum was studied to interpret the chemical nature of the bioactive compound [14].

2.3.3. GC-MS analysis.

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used, Helium gas was used as the carrier gas at a constant flow rate of 1ml min^{-1}. The injector and MS transfer line temperature was set at 280℃.

The oven temperature was programmed at an initial temperature of 50℃ (hold 2 min) to150˚C at an increasing rate of 7˚C min^{-1}. Then to 270 ℃ at an increasing rate of 5˚C min^{-1} (hold 2min) then to 310 as a final temperature at an increasing rate of 3.5˚C min^{-1} (hold 10 min).

The quantification of all the identified components was investigated using a percent relative peak area. Tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system.

2.4. Antimicrobial activity of pyocyanin.

2.4.1. Test microorganisms.

The inhibitory effect of pyocyanin extract was carried out on five species of pathogenic bacteria. Four Gram-positive bacteria (*Bacillus cereus* EMCC 1080, *Staphylococcus aureus* ATCC 13565, *Staphylococcus sciuri* 2-6 and *Lactococcus lactis* subsp. *lactis*) and five Gram-negative bacteria (*Salmonella typhi* ATCC 25566, *S. enterica* SA19992307, *Escherichia coli* 0157 H7 ATCC 51659, *Pseudomonas fluorescens* FP10 and *Klebsiella pneumoniae* LMD 7726). The stock cultures were grown on nutrient agar slant at 37˚C for 24 h and then kept in the refrigerator until use. Ten fungal species were used for the antifungal assay, *Aspergillus*

flavus NRRL 3357, *A. parasiticus* SSWT 2999, *A. westerdijikia* CCT 6795, *A. styni* IBT LKN 23096, *A. ochraceus* ITAL 14, *A. niger* IMI288550, *A. carbonarus* ITAL 204, *Fusarium verticelloides* ITEM 10027, *F. proleferatum* MPVP 328 and *Penicillium verrucosum* BFE 500. The stock cultures were grown on potato dextrose agar slant at 25˚C for 5 days and then kept in the refrigerator until use.

2.4.2. Disc diffusion technique.

From the 24 h incubated nutrient agar slant of each bacterial species, a loop full of the microorganism was inoculated in a tube containing 4 to 5 ml of tryptic soy broth. The broth culture is incubated at 35ºC for 2 - 6 h until it achieves the turbidity of 0.5 McFarland $BaSO_4$ standards. The density of the turbidity standard was determined by using a spectrophotometer at 625 nm. The absorbance at 625 nm should be (0.008 to 0.1) for the 0.5 McFarland standards. $BaSO_4$turbidity standard should be vigorously agitated on a mechanical vortex mixer before each use and inspected for a uniformly turbid appearance.

The sensitivity test of pyocyanin extract was determined with different bacterial cultures using the disc diffusion method by Kirby-Bauer technique [15,16]. Petri dishes were prepared with 20 ml nutrient agar, and the bacterial cultures were uniformly from tryptic soy broth using cotton swabs. Pyocyanin extract was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to give 0.1, 0.5, 1.0, 2.5, and 5 mg ml^{-1} for pyocyanin. Sterilized discs (6 mm) from Whatman No. 1 filter paper were loaded by extract and entirely dried under sterile conditions. The discs were placed on the seeded plates by using a sterile forceps. DMSO represented as a negative control and tetracycline (500 µg ml^{-1}) was used as a positive control. Plates were incubated at 37°C for 24h. The inhibition zones were measured and expressed as the diameter of the clear zone, including the diameter of the paper disc.

The fungal strains were plated onto potato dextrose agar (PDA) and incubated for 5 days at 25°C. The spore suspension of each fungus was prepared in 0.01% Tween 80 solution. The fungal suspension was compared with the 0.5 McFarland standard. Petri dishes of the YES medium were inoculated with 50 µl of each fungal culture and uniformly spread using a sterile L- glass rod. Pyocyanin was loaded discs and placed on the seeded plates by using a sterile forceps. Negative control was prepared by using DMSO, and the commercial fungicide Nystatin (1000 Unit ml^{-1}) was used as a positive control. Plates were incubated at 25°C for 24 - 48 h. At the end of the period, antifungal activity was evaluated by measuring the zone of inhibition (mm) against the tested fungus [17,18].

2.4.3. MIC of pyocyanin.

The determination of minimum inhibitory concentration (MIC) was conducted using the tube dilution method [19,20]. A 24 h culture of the tested bacterial species was diluted in 10 ml of tryptic soy broth (TSB) about the 0.5 McFarland standard to achieve inocula of 10^8 CFU ml^{-1}. In culture tube containing nine different concentrations of pyocyanin (0.015, 0.025, 0.05, 0.075, 0.1, 0.15, 0.25, 0.5, 0.75 and 1.0 mg ml^{-1} in DMSO) were prepared. Each tube was inoculated with 100µl of bacterial cell suspension and incubated at 37ºC for 24h. The growth of the inoculum in broth is indicated by turbidity of the broth, and the lowest concentration of the extract, which inhibited the growth of the test organism, was taken as the minimum inhibitory concentration (MIC).

MIC against fungi was performed by using the technique of Perrucci *et al*. [21]. Different concentrations of pyocyanin were separately dissolved in 0.5 ml of 0.1% Tween 80

(Merck, Darmstadt, Germany), then mixed with 9.5 ml of melting, 45ºC, PDA, and poured into Petri dish (6 cm). The prepared plates were centrally inoculated with 3µl of fungal suspension. The plates were incubated at 25°C for 24-48h. At the end of the incubation period, mycelial growth was monitored, and the MIC was determined.

2.5. Toxicity assessment of pyocyanin.

2.5.1. Brine shrimp bioassay.

Toxicity of pyocyanin was tested using *Artemia salina* leach nauplii (Brine shrimp). *Artemia salina* was supplied by Avocet Artemin Inc., Utah, USA. Dried cysts were hatched (1g cyst per liter) in filtered seawater at 27-30°C with strong aeration, under a continuous light regime. Approximately 12 hours after hatching, the phototrophic nauplii were collected with a pipette and concentrated in a small vial containing 5 ml seawater. Each test consisted of exposing groups of 10 nauplii to various concentrations (0, 10, 25, 50, 100, 200, 250, 300, 350, 400, 450, 500, 750 and 1000µg ml^{-1}) of pyocyanin. The toxicity was determined after 12 and 24 hours of exposure by counting the number of survivors and calculating the percentage of mortality [22,23]. Larvae were considered dead if they did not exhibit any movement of appendages. All data were subjected to probit analysis using SPSS software.

2.5.2. Mouse bioassay.

Various concentrations (100, 250, 300, 350, 400, 450 and 500 µg ml^{-1}) of pyocyanin were dissolved in 1:1 (w/v) 0.1 Molar acetic acid. To determine the dose-response curve, male Albino Swiss mice weighting 20±2 g were obtained from the Animal Housing Division, National Research Center, Cairo, Egypt. Potency was expressed as Mouse Units (MU), where 1 MU is defined as the amount of toxins required for killing a 20 g mouse in 15 minutes. The survival time was measured from the completion of the intraperitoneal (i.p.) injection to the last breath [24]. Preliminary toxicity determination was performed using 3 mice for each sample preparation dose. Toxicity was observed, and death times were recorded.

2.6. Statistical analysis

Results were subjected to a one-way analysis of variance (ANOVA) of the general linear model (GLM) using the SAS statistical package. The results were the average of three replicates ($p \leq 0.05$).

3. Results and Discussion

3.1. Purity confirmation of pyocyanin.

Like most low molecular weight compounds, the crude pyocyanin from selected *P. aeruginosa* strain was purified using column chromatography. Ohfuji *et al.* [12] purified pyocyanin produced by *P. aeruginosa* using a silica gel column equilibrated with 1% methanol in chloroform. Pyocyanin was fractionated into yellow, red, and dark blow bands, a yellow-green band tightly bound to stationary phase after elution with 15% methanol in chloroform. In contrast, blue band easily eluted as pure pyocyanin.

3.1.1. UV-Vis spectrophotometer.

The absorbance spectrum of pyocyanin was monitored from 200 to 800 nm using a UV-Vis spectrophotometer (Figure 1). UV-visible spectroscopic analysis of purified pyocyanin showed two maxima at wavelengths 370 nm and 691 nm. This peak, 370 nm, indicates the presence of the pyocyanin compound. Parsons *et al*. [25] reported that pyocyanin was confirmed and quantitated by its characteristic absorption at 370 and 690 nm. While, Priyaja [26] found that UV-visible spectroscopic analysis of purified pyocyanin observed four absorption maxima, at wavelengths of 699, 529, 310 and 254.5 nm in the chloroform solvent and five absorption maxima in 0.1 N HCl at wavelengths of 553, 390, 284, 246 and 246 nm. This was comparable to the absorption maxima obtained by the pyocyanin standard at wavelengths such as 691, 529, 306, and 255.5 nm in chloroform and 555, 388, 284, 247 and 225 nm in 0.1 N HCl. On the other hand, Sudhakar *et al*. [27] indicated that the partially purified of pyocyanin was subjected to UV-spectrophotometer, and the absorbance of this solution was maximum at 278 nm.

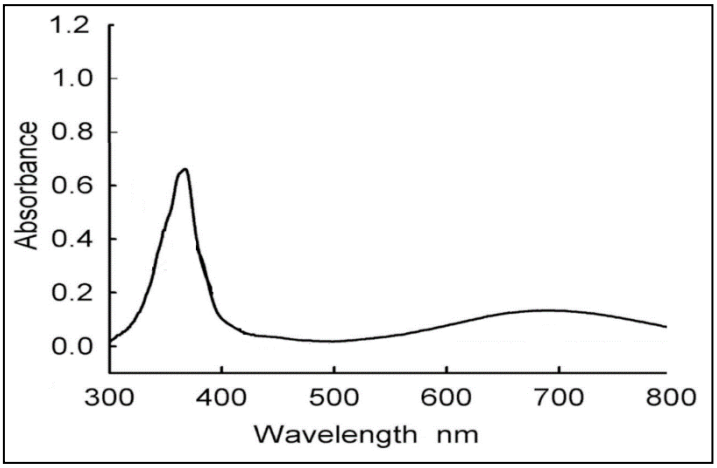

Figure 1. UV absorption spectra of pyocyanin.

3.1.2. Fourier Transform Infra-Red Spectroscopy (FTIR).

FTIR is the most preferred method of infrared spectroscopy. In infrared spectroscopy, IR radiation is passed through a sample. Some of the radiation is absorbed by the sample, and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption and transmission, which creates a molecular fingerprint of the sample. This is unique for every sample. This makes infrared spectroscopy useful for several types of analysis [14].

Further characterization of pyocyanin was by analyzing their IR spectrum. The pyocyanin spectrum in Figure (2) indicated the presence of phenazine as specified by side chains of the molecule. The peak at 3430.59 cm^{-1} shows the presence of O-H bond. The peak at 2365.26 cm^{-1} relates to the C-Haromatic bond. The peak shown at 1633.41 cm^{-1} represents C=N bond, and the peak at 1252.54 cm^{-1} corresponds to C-O bond. This is comparable to the reports of standard FTIR spectra.

Pyocyanin was extracted using chloroform, and the presence of secondary metabolites were confirmed by the addition of 0.2 N HCl partial purification of the pigment was carried out by column chromatography and subjected to UV-Vis spectrophotometer, and the structure elucidation of pyocyanin was done using ^{1}H NMR and FTIR [13,27]. Nansathit *et al*. [28] found that IR spectrum of pyocyanin showed bond at 3450 and 1557 cm^{-1} for N-H and the peak

at 1971 cm^{-1} of C=O, characteristic of the amide group. For C=C and C=N were exhibited at the regions1600 – 1400 cm^{-1}. Peaks at 900 -690 cm^{-1} were also presented C-H of aromatic in the molecule. Laxmi and Bhat [29] reported that the pyocyanin FTIR spectrum showed peaks at 3448.59 cm^{-1} shows the presence of O-H bond. The peak at 2951.18 cm^{-1} due to the C-Haromatic bond and the peak shown at 1637.34 cm^{-1} represents C=N bond, and the peak at 1307.02 cm^{-1} corresponds to C-O bond.

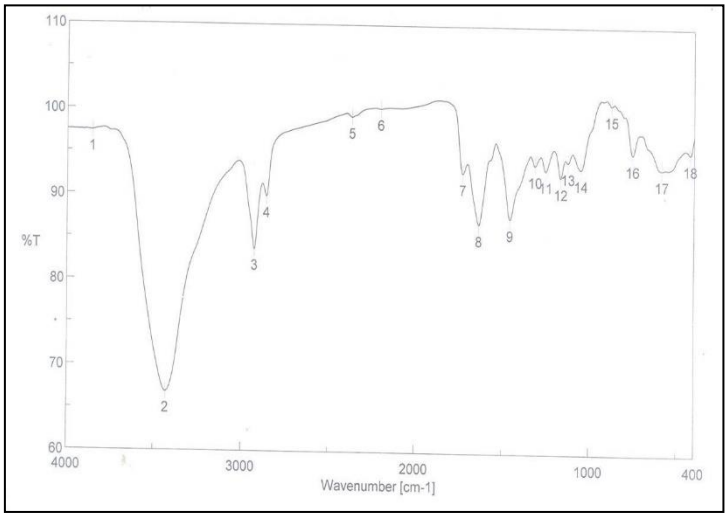

Figure 2. FTIR spectrum of pyocyanin produced by *P. aeruginosa***.**

3.1.3. GC-MS analysis.

The GC-MS analysis of *P. aeruginosa* pigment showed that pyocyanin compound had retention time 53.08 min (Figure 3) and GC-MS spectrum (Figure 4) analysis revealed that the molecular weight 211 Dalton typical to as close as published for pyocyanin ($C_{13}H_{10}N_2O$) [30,31].

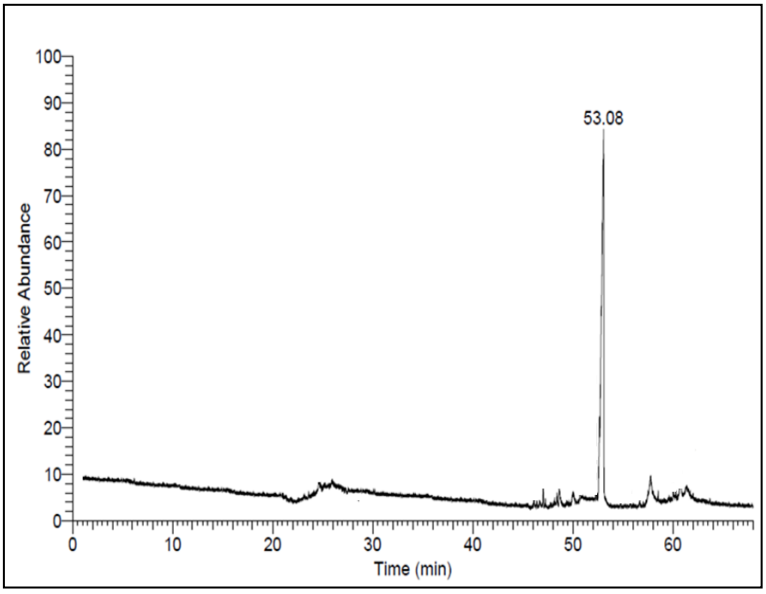

Figure 2. GC-MS chromatogram of pyocyanin produced by *P. aeruginosa*.

The results obtained were in agreement with results observed by Sudhakar *et al*. [27] they reported that the partially purified pyocyanin compound which was synthesized by *P. aeruginosa* and subjected to GC-MS which had the intense molecular ion at m/z 210.23 defining the molecular weight as 210 Dalton.

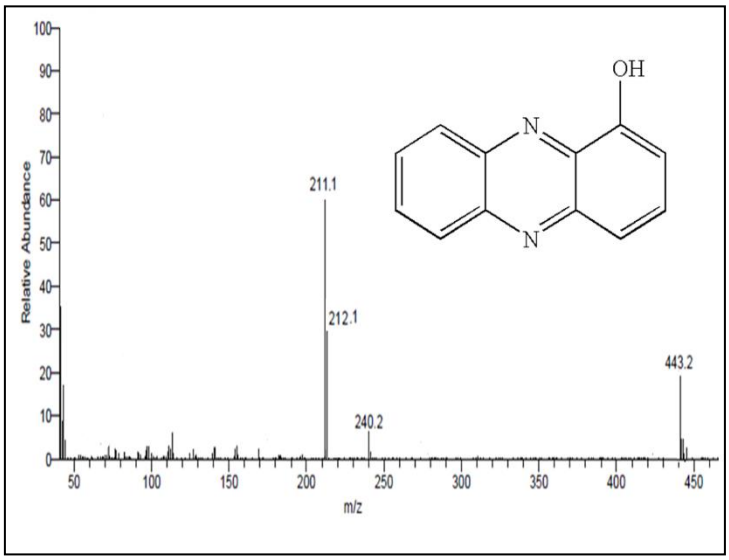

Figure 4. Chemical structure and GC-MS spectrum of pyocyanin.

The mass spectrum showed intense ions at m/z 210 and other ions125, 140, 168, 194, 181, 156, 108, and 92. Also, the spectrum was in agreement with pal and Revathi. [32]. The pyocyanin GC-MS was done, and the retention time was found at 11.04 mins. The molecular weight of the pyocyanin compound was about 210.23 kDa [33]. Priyaja *et al*. [34] reported that mass spectroscopic analysis of the purified compound and pyocyanin standard demonstrated a protonated molecular ion at m/z 211 for confirmation of the purified pyocyanin compound.

3.2. Antimicrobial activity of pyocyanin.

3.2.1. Antibacterial activity of pyocyanin.

The antibacterial activities of different pyocyanin concentrations against 9 species of foodborne pathogenic bacteria are illustrated in (Table 1). All different concentrations of pyocyanin showed antibacterial activity against all tested bacteria ranged between 10.8 to 32.2 mm inhibition zones. The antibacterial activity of pyocyanin against tested bacteria increased by increasing of the pyocyanin concentrations (0.1, 0.5, 1, 2.5, 5 mg ml^{-1}, respectively).

The inhibition zone of *B. cereus* ranged from 16.2 to 32.2 mm, but the inhibition zone of *Staph. aureus* and *Staph. sciuri* ranged from 14.0 to 30.8 and 15.8 to 28.6 mm, respectively. The antibacterial activity of pyocyanin against *E. coli* showed an inhibition zone increased by increasing in pyocyanin concentrations; it ranged from 16.8 to 26. 7 mm. The inhibition zones of *S. typhi* ranged from 14.7 to 31.6 mm and from 15.2 to 25.8 mm against *S. entrica*. However, the inhibition zone of pyocyanin against *P. aeruginosa* and *P. fluorescens* ranged between 12.7 – 22.6 mm. Antibacterial activity of pyocyanin against *K. pneumonia*, and *L. lactis* showed zone of inhibition ranged from 10.8 to 26.0 and 11.1 to 18.3 mm, respectively.

Pyocyanin at concentration 1 mg ml^{-1} showed antibacterial activity against *E. coli* higher than antibiotic standard, while required 2.5 mg ml^{-1} to observe activity higher than antibiotic standard against *B. cereus*, *S. typhi*, *P. aeruginosa* and *K. pneumoniae*. Whereas, it required5 mg ml^{-1} of pyocyanin to showed antibacterial activity higher than tetracycline standard against *S. aureus*, *S. sciuri*, *S. entrica*, *P. fluorescens*, and *L. lactis*. The highest antibacterial activities were recorded against, *B. cereus* followed by *S. typhi* and *S. aureus*, which had inhibition zones 32.2, 31.6, and 30.8 mm, respectively.

Alzahrani and Alqahtani [35] indicated pyocyanin pigment of *P. aeruginosa* showed a greater effect against all Gram-positive and Gram-negative tested bacteria, and the antagonistic activity against tested bacteria increased by increasing of the pyocyanin pigment concentration. Also, El-Shouny *et al.* [36] found that the growth of all tested Gram-positive bacteria and *Candida* spp. were entirely inhibited by pyocyanin, whereas Gram-negative bacteria, including *S. typhi* and *Pseudomonas mirabilis*, were intermediately affected and *K. pneumonia* was resistant to pyocyanin. Saha *et al*. [37] reported that pyocyanin has antagonistic activity against pathogenic bacteria like *Salmonella paratyphi, E. coli*, and *Klebsiella pneumonia*. Rahman *et al*. [38] indicated that pyocyanin from *P. aeruginosa* DSO-129 has an antimicrobial effect on organisms like *S. aureus, Staphylococcus epidermis, Bacillus subtilis, Micrococcus luteus* and *Saccharomyces cerevisiae*.

Rane *et al.* [39] found that antimicrobial activity of the pyocyanin against strains of *B. subtilis, C. albicans*, and *E. coli*. El-fouly *et al.* [40] indicated that pyocyanin had high antimicrobial activity against *S. aureus, E. coli, Klebsiella* sp., *S. typhi, Shigella* sp. and *C. albicans*. Mathew *et al*. [41] found that pyocyanin had antibacterial activity against *Bacillus thuringiensis, B. coagulans, B. subtilis, Staphylococcus aureus*, and *Pseudomonas fluorescence* B21. However, no antimicrobial activity was noted against *E. coli, P. aeruginosa* SPC B 65 and *P. fluorescens* SPC B 60. Onbasli and Aslim [42] found that pyocyanin from *Pseudomonas* inhibits the *E. coli* isolates from sugar beet molasses.

3.2.2. MIC of pyocyanin against foodborne pathogenic bacteria.

As shown in Figure (5), the pyocyanin has a high antibacterial activity against all tested pathogenic strains and acts as a broad-spectrum antibiotic. The highest activity of pyocyanin was recorded against *S. typhi* with MIC value 21.7 µg ml^{-1}, followed by *B. cereus, Staph. sciuri, Staph. aureus* and *S. entrica* with MIC values of 33.3, 41.7, 58.3, and 66.7 µg ml^{-1}, respectively. Whereas, the lowest activity was showed against *K. pneumoniae, L. lactis*, and *P. fluorescence* with MIC values 183.4, 200, and 233.3 µg ml^{-1}, respectively. Pyocyanin recorded the same MIC value (91.7 µg ml^{-1}) against both *E. coli* and *P. aeruginosa*. Nowroozi *et al*. [43] revealed that the growth of *S. aureus* and *E. coli* were inhibited by the same concentration of pyocyanin with MIC value 56 µg ml^{-1}.

3.2.3. Antifungal activity of pyocyanin.

Table 2 illustrates the antifungal activity of *P. aeruginosa* pyocyanin extract against different species of mycotoxigenic fungi. The highest antifungal activity of pyocyanin was shown against *A. stynii* followed by *F. proliferatum* and *A. parasiticus* with a zone of inhibition values 17.6, 16.7 and 16.5 mm at concentration 0.1 mg ml^{-1}. The pyocyanin concentration 0.1 mg ml^{-1} showed antifungal activity against *A. flavus, A. carbonarus, A. stynii, F. verticelloides, F. proleferatum,* and *P. verrucosum* with inhibition zone value ranged from 7.2 to 8.7 mm, while *A. parasiticus, A.niger, A. ochraceus, A. westerdijikia*, and *.F. proleferatum* showed resistance. The negative control (DMSO) showed no inhibition whereas positive control, Nystatin at 1000 unit ml^{-1} showed inhibition of 12.8, 16.0, 15.8, 15.4, 13.2, 15.4, 16.6, 11.5, 13.5 and 15.8 mm against *A. flavus, A. parasiticus, A. carbonarus, A. niger, A. stynii, A. ochraceus, A. westerdijikia, F. proliferatum, F. verticillioides* and *P. verrucosum*, respectively.

Table 1. Antibacterial activity of different concentrations of pyocyanin against different foodborne pathogenic bacteria species.

Bacteria	Inhibition zone mm (Mean±*S.E)						
	Negative control	Positive control	5 mg ml^{-1}	2.5 mg ml^{-1}	1 mg ml^{-1}	0.5 mg ml^{-1}	0.1 mg ml^{-1}
B. cereus	0	26.8±1.04^{bc}	32.2±2.08^{a}	28.3±1.28^{b}	24.6±0.86^{c}	18.6±1.36^{d}	16.2±1.04^{e}
S. aureus	0	25.7±0.58^{b}	30.8±2.14^{a}	25.6±1.60^{b}	22.6±2.04^{bc}	20.2±1.58^{c}	14.0±1.00^{d}
S. sciuri	0	26.5±1.28^{ab}	28.6±0.76^{a}	24.8±1.58^{b}	22.3±0.87^{c}	21.0±1.00^{c}	15.8±0.48^{d}
E. coli	0	18.8±0.86^{c}	26.7±1.16^{a}	24.3±1.04^{a}	21.0±1.00^{b}	20.6±2.11^{bc}	16.8±1.35^{d}
S. typhi	0	25.4±2.02^{c}	31.6±2.14^{a}	30.5±1.00^{b}	25.8±1.58^{c}	22.0±1.00^{d}	14.7±0.86^{e}
S. enterica	0	24.8±1.15^{ab}	25.8±1.36^{a}	24.2±1.21^{ab}	22.3±0.58^{b}	19.6±0.96^{c}	15.2±1.08^{d}
P. aeruginosa	0	18.9±0.76^{b}	22.6±1.28^{a}	20.7±0.86^{ab}	18.4±0.58^{b}	16.8±0.78^{c}	12.7±1.04^{d}
K. pneumoniae	0	21.3±1.26^{c}	26.0±1.86^{a}	24.7±1.58^{b}	21.8±1.04^{c}	18.0±1.00^{d}	10.8±0.36^{e}
L. lactis	0	18.5±1.16^{a}	18.3±0.76^{a}	16.0±1.00^{b}	14.6±1.14^{bc}	14.0±2.04^{c}	11.1±1.28^{d}

n=3, *S.E: standard error, different subscripts within the row are significantly different at the 5% level, 0: No inhibition, negative control: DMSO, positive control: tetracycline.

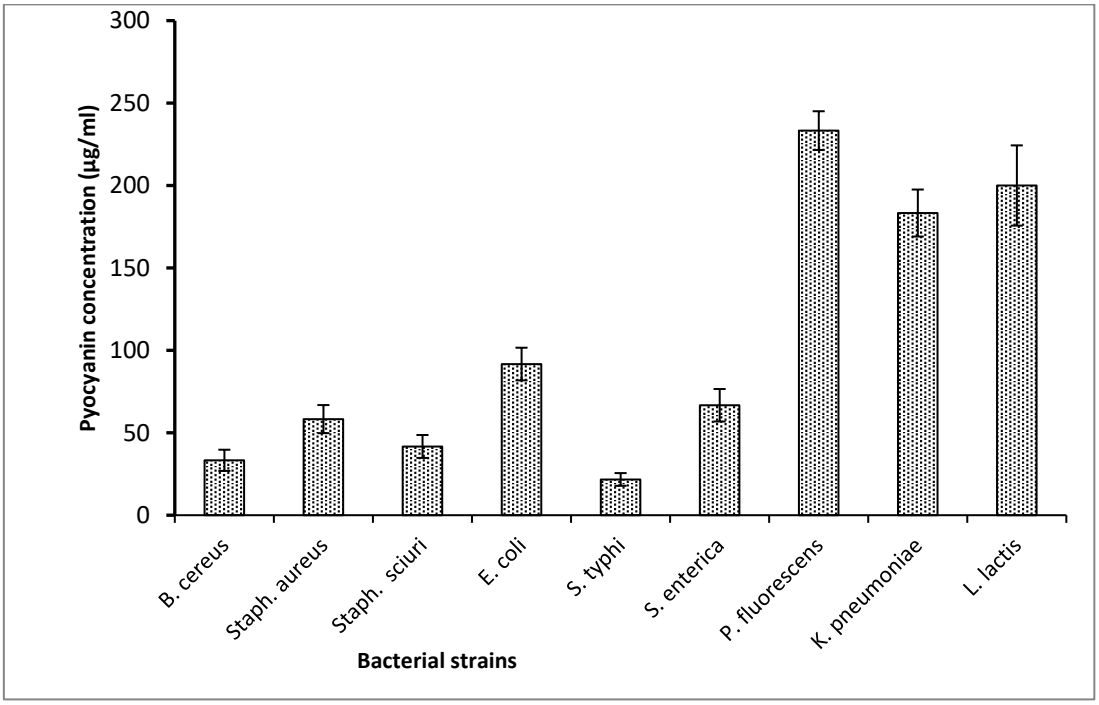

Figure 5. Minimum Inhibitory concentration of pyocyanin against different strains of foodborne pathogenic bacteria.

Wilson et *al.* [44] reported that pyocyanin, which is produced by *P. aeruginosa*, has the ability to disrupt the electron transport chain of fungi, and this reveals the antifungal effect. Kerr [45] reported that *P. aeruginosa* inhibited the growth of *Candida albicans in vitro*.

Table 2. Antifungal activity of different concentrations of pyocyanin against different mycotoxigenic fungi.

Fungi	Inhibition zone mm (Mean±*S.E)						
	Negative control	Positive control	5 mg ml^{-1}	2.5 mg ml^{-1}	1 mg ml^{-1}	0.5 mg ml^{-1}	0.1 mg ml^{-1}
A. flavus	0	12.8±0.86^{b}	14.8±1.28^{a}	11.2±1.12^{c}	8.2±0.36^{d}	8.0±1.00^{d}	7.2±0.58^{e}
A. parasiticus	0	16.0±1.00^{a}	16.5±1.04^{a}	10.5±0.96^{b}	8.7±0.58^{c}	7.5±0.21^{d}	0
A. carbonarus	0	15.8±0.48^{a}	12.6±0.86^{b}	12.0±1.00^{bc}	11.8±1.11^{c}	10.3±1.21^{d}	7.6±0.67^{e}
A.niger	0	15.4±0.76^{a}	11.8±0.75^{b}	9.5±0.32^{c}	8.7±0.86^{cd}	8.0±1.00^{d}	0
A. stynii	0	13.2±0.36^{bc}	17.6±1.58^{a}	14.2±1.08^{b}	12.6±0.58^{c}	12.0±1.00^{c}	8.7±0.48^{d}
A. ochraceus	0	15.4±1.04^{a}	10.8±0.48^{b}	9.7±1.14^{c}	7.8±0.21^{d}	7.0±0.50^{d}	0
A. westerdijikia	0	16.6±0.92^{a}	13.2±1.21^{b}	11.8±0.86^{c}	8.4±0.58^{d}	8.2±1.04^{d}	0
F. proliferatum	0	11.5±0.86^{b}	16.7±1.08^{a}	9.2±0.58^{c}	8.8±0.86^{c}	7.5±0.50^{d}	0
F. verticillioides	0	13.5±0.58^{a}	10.4±0.48^{b}	8.8±0.28^{c}	8.0±1.00^{d}	7.7±0.48^{d}	7.2±0.21^{e}
P. verrucosum	0	15.8±1.14^{a}	14.7±1.28^{b}	9.6±1.58^{c}	8.8±1.14^{d}	8.2±0.76^{e}	8.0±1.00^{e}

n=3, *S.E: standard error, different subscripts within the row are significantly different at the 5% level, 0: No inhibition, negative control: DMSO, positive control: Nystatin.

Also, Kerr *et al.* [46] revealed that *Pseudomonas* spp. produces a variety of metabolites, of which some exhibit the antimicrobial activity of which antimicrobial substances pyrrolnitrin has been known to possess antifungal activity. Karpagam *et al*. [13] found that pyocyanin produced by *P. aeruginosa* possesses antifungal activity against *Candida albicans, C. krusei, C. glabrata, C. tropicalis* and *Cryptococcus neoformans*. Anjaiah et al. [47] reported that *P. aeruginosa* PNA1 produced phenazine antibiotics, pyocyanin, and phenazine-1-carboxylic acid, when grown in culture and inhibited the mycelial growth of *Fusarium oxysporum*, *Penicillium splendens* and other phytopathogenic fungi. Sudhakar *et al*. [27] indicated that pyocyanin from *P. aeruginosa* WS1 had MIC activity about 64 µg ml^{-1} against *Aspergillus flavus* and *A. fumigatus* and 128 µg ml^{-1} against *Candida* sp.

Hassanein *et al*. [48] reported that *Pseudomonas aeruginosa* Sha8 chloroform extract had antifungal activity against *Aspergillus niger, Helminthosporium* sp., *Fusarium oxysporium, Candida albicans*, and *Saccharomyces cerevisiae*. Bakthavatchalu *et al.* [49] reported that pyocyanin produced by *P. aeruginosa* plays an essential role as an indicator of phytopathogens pathogens like *R. solani*. Jayaseelan *et al.* [50] found that pyocyanin extract of *P. aeruginosa* was isolated from rhizosphere soil can be used as biosupplement antagonism against fungal rice pathogens such as *Helminthosporium oryzae*, *Pyricularia oryzae* and *Rhizoctonia solani*. Audenaert *et al*. [51] reported the role of pyocyanin from *P. aeruginosa* 7NSK2 strain, in inducing resistance to Botrytis cinerea that causes infection in tomato and grapevine. Mallesh [52] found that *Pseudomonas* strains isolated from the rhizosphere plant were used to treat against various species of *Fusarium*, *Ralstonia* and *Meloidogyne* which cause wilting disease in coleus and ashwagandha species. Also, Audenaert *et al*. [51] reported that pyocyanin from *P. aeruginosa* 7NSK2 strain-induced resistance to *Botrytis cinerea* that causes infection in tomato and grapevine.

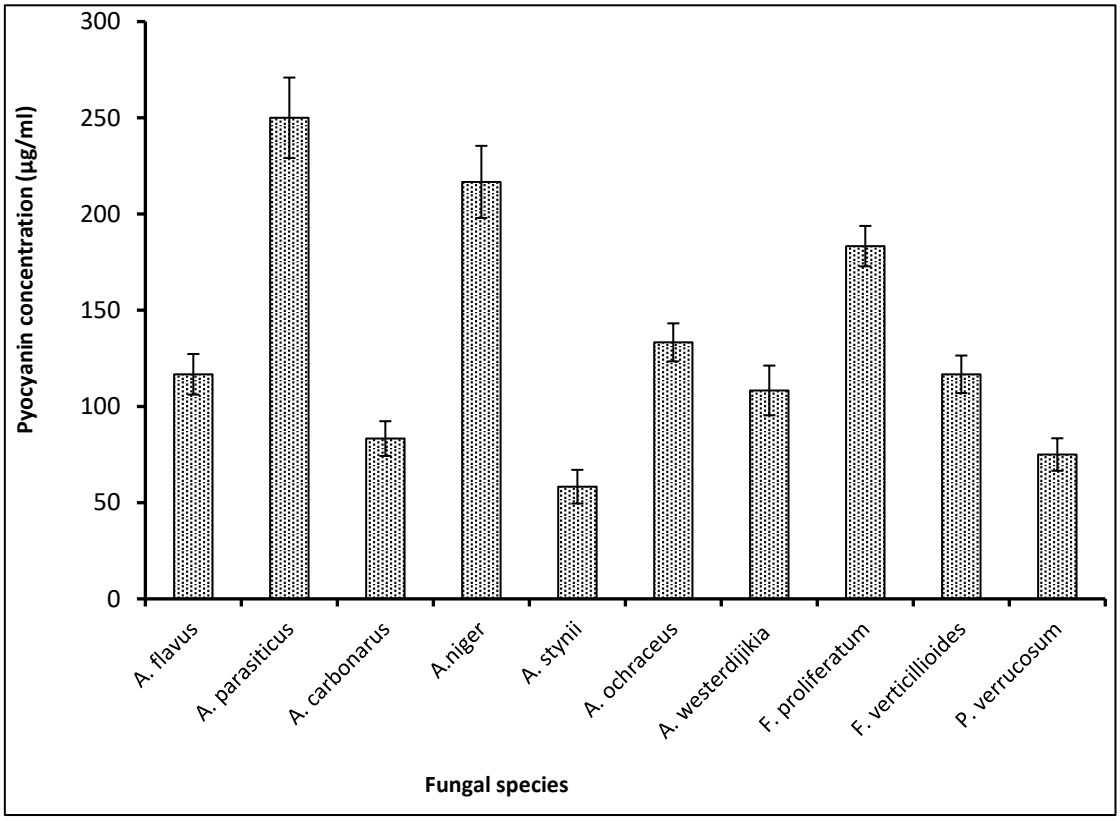

Figure 6. Minimum Inhibitory concentration of pyocyanin against different strains of Mycotoxigenic fungi.

3.2.4. MIC of pyocyanin against mycotoxigenic fungi.

As shown in Figure (6) the minimum inhibitory concentration of pyocyanin against 10 species of mycotoxigenic fungi. The lowest MIC value of purified pyocyanin (58.3 µg ml^{-1}) was observed against *A. styni* followed by *P. verrucosum* and *A. carbonarus* with MIC values 75.0 and 83.3 µg ml^{-1}, respectively. Whereas the highest MIC was recorded by *A. parasiticus*, *A. niger*, *F. proliferatum* and *A. ochraceus* with values 250, 216.7, 183.3, and 133.3 µg ml^{-1}, respectively. Purified pyocyanin recorded the same MIC value (116.7 µg ml^{-1}) against both *A. flavus* and *F.*, *F. verticillioides*, while *A. westerdijikia* needed 108.3 µg ml^{-1} to observe MIC value by pyocyanin.

Kerr *et al.* [46] reported that the significant antifungal agent of *P. aeruginosa* was found to be pyocyanin and pyocyanin MICs for *Candida albicans* and *Aspergillus fumigatus* were about 64 µg ml^{-1}. Also, Sudhakar *et al*. [27] indicated that pyocyanin from *P. aeruginosa* WS1 had MIC activity about 64 µg ml^{-1} against *Aspergillus flavus* and *A. fumigatus* and 128 µg ml^{-1} against *Candida* sp. Rane *et al.* [39] reported that the minimal inhibitory concentration of pyocyanin was 29 µg/ml for *Sclerotium rolfsii* and also inhibited the growth of phytopathogens such as *Aspergillus niger*, *Fusarium oxysporum*, *S. rolfsii* and *Colletotricum falcatum*.

3.3. Toxicity evaluation of pyocyanin.

3.3.1. Brine shrimp bioassay.

The use of aquatic organisms for biomonitoring is an important tool in aquatic ecotoxicology, allowing the detection and evaluation of the potential toxicity [53]. Brine shrimp bioassay was used firstly as an easy test to detect toxicity; however, it is not a specific test. Pyocyanin-induced toxicity on brine shrimp was studied for 12 and 24 hours by exposing 10 brine shrimp larvae to different concentrations of pyocyanin. Percentage mortalities amongst groups of brine shrimp exposed to pyocyanin for 12 and 24 hours are shown in Fig. (7). LC_{50} values of pyocyanin on brine shrimp nauplii were 368.34 ± 41.67 µg ml^{-1} and 292.11 ± 33.24 µg ml^{-1} for exposure times of 12 and 24 h, respectively. At higher concentrations (500, 750, and 500 µg ml^{-1}) within 12 and 24 hours of exposure, the toxicity was 100%, indicating that the toxicity was concentration and time-dependent.

Environmental isolates of *P. aeruginosa* and the inhibitory compound produced by them (pyocyanin) have been accepted as probiotics as well as antagonistic compounds against pathogenic *Vibrio* spp. in aquaculture [54-56]. The potential degradation and the environmental detoxification of pyocyanin and its precursors have been well reported [57,58]. Yang et *al*. [59] revealed that phenazine-1-carboxylic acid (PCA) got degraded entirely within 40 hours by soil organism *Sphingomonas* sp. DP58. However, the pyocyanin induced toxicity is still a controversial issue as the studies have been mainly performed with clinical isolates focusing on its dimensions clinically [10].

Brine shrimp nauplii and shrimp larvae and post-larvae have been used for toxicity assays in a dose-response manner [60, 61]. In literature, no study has ever been reported on the toxicity of pyocyanin on biological systems other than the study conducted by Chythanya *et al*. [62] on the toxicity of chloroform extract of *Pseudomonas* 1-2 in *P. monodon* post-larvae PL-18 and Preetha *et al*. [56] on *in vitro* toxicity studies of pyocyanin from *Pseudomonas* MCCB102 in primary hemocyte culture of *P. monodon*.

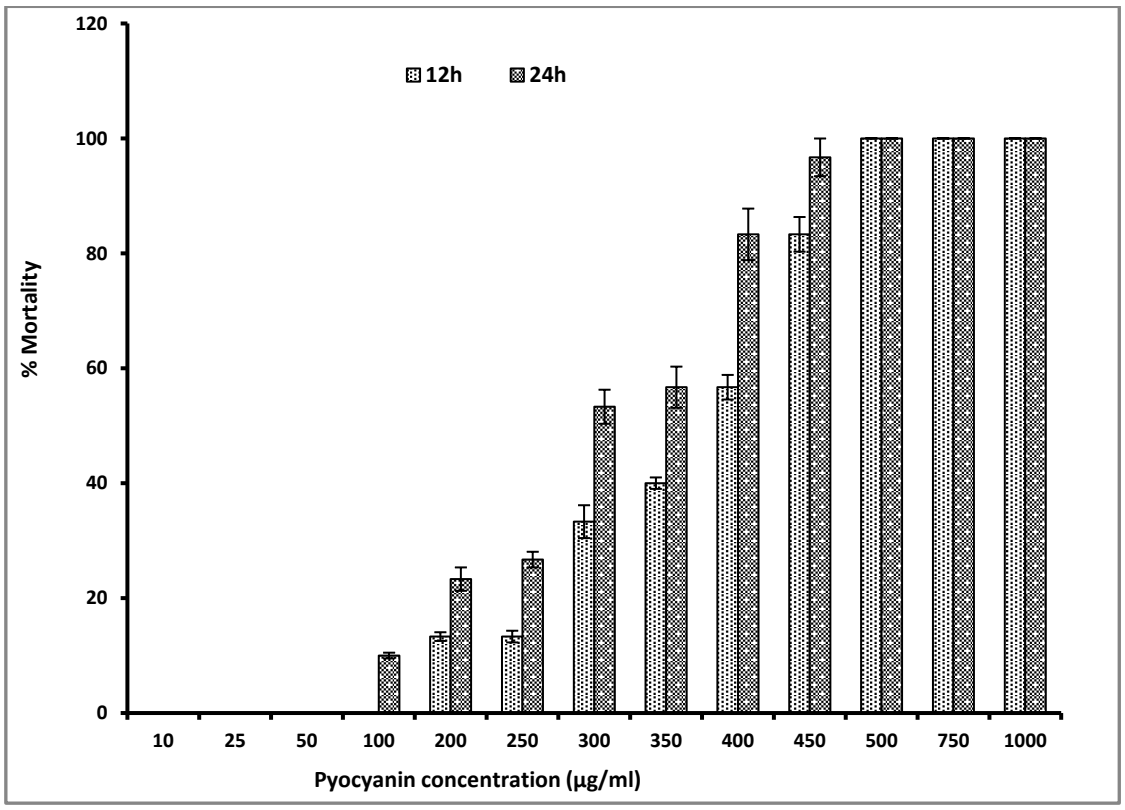

Figure 7. Brine shrimp assay using *Artemia salina* against pyocyanin.

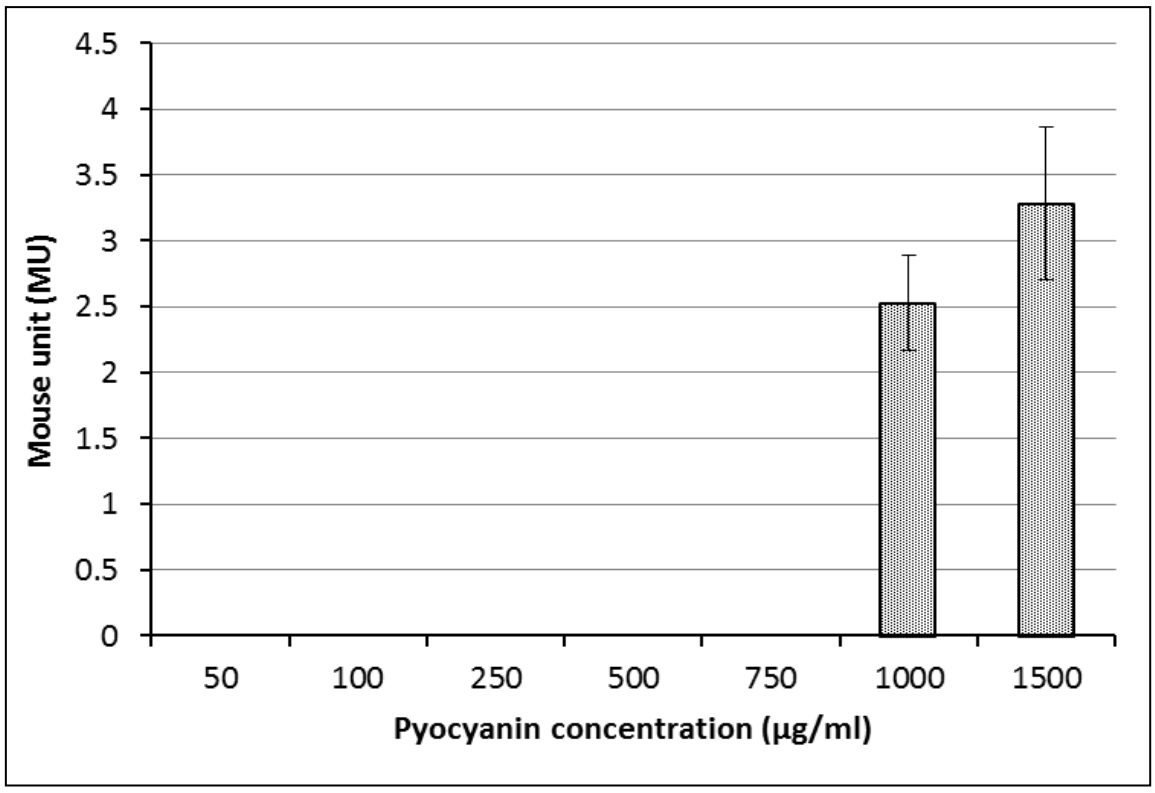

Figure 8. Mouse bioassay of pyocyanin produced by *P. aeruginosa.*

Vijayan *et al*. [63] studied the pathogenicity of environmental isolates of *Pseudomonas* PS-102 and reported that it did not cause any lethality to shrimp larvae (Pl-9) upon challenge even at a dosage of 10^7 cells, and a higher LD_{50} to BALB/c mice (10^9 cells) suggested its safety to the mammalian system. Subsequently, this isolate was deposited with the Culture Collection of National Central for Aquatic Animal, Cochin University of Science and Technology, India,

as *Pseudomonas*MCCB102, and the same could be used by Preetha *et al*. [56] and concluded that the inhibitory compound produced by the organism was pyocyanin. In the present study, *Pseudomonas* MCCB102 has been identified as *Pseudomonas aeruginosa*. This has been commercialized as the probiotics preparation PS -1TM for shrimp aquaculture systems against pathogenic *Vibrio* spp.

3.3.2. Mouse bioassay of pyocyanin.

The toxicity of pyocyanin was tested using the mouse bioassay technique (Fig. 8). The highest toxicity 2.53 MU (mouse unit) was recorded using purified pyocyanin at a concentration of 1000 µg ml^{-1}, and 3.28 MU was observed at a concentration of 1500 µg ml^{-1}. On the other hand, there were no toxic effects and symptoms observed in the mice that injected with pyocyanin at the concentration 50, 100, 250, 500, and 750 µg ml^{-1} (Figure 8).

4. Conclusions

The present study revealed that pyocyanin showed abroad spectrum antimicrobial activity against foodborne pathogenic bacteria and mycotoxigenic fungi. Also, based on the toxicity evaluation, brine shrimp assay, and mouse bioassay, pyocyanin can use food industries application as a promising source in food colorant and food biopreservatives ingredients.

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Conflicts of Interest

The authors declare no conflict of interest.

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