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Pyocyanin Isolated from *Pseudomonas aeruginosa*: Characterization, Biological Activity and Its Role in Cancer and Neurodegenerative Diseases

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HIGHLIGHTS

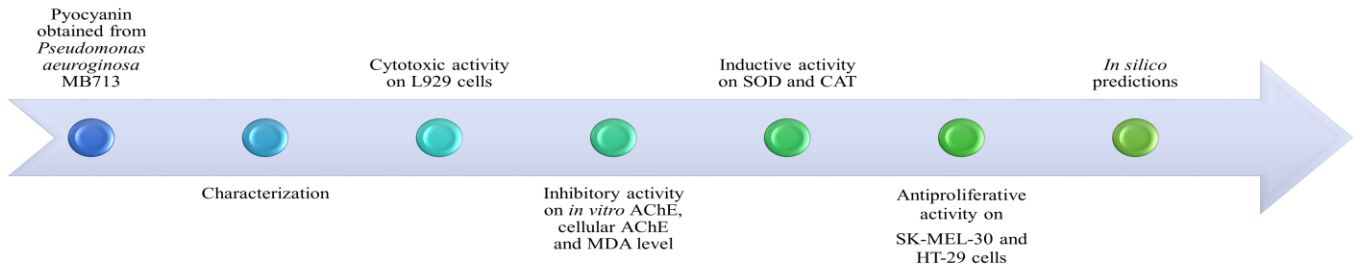
- The maximum production of the pyocyanin was found to occur in MFDM and with shaking.
- UV-VIS spectroscopy, GC-MS, and FT-IR analyses showed the presence of pyocyanin.
- Pyocyanin had no cytotoxic impact on L929 cells (except 50-100 µM concentrations).
- Pyocyanin had an antiproliferative impact on SK-MEL-30 cells.
- Pyocyanin had in vitro and cellular AChE inhibitory and antioxidant properties.
- The use of the pyocyanin as a neuroprotective agent or an anticancer agent is advised.

Abstract: This study aimed to find a pigment of bacterial origin with protective/preventive activity against neurological injury and carcinogenesis, which may be used as a natural food additive in food activity or an active drug ingredient of an anti-neural injury/anticancer agent in the pharmaceutical industry. Within this scope, the *Pseudomonas aeruginosa* MB713 strain was used to produce pyocyanin. Characterization of the pyocyanin was carried out using UV-VIS, FT-IR, and GC-MS analyses. In addition, its inhibitory action on AChE and antioxidant activities were determined. The cytotoxic activities of the pyocyanin as well as its antiproliferative activities were detected. It was determined that the maximum production of the pyocyanin (51 µg/mL). The pyocyanin did not cause a cell death rate above 50% in L929 cells. The pyocyanin was found to exert an inhibitory effect on the AChE. The pyocyanin was found effective on all antioxidant parameters tested. The IC_{50} values of the pyocyanin on SK-MEL-30 and HT-29 cells were calculated as 72 and 179 µM, respectively. And due to the pyocyanin's antioxidant and inhibitory effects on AChE, antiproliferative effects, and protective effects against neurodegenerative injury, the use of the pyocyanin as

an active drug ingredient or a preservative food additive may be predicted. This situation can be clarified by conducting advanced molecular studies.

Keywords: Biological activity; Cancer; FT-IR spectroscopy; GC-MS analysis; Neurodegenerative diseases; *Pseudomonas aeruginosa*; Pyocyanin; UV-VIS specroscopy.

GRAPHICAL ABSTRACT



INTRODUCTION

Cancer encompasses various diseases with different pathobiologies that result from permanent homeostatic and functional abnormalities [1]. It is the second leading cause of mortality around the world, being responsible for an annual number of deaths exceeding eight million. Moreover, it is expected to be 50% more prevalent within several decades [2]. It is increasingly clear that, apart from the neuronal functions of acetylcholinesterase (AChE), it also regulates cell proliferation, differentiation, apoptosis, and cell-cell interaction. Aberrant AChE expression has been shown in a number of cancers, suggesting that AChE takes part in the regulation of oncogenesis. In light of this information, it is plausible to use some AChE inhibitors as anticancer drugs [3].

A variety of human diseases including neurodegenerative diseases increase their incidence with aging and thus affect increasingly more elderly populations each passing day. Alzheimer's disease (AD) and other neurodegenerative diseases cause progressive neuronal loss, motor and cognitive dysfunction, and abnormal protein aggregates [4]. Several neurodegenerative disorders are closely linked to abnormal AChE expression. Mounting evidence suggests that AChE degrades cerebral acetylcholine (ACh), which in turn plays a central role in AD by hastening the aggregation of amyloid beta peptides into fibrils. Indeed, symptoms of AD are mainly treated by aiming to inhibit AChE activity to increase cerebral ACh levels, thereby promoting cholinergic signal conduction [5].

Oxidative stress is traditionally defined as a relative increase in the levels of reactive oxygen species (ROS) compared with those of antioxidants; it has been related to oncogenesis, neuronal degeneration, and a variety of other disorders [6]. Malondialdehyde (MDA) is considered an important endogenous source of oxidative stress; it reportedly induces cellular mutations in humans and potentiates other substances' detrimental activities [7]. Humans possess particular defense systems against oxidative stress, including the enzymes superoxide dismutase (SOD) and catalase (CAT). The expression of SOD and CAT can be triggered or augmented by some natural substances [8].

Traditional anticancer and antineurodegenerative treatments are not only partially effective, but they are also sometimes more detrimental than beneficial due to their adverse effects. Since bacterial pigments offer a natural, safe, and nutritionally rich vitamin like alternative to chemical drugs, the scientific interest in them has been increasing recently [11]. Bacterial pigments are specific to bacterial strains, having different colors, chemical characteristics, and mechanisms of action [9,10]. The pyocyanin is an extracellular pigment derived from phenazine produced by *Pseudomonas aeruginosa* as a secondary metabolite, which contains nitrogen ($C_{13}H_{10}N_2O$) and is soluble in aqueous solutions. It exerts a variety of biological actions including antimicrobial, anticancer, antimalarial, antiparasitic, immunosuppressive, antioxidant, and antibiofilm properties [11]. These features of the pyocyanin make it an attractive candidate for use in medical, pharmaceutical, food, textile, biocontrol, nanotechnology, and physicochemical fields [12]. Its activities on neurodegenerative disorders, however, have not been studied.

This study, on the basis of the aforementioned properties of the pyocyanin, thus aimed to study the anticancer and antineurodegenerative activities of pyocyanin derived from *Pseudomonas aeruginosa* on HT-29 human colon cancer, SK-MEL-30 human melanoma, and SH-SY5Y human neuroblastoma cells. Pyocyanin was characterized to determine its structure and possible effects. We contributed to the insight

and knowledge of pyocyanin's anticancer effects and protective properties against neurodegenerative diseases.

MATERIAL AND METHODS

Microorganism

We obtained and cultured the MB713 strain of *Pseudomonas aeruginosa* from Gazi University, Faculty of Science, Department of Biology, Biotechnology Laboratory Collection. The culture was a nutrient broth (NB) (Merck) culture; the strain was then kept in NB with glycerol (Sigma-Aldrich) at -80°C and subjected to the regeneration process twice before its use. Aerobic conditions were used for incubation; the medium temperature was 37°C and the incubation period was 16-18 h, and it was shaken at 150 rpm.

Extraction, partial purification and quantitative assay of pyocyanin

The pyocyanin production was performed with an initial concentration of 10^8 CFU/mL of the strain. Sterile NB and modified Frank and De Moss (MFDM) were used to dilute the strain to a 2:100 ratio, followed by its 6-day incubation at 30°C under shaking and non-shaking conditions at 150 rpm. At the end of incubation, the culture was centrifuged at 4,200 rpm for 25 min, followed by the separation of the supernatant and the addition of a 1:2 ratio of chloroform (Merck). After the new solution was poured into a new test tube, 0.2 M hydrochloric acid (HCl) (Merck) was added. The mixture was then admixed with 0.4 M borate (Merck)-sodium hydroxide (NaOH) (Merck) until the formation of a blue layer. Thereafter, a 1:2 ratio of chloroform was added and a 2 hour incubation was repeated. The resulting mixture in the supernatant was then placed into a 500 mL vacuum rotary flask to concentrate it in a vacuum rotary evaporator at 45°C. The pyocyanin concentration was calculated as µg/mL according to the formula: The pyocyanin concentration (µg/mL) = Optic density $(OD)_{520}$ × 17.072. The molecular weight of the pyocyanin pigment was used to perform the molar calculation. The analysis of partially purified pyocyanin was carried out with thin layer chromatography (TLC) with the help of a silica gel 60 F254 aluminum sheet (Merck). The mobile phase used in the process was chloroform, with 200 ng purified pyocyanin being used on the TLC plate [13-17].

Characterization

UV-VIS spectroscopy

A solution of 2 mg the pyocyanin was prepared in 95% ethanol (Merck) or pure water, and its UV-VIS absorption spectrum was determined over the range of 200–800 nanometer (nm) with the help of a UV-1800 spectrophotometer (Shimadzu). The analysis was performed by Düzce University, Scientific and Technological Research and Application Center, Environmental and Chemistry Laboratory, Düzce, Turkey [18].

FT-IR spectroscopy

In order to investigate different functional groups, the FT-IR spectrum for the pyocyanin was determined by Shimadzu IRprestige-21 in transmittance mode. Compressed discs with a diameter of 3 mm were formed by the addition of 2 mg pyocyanin to 200 mg potassium bromide (KBr) (Merck), and the spectrum was corrected for the KBr background. This was followed by scanning of the pellets in the range of 3500-750 cm^{-1} using a resolution of 4 cm^{-1} and 32 scans. The analysis was performed by Düzce University, Scientific and Technological Research and Application Center, Environmental and Chemistry Laboratory, Düzce, Turkey [18].

GC-MS analysis

GC-MS analysis of pyocyanin was performed by Agilent 6890N Network GC system combined with Agilent 5973 Network mass selective detector system, under these conditions: column, HP Innowax FSC (60.0 m × 0.25 mm × 0.25 µm); oven temperature program, the column held initially at 65 °C; then increased to 200 °C with a 6 °C/min heating ramp and kept for 5 min and increased to 250 °C with 6°C/min heating ramp and kept for 30 min. The injection was operated in splitless mode. The rest of the conditions were as follows; injector temperature: 250 °C; carrier gas: He; and injected volume: 2.0 µL; ionization energy: 70 eV and mass range: 34–450 atomic mass units. The identification of the peaks were performed by using computer matching of mass spectral database using library search systems (WILEY7 and NIST libraries).

In vitro AChE inhibitory activity

The anti-AChE activity of the pyocyanin was quantified by the modified Ellman's method. The spectrophotometric assay was used to assess the inhibitory activity of the pyocyanin on the AChE enzyme (Sigma-Aldrich) originated from an electric eel. The substrate was acetylthiocholine iodide (Sigma-Aldrich). 80 µL of phosphate buffer with a pH of 7 (Merck) was transferred into each test tube. Then, test sample solutions with concentrations of 10, 25, 50, 75, and 100 µM were added first, followed by the addition of 20 µL enzyme. The resulting admixture was kept in an erect position for a duration of 10 minutes. The coloring reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was added to yield a yellow anion color of 5-thio-2-nitrobenzoic acid (Sigma-Aldrich). Then, 50 µL substrate was added to the left for 10 min incubation. The absorbance level was determined at 412 nm. The percentage of the inhibitory action of the pyocyanin was determined by the formula: AChE inhibition activity (%) = Absorbance (control) – Absorbance (test) / Absorbance (control) × 100 [19,20].

Cell culture

Cell cultures of HT-29 human colon cancer and SH-SY5Y human neuroblastoma cells were performed using Dulbecco's modified eagle medium (DMEM) (Gibco) that was added 10% heat inactivated fetal bovine serum (FBS) (Gibco), 1% penicillin streptomycin (Gibco), 1% L-Glutamine (Sigma-Aldrich) and 40% MCDB-201 (Sigma-Aldrich). Cultures of L929 healthy mouse fibroblast cells were cultivated in a DMEM culture medium that was added 10% heat-inactivated FBS and 1% penicillin streptomycin. Cultures of SK-MEL-30 human melanoma cells were cultivated in a DMEM culture medium that was added 10% heat inactivated FBS, 1% penicillin streptomycin, and 1% nonessential amino acid (Sigma-Aldrich). Humidified atmosphere composed of 5% carbon dioxide (CO_2) and 95% air at 37°C was used to maintain the cultures, with the culture medium having been renewed at 2-3 day intervals. HT-29 human colon cancer, L929 healthy mouse fibroblast, SH-SY5Y human neuroblastoma, and SK-MEL-30 human melanoma cells were seeded into 96 plate or 6 plate inserts (Corning); after equilibrium was reached at 24 h, the experiments were begun. The pyocyanin solution in serum free medium at different concentrations (10, 25, 50, 75, and 100 µM) was used to treat cells for 18 h. The control cells were supplemented with the same medium [21-23].

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was assessed by the MTT assay. After incubation with pyocyanin, serum free media containing 0.5 mg/mL of MTT (Sigma-Aldrich) was used to replace all media in 96 well plates. The plate was subjected to incubation further with 5% CO_2 for 4 h at 37°C; MTT solution was then removed, and the cells were lysed with 200 µL dimethyl sulfoxide (DMSO) (Sigma-Aldrich) in the respective order. The absorbance of the plate at 570 nm was read by a microplate reader (Biotek Instruments) after the plate was shaken by an orbital shaker (VWR) for 30 min. The absorbance values were determined as percent absorbance of the untreated control cells (control = 100%) [24-28].

Cellular AChE inhibitory activity

Initially, SH-SY5Y human neuroblastoma cells were seeded and incubated for a period of 18 h with 10, 25, 50, 75, and 100 µM pyocyanin; they were then subjected to 150 µM hydrogen peroxide (H_2O_2) (Merck) again for 18 h. They were then incubated at 37°C and 5% CO_2, and all cells were irrigated twice with 300 µl phosphate buffered saline (PBS) (Merck) at a pH of 7.4. A sterilized scrapper (Gibco) was used to detach the cells; a 25 mmol/L Tris (Sigma-Aldrich)–HCl lysis buffer lysed all the cells by not using any protease or phosphatase inhibitor. The cell lysate was sonicated on ice (10 sec pulse), followed by the centrifugation of the homogenate at 11.000 rpm for 15 min as the final step. The resultant cell lysate was picked up and preserved at −20°C for analyses of cellular AChE inhibitory activity. Protein concentration of the cell lysate was measured by a standard method [19,20].

SOD, CAT activities, and MDA level

As for the activities of SOD and CAT, and the MDA level of the SH-SY5Y human neuroblastoma cells, the cells were treated with 150 µM of H_2O_2 for 18 h after being seeded and incubated with 10, 25, 50, 75, and 100 µM pyocyanin for 18 h. After incubation with 5% CO_2 at 37°C, all cells were irrigated twice with 300 µL PBS at pH 7.4. The cells were then detached using a sterilized scrapper and lysed by a 25 mmol/L Tris–HCl lysis buffer without employing any protease or phosphatase inhibitor. In the final steps, the homogenate was sonicated on ice (10 sec pulse) and centrifuged at 11.000 rpm for 15 min. The resulting cell lysate was

picked up and stored at −20°C until studies for SOD, CAT activities, and MDA level, as per instructions provided by the manufacturer (Cayman Chemical and FineTest). The cell lysate's protein concentration was measured with a standard method [29].

AdmetSAR predictions

AdmetSAR (ADMET structure activity relationship) database serves as an online database on the mutagenicity, carcinogenicity, and toxicity of candidate drugs. This information makes a significant contribution to the discovery of novel agents [30,31].

The biological and pharmacological utilities of a drug candidate which reflects the interaction of the drug candidate with various biological entities can be explored using prediction of probable activity spectra of substances (PASS) analysis, a computer based online software. This helps to screen the biological activities of chemical structures based on the structure activity relationship. The biological activity is expressed in terms of probability values probable activity (Pa) and probable inactivity (Pi). It has been proposed that structures with Pa greater than Pi were the only compounds considered for a particular pharmacological activity [32].

Statistical analysis

SPSS version 21.0 was used for all statistical analyses. Descriptive statistics were reported as mean ± standard deviations (SDs). One-way analysis of variance (ANOVA) test with posthoc Tukey's test was used to perform inter group analyses. A p value of less than 0.05 was considered statistically significant. Reproducible results of at least three independent studies were obtained and used as the study data.

RESULTS

Quantity of the pyocyanin pigment

In this study, the amounts of the pyocyanin pigment produced by *Pseudomonas aeruginosa* MB713 strain in different growth media (MFDM and NB) and under shaking/non-shaking conditions were determined. The maximum production of the pyocyanin pigment was found to occur in MFDM growth culture and under shaking conditions (51 µg/mL). Statistical analyses determined that NDFM growth medium and shaking condition increased pyocyanin pigment production significantly (p<0.05). The results are presented as the average of n:3 and ± SD in Table 1.

Table 1. The amount of pyocyanin pigment produced by *P. aeruginosa* MB713 strain according to different medium and shaking conditions

Medium	Shaking condition	Quantity of pyocyanin pigment (µg/mL)
NB	Without shaking	10 ± 0.4
NB	With shaking	23 ± 0.8
MFDM	Without shaking	41 ± 0.5
MFDM	With shaking	51 ± 0.7

Characterization of the pyocyanin pigment

UV-VIS spectroscopy analysis

A UV-VIS spectroscopy analysis was performed to determine the wavelength absorption of the pyocyanin pigment produced by *P. aeruginosa* MB713 strain. The UV absorption spectrum images of the pyocyanin pigment in two different solvents, namely pure water and 95% ethanol, are given in Figure 1. The peak produced by the pyocyanin pigment in pure water in the wavelength marked with a black arrow and the peak produced by the pyocyanin pigment in 95% ethanol in the wavelength marked with a black arrow in Figure 1 both indicates the presence of the pyocyanin pigment. The maximum peak of the pyocyanin pigment is observed between 300 nm and 400 nm for both solvents. The maximum peak was determined as 300 nm for water and 370 nm for 95% ethanol.

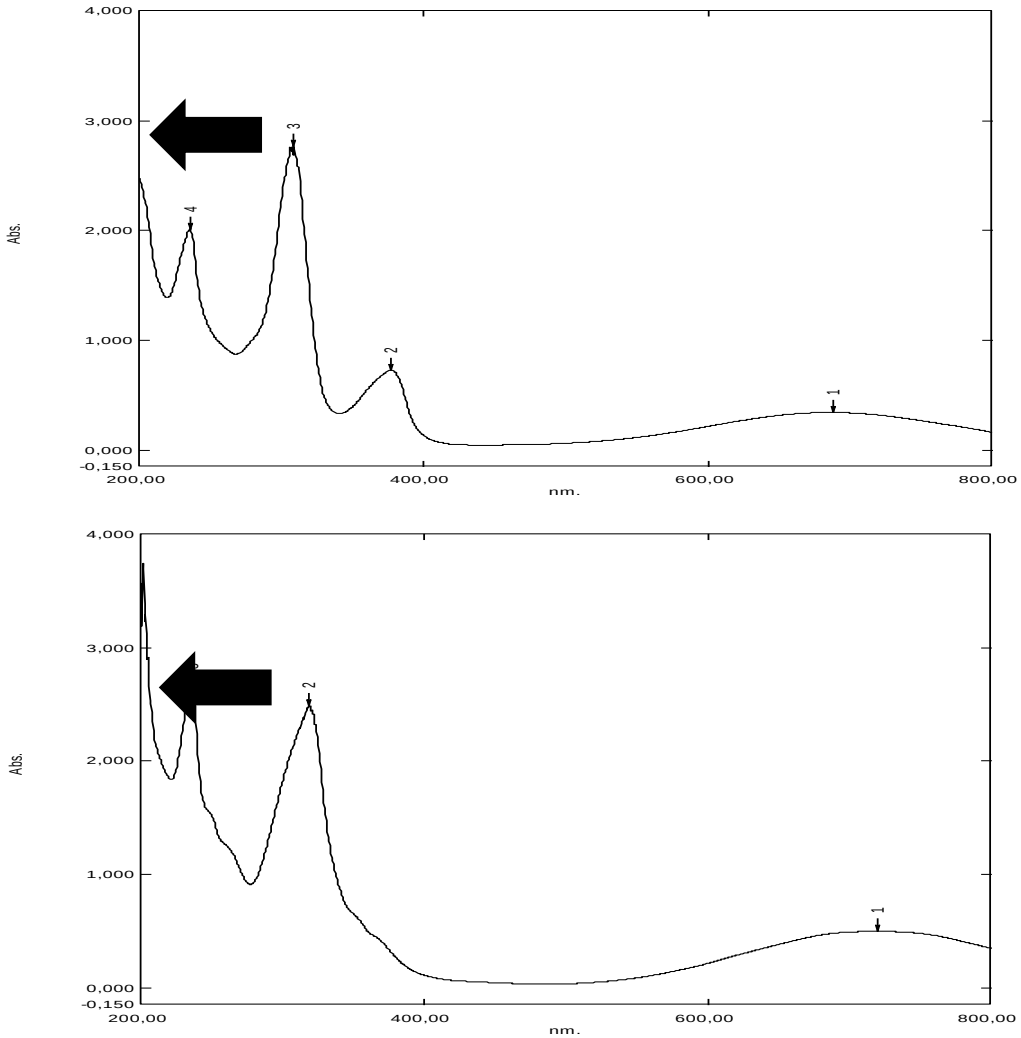


Figure 1. UV absorption spectrum in water (A) and 95% ethanol (B) of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain

GC-MS analysis

GC-MS analysis, an analytic method combining the features of gas chromatography and mass spectrometry, was performed to identify different substances in the pyocyanin pigment produced by *P. aeruginosa* MB713 strain. The analysis of pyocyanin pigment by GC-MS analysis determined dense molecular ion peaks at 168 m/Z and 196 m/Z. While phenazine exhibited a sharp peak at 168 m/Z, 1-hydroxyphenazine (hemi pyocyanin), on the other hand, exhibited a sharp peak at 196 m/Z. The presence of simultaneously detected peaks at 168 m/Z and 196 m/Z in the GC-MS analysis indicates the presence of the pyocyanin pigment converted into 1-hydroxyphenazine (Figure 2).

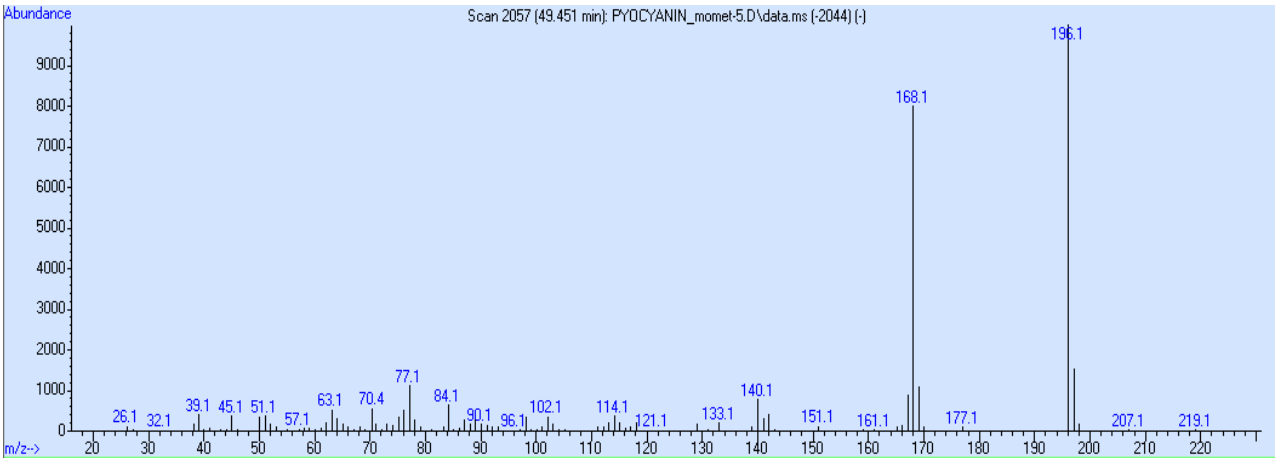


Figure 2. GC-MS spectrum of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain

FT-IR analysis

FT-IR analysis was performed to determine the functional groups of the pyocyanin pigment produced by *P. aeruginosa* MB713 strain. The FT-IR spectrum of the pyocyanin pigment is given in Figure 3. The peak at 3462 cm^{-1} shows the presence of an O-H bond while the peak at 2953 cm^{-1} indicates the presence of a C-H aromatic bond. While the peak shown at 1620 cm^{-1} represents a C=N bond, it exhibited absorption at the regions of 1500-1400 cm^{-1} and 1600-1585 cm^{-1} due to the stretching vibrations of C-C bonds in the C-C aromatic ring. In the presence of CH_3, the C-H bond of the alkyl group (methyl) at 1380-1400cm^{-1} stretched to form a peak. The peak at 1301 cm^{-1} corresponds to the C=O bond. The FT-IR spectrum showed the presence of phenazine and confirmed that this pigment containing aromatic ring was the pyocyanin pigment.

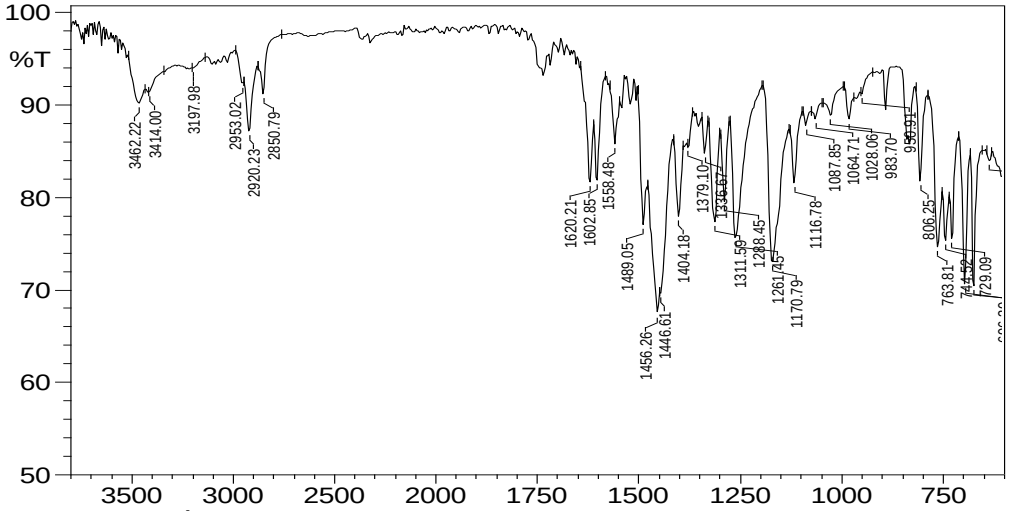


Figure 3. FT-IR spectrum of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain

Cytotoxic activity of the pyocyanin pigment on L929 healthy mouse fibroblast cells

This study aimed to determine with the MTT method whether the pyocyanin pigment would show any cytotoxic activity on L929 healthy mouse fibroblast cells. It was determined that the cytotoxic activity of the pyocyanin pigment increased with its increasing concentrations (10-100 µM (p<0.05)). The IC_{50} value of the pyocyanin pigment was 101 µM. According to these findings, since the pyocyanin pigment did not cause a cell death rate above 50% in L929 healthy mouse fibroblast cells. The results of all studies are given as the average of n:5 and ±SD in Figure 4.

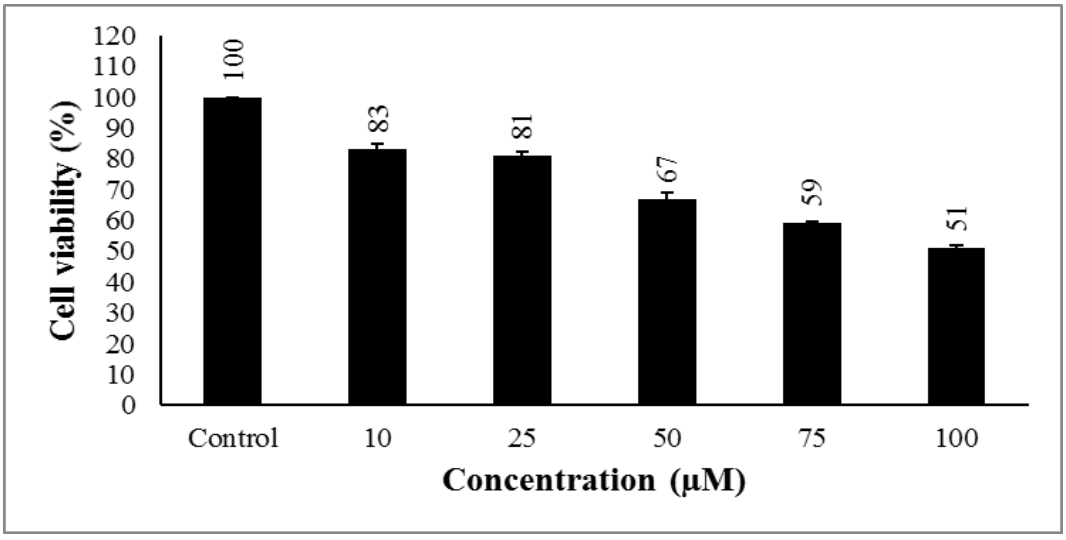


Figure 4. Cell viability of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain on L929 healthy mouse fibroblast cells

Inhibitory activity of the pyocyanin pigment on in vitro AChE enzyme activity

In this study, it was aimed to determine the inhibitory activity of the pyocyanin pigment on *in vitro* AChE enzyme activity using the Ellman method. The inhibitory activity of the pyocyanin pigment on the AChE enzyme, one of the neural death factors in neurodegenerative diseases, was determined, showing that this activity increased with increasing concentrations of the pyocyanin pigment (p<0.05). The maximum inhibitory effect on AChE enzyme activity was observed with the application of 100 µM pyocyanin pigment (38%). The results are shown as the average of n:9 and ±SD in Table 2. Based on the inhibitory action of the pyocyanin pigment on *in vitro* AChE enzyme activity, it can be suggested that the pyocyanin pigment may be used in the treatment of neurodegenerative diseases and cancer.

Table 2. *In vitro* AChE inhibition of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain

Treatments	*in vitro* AChE inhibition (%)					
	Control	10 µM	25 µM	50 µM	75 µM	100 µM
Pyocyanin	0 ± 0	23 ± 0	27 ± 1	31 ± 0	35 ± 0	38 ± 1

Inhibitory activity of the pyocyanin pigment on H_2O_2 stimulated cellular AChE enzyme activity

In this study, it was aimed to determine the inhibitory activity of the pyocyanin pigment on H_2O_2 induced cellular AChE enzyme activity in SH-SY5Y human neuroblastoma cells using the Ellman method. AChE enzyme activity in the H_2O_2 group was increased by 43% compared with the control group. It was determined that the pyocyanin pigment applied in increasing concentrations exerted an inhibitory action on AChE enzyme activity (p<0.05). The pyocyanin pigment showed its highest inhibitory action at a concentration of 100 µM. It was even observed that the pyocyanin pigment at this concentration reduced H_2O_2 induced AChE enzyme activity to a lower level than that of the control group. These results suggest that the pyocyanin pigment significantly suppressed H_2O_2 induced AChE enzyme activity in SHSY-5Y cells, exhibiting a neuroprotective activity against neural injury caused by AChE activity. The results were presented as the average of n:9 and ±SD in Figure 5.

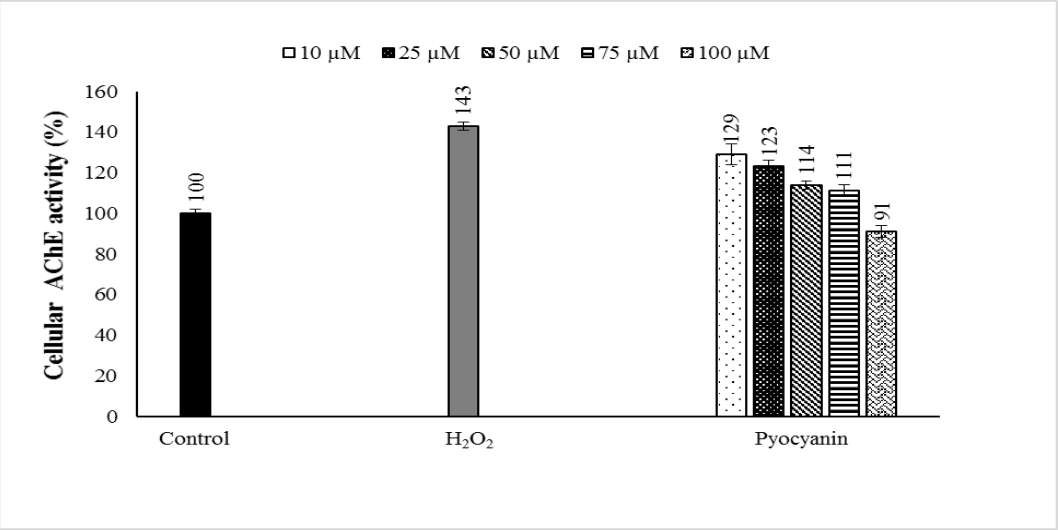


Figure 5. Effect of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain on cellular AChE activity in SH-SY5Y human neuroblastoma cells

Inhibitory activity of the pyocyanin pigment on MDA level against oxidative damage caused by H_2O_2

In this study, it was aimed to determine the suppressive activity of the pyocyanin pigment on MDA level against H_2O_2 induced oxidative injury using an ELISA kit in SH-SY5Y human neuroblastoma cells. The MDA level was induced by H_2O_2 and increased by 29% (p<0.05). Increasing the pyocyanin pigment concentrations suppressed H_2O_2 induced MDA level (p>0.05), which showed that the pyocyanin pigment protected SH-SY5Y human neuroblastoma cells against H_2O_2 induced oxidative injury. The results were presented as the average of n:5 and ±SD in Figure 6.

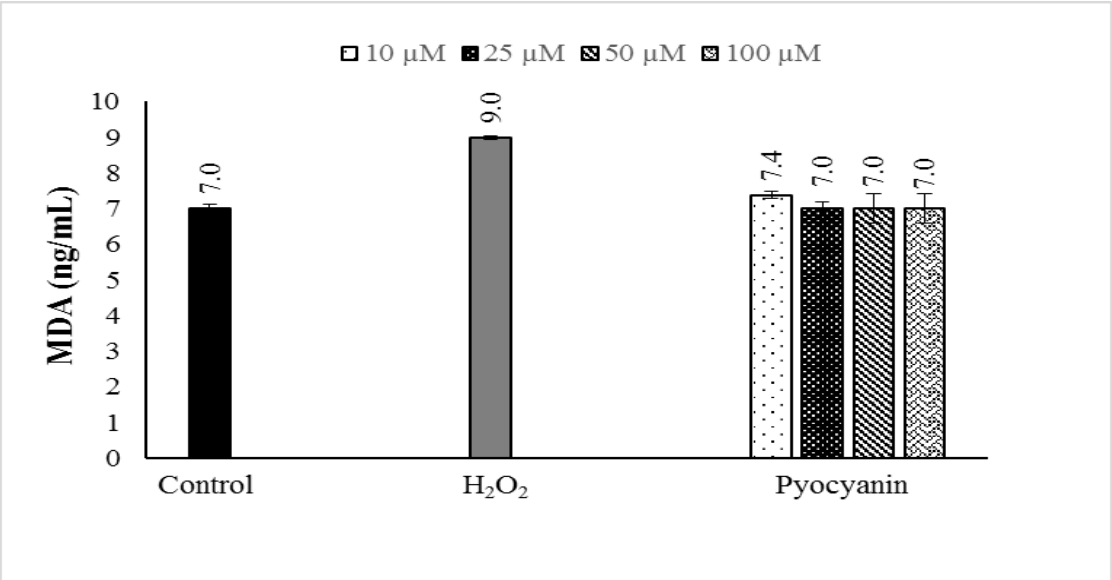


Figure 6. Effect of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain on MDA level in SH-SY5Y human neuroblastoma cells.

Inductive activity of the pyocyanin pigment on SOD and CAT antioxidant enzyme activities against oxidative damage induced by H2O2

In this study, it was aimed to determine the inductive activity of the pyocyanin pigment on SOD and CAT antioxidant enzyme activities against H_2O_2 induced oxidative stress using an ELISA kit in SH-ST5Y neuroblastoma cells. While SOD enzyme activity of the control group that was not treated by any substance other than the cell growth medium was determined 13 U/mL; this activity was found to be reduced to 5 U/mL in 150 µM in H_2O_2 applied cells (p<0.05). It was determined that oxidative stress formed by reduced activities of the SOD and CAT enzymes was eliminated by an increased pyocyanin pigment concentration (p<0.05). It was shown that 100 µM was the concentration at which the best antioxidant activity was seen. SOD and CAT enzyme activities, which were suppressed to 5 U/mL by H_2O_2, were increased by ~50% after the pyocyanin pigment was applied. The results were presented as the average of n:5 and ±SD in Figure 7.

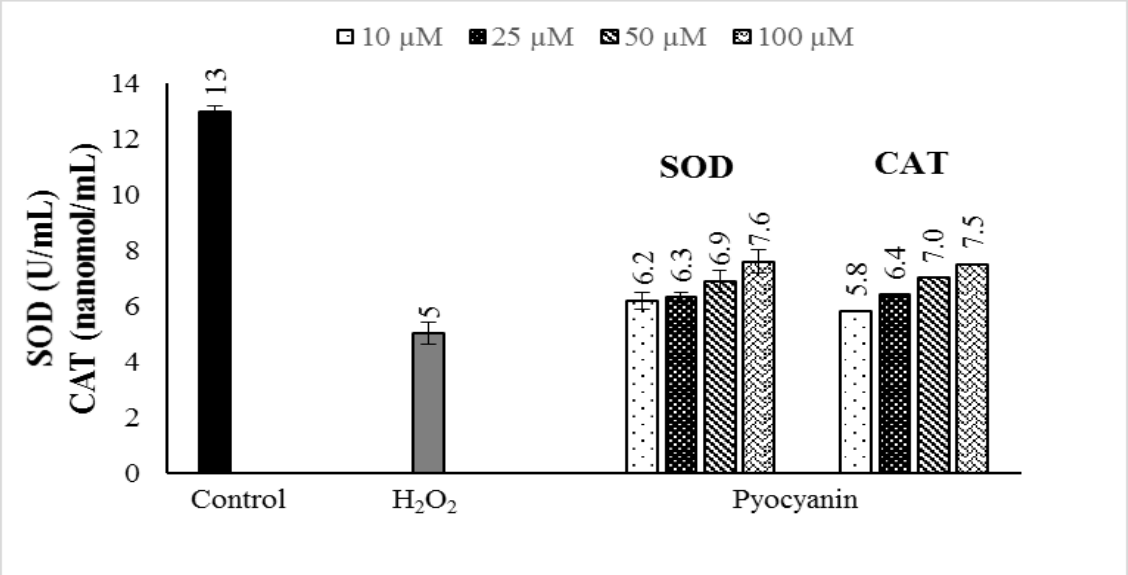


Figure 7. Effect of pyocyanin pigment isolated from *P. aeruginosa* MB713 strain on SOD and CAT enzyme activity in SH-SY5Y human neuroblastoma cells

Antiproliferative activity of the pyocyanin pigment on SK-MEL-30 human melanoma and HT-29 human colon cancer cells

In this study, it was aimed to determine with the MTT method whether the pyocyanin pigment had an antiproliferative activity against SK-MEL-30 human melanoma cells and HT-29 human colon cancer cells. It was determined that the antiproliferative activity of the pyocyanin pigment on SK-MEL-30 human melanoma cells increased as its concentration increased (p<0.05), with the maximum antiproliferative activity being observed for the concentration of 100 µM. The IC_{50} value of the pyocyanin pigment for SK-MEL-30 human melanoma cells was 72 µM. Although it was determined that the antiproliferative activity of the pyocyanin pigment on HT-29 human colon cancer cells increased with increasing concentrations (p<0.05), it was observed that the antiproliferative activity of the pyocyanin pigment on HT-29 human colon cancer cells was negligible. The IC_{50} value of the pyocyanin pigment for HT-29 human colon cancer cells was calculated as 179 µM. The results of all studies were presented as the average of n:5 and ±SD in Figure 8.

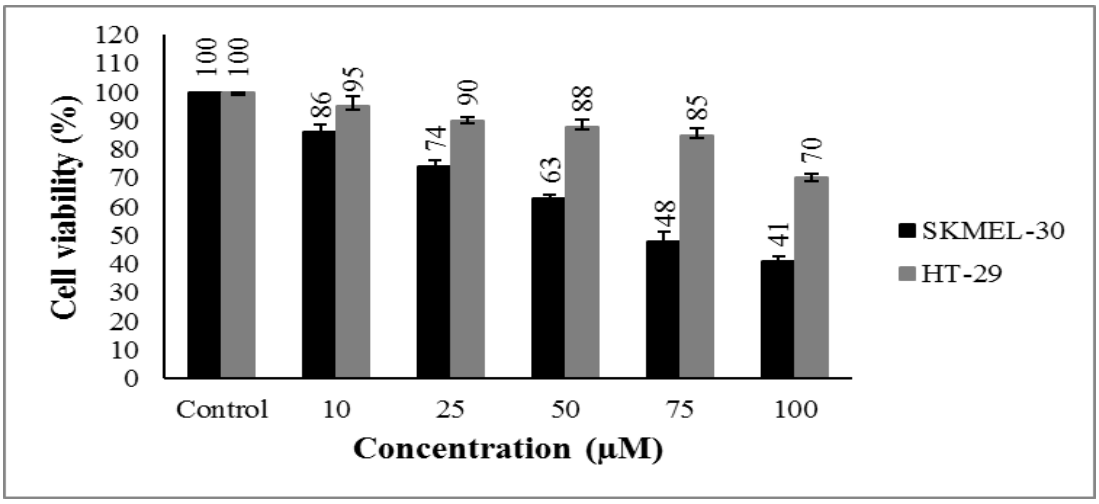


Figure 8. Antiproliferative effects of pyocyanin pigment isolated *from P. aeruginosa* MB713 strain on SK-MEL-30 human melanoma cells and HT-29 human colon cancer cells.

In silico predictions

The results showed that the pyocyanin pigment is non-mutagenic, non-carcinogenic in healthy cells, and non-toxic in acute oral, fish aquatic, and *Tetrahymena pyriformis* (Table 3). After *in silico* predictions are confirmed by experimental-based assays, it is thought that pyocyanin pigment can be used safely as a drug candidate in the treatment of cancer and neurodegenerative diseases.

A rigorous analysis based on structure activity relationship was used to film the pyocyanin pigment *in silico*. Table 4 lists the most likely biological activities of the pyocyanin pigment as determined by a computer based PASS online program.

Table 3. Summary of AdmetSAR studies

Property	Value
Acute oral toxicity	1.63 kg/mol
Ames mutagenesis	-0.5700
Carcinogenicity	-0.9286
Fish aquatic toxicity	-0.8979
Tetrahymena pyriformis toxicity	1.546 $pIGC_{50}$ (ug/L)

Table 4. Summary of PASS online studies

Activities	Pa values	Pi values
Antineoplastic	0.763	0.017
Antineoplastic alkaloid	0.421	0.013
Antineoplastic (brain cancer)	0.310	0.022
Antineoplastic (cervical cancer)	0.578	0.004
Antineoplastic (colorectal cancer)	0.614	0.008
Antineoplastic (lymphocytic leukemia)	0.411	0.008
Antineoplastic (lung cancer)	0.445	0.018
Antineoplastic (melanoma)	0.645	0.004
Antineoplastic (pancreatic cancer)	0.345	0.033
Antineoplastic (renal cancer)	0.352	0.008
Antineoplastic (small cell lung cancer)	0.310	0.025
Caspase 3 stimulant	0.310	0.116
Caspase 8 stimulant	0.362	0.078
Dementia treatment	0.397	0.054
JAK2 expression inhibitor	0.447	0.069
MAP kinase kinase 4 inhibitor	0.317	0.030
Mitochondrial processing peptidase inhibitor	0.378	0.056
MMP9 expression inhibitor	0.318	0.089
Nootropic	0.389	0.228
Preneoplastic conditions treatment	0.380	0.110
TP53 expression enhancer	0.558	0.064

DISCUSSION

Since microbial pigments are easily produced, can be extracted using simple solvents, do not show any cytotoxic and carcinogenic activities on healthy cells, and possess many bioactive properties, their use in food and health industries is considered important [33,34].

In order to determine the medium of maximum pigment production for *P. aeruginosa*'s MB713 strain, NB and MFDM growth media were used in the present study. While there is only a nitrogen source (yeast extract and meat extract) and sodium chloride (NaCl) in the NB growth medium, there are a nitrogen source (L-phenylalanine), a carbon source (glycerol), and inorganic salts ($FeCl_2$, K_2HPO_4, K_2SO_4, and $MgCl_2.6H_2O$) in the MFDM growth medium. Maximum pigment production was determined in the MFDM growth medium (51 µg/mL). It is thought that iron (Fe^{2+}), phosphate (PO_4^{3-}), sulfate (SO_4), and magnesium (Mg^{2+}) compounds induce pigment production in the MFDM growth medium. It has been reported in the literature that the PO_4^{3-} compound added to the medium induces the production of secondary metabolites such as pigments [13,35]. When an adequate amount of SO_4^{2-} ion is added to a growth medium where a low concentration of PO_4^{3-} ion is found, the pyocyanin pigment production is induced [36]. The synthesis of this pigment also appears to be controlled by Fe^{2+} concentration because adding Fe^{2+} to a medium containing low PO_4^{3-} ion induces the synthesis of pyocyanin pigment and related phenazine derivative pigments by other bacteria species [36,37]. Li and coauthors [37] demonstrated a phenomenon where the presence of the Mg^{2+} ion in the medium in addition to these ions also has a positive effect on pyocyanin pigment production. It is reported that the substances found in the growth media enabling a maximum pyocyanin pigment production are Fe^{2+}, PO_4^{3-}, Mg^{2+}, glycerin, leucine, and DL-alanine [13,38,39]. In a study where Barakat and coauthors [40] used NB, tryptone water, peptone water, and King's B broth growth media, the authors reported pyocyanin pigment amounts of 5.28, 9.55, 11.7, and 25.5 µg/mL, respectively. El Fouly and coauthors [16] determined pyocyanin pigment productions between 1.7 µg/mL and 6.3 µg/mL in the NB growth medium with glycerol supplementation for 9 different *P. aeruginosa* strains. Gahlout and coauthors [41] determined the pyocyanin pigment productions of 12 different *P. aeruginosa* strains ranging between 0.52 µg/mL and 10.9 µg/mL at a period of 48-96 hours in the King's B broth growth medium. In our study, the amount of pyocyanin pigment production by *P. aeruginosa's* MB713 strain in the MFDM growth medium was higher than those reported in the literature. The difference in pigment production is thought to have stemmed from the difference in the strain and growth medium used in the study.

In the present study, we observed differences in the amount of pyocyanin pigment production between the NB and MFDM growth media, and between the shaking and nonshaking conditions; it was determined that the pyocyanin pigment production was greater under shaking conditions in both growth media. The highest pyocyanin pigment production was found to occur in the MFDM growth medium, under shaking conditions (51 µg/mL). Since the growth medium's surface contacts air, oxygen enters by diffusion through here and melts, making the upper parts of a medium richer in oxygen than its bottom parts. Therefore, the

surface layers of fluid growth media contain more oxygen than their bottom layers. Growth media thus need to be mildly shaken at certain intervals (aeration) so that aerobic microorganisms are equally distributed in all parts of a fluid medium and proliferate better. As *P. aeruginosa's* MB713 strain is an aerobic bacterium, oxygen turnover achieved by shaking increased both bacterial growth and pigment production. Kurachi [42] determined that both bacterial growth and pyocyanin pigment production could be augmented by oxygenation achieved by aerating the medium (aeration or shaking). Suryawanshi and coauthors [18] also determined that shaking was influential on pigment production, supporting our findings.

In the present study, UV-VIS spectroscopy, FT-IR, and GC-MS analyses were performed to confirm the pigment produced under optimum conditions. In the UV-VIS spectroscopy used to characterize pigments, the maximum peak of pyocyanin pigment appeared at a range of 300 to 400 nm for both solvents. While the maximum peak for pure water was 300 nm, it was 370 nm for 95% ethanol. Moayedi and coauthors [43] determined a maximum peak of 308-387 nm for the pyocyanin pigment. The maximum peak for chloroform and 0.1 M HCl was 308 nm and 387 nm, respectively. Glasser and coauthors [44] reported a maximum peak of 387 nm for the pyocyanin pigment in 2% methanol solvent. It is thought that the absorbance differences in the maximum peak of the pigment result from the differences in the solvents used. Our results are in agreement with the literature data.

Dense molecular ion peaks were determined at 168 m/Z and 196 m/Z in the GC-MS analysis of pyocyanin pigment. While phenazine showed a sharp peak at 168 m/Z, 1-hydroxyphenazine (hemi pyocyanin) showed a sharp peak at 196 m/Z. The presence of simultaneously detected peaks at 168 m/Z and 196 m/Z in the GC-MS analysis indicate the presence of the pyocyanin pigment that was converted to 1-hydroxyphenazine. Watson and coauthors [45], Kerr and coauthors [46], Barakat and coauthors [40], and Abdul Hussein and Atia [47] found that the pyocyanin pigment showed two peaks at 168 m/Z (phenazine) and 196 m/Z (1-hydroxyphenazine) in the GC-MS analysis. Literature studies corroborate the results of our GC-MS analysis of pyocyanin pigment.

The peak at 3462 cm^{-1} in the FT-IR spectrum of pyocyanin pigment shows the presence of an O-H bond, and the peak at 2953 cm^{-1} shows the presence of a C-H aromatic bond. The peak at 1620 cm^{-1} represents a C=N bond that is specific to the pyocyanin pigment. Due to stretching vibrations of C-C in the aromatic ring, it showed absorption at the regions of 1500-1400 cm^{-1} and 1600-1585 cm^{-1}. In the presence of CH_3, the C-H bond of the alkyl group (methyl) at 1380-1400cm^{-1} was stretched and formed a peak. The peak at 1301 cm^{-1} corresponds to a C=O bond. The FT-IR spectrum showed the presence of phenazine, confirming that this pigment-containing aromatic ring was the pyocyanin pigment. The peaks obtained by the FT-IR analysis and the bonds detected at these peaks were supported by literature data establishing the characterization of the pyocyanin pigment by FT-IR analysis and showed the presence of the pyocyanin pigment as the main molecule in the extract [48,49].

The nontoxic character of the pyocyanin pigment used in the study was considered important for interpreting the concentrations and their activities applied in the studies. The cytotoxicity of the pyocyanin pigment on L949 healthy mouse fibroblast cells was studied to provide an insight for future studies on the same subject. Our study determined whether the pyocyanin pigment had cytotoxic action by studying its activity on the viability of L929 healthy mouse fibroblast cells. Our results confirmed that the pyocyanin pigment reduced cellular viability, proportional to its concentration ($p<0.05$). According to the ISO 10993-5 standards, quantitative classification groups were used as a base to determine the levels of cytotoxic activity of *in vitro* tested chemicals. Accordingly, substances showing a cytotoxic activity cause a death rate above 30% of the total cell concentration. According to the above mentioned ISO standards, 3 concentrations of pyocyanin pigment (50-100 µM) were considered to have cytotoxic potential. According to a study by Laxmi and Bhat [48], where different concentrations of the pyocyanin pigment were applied on L929 healthy mouse fibroblast cells, different concentrations (6.25-50 µg/mL) of the pyocyanin pigment showed no cytotoxic effects on L929 healthy mouse fibroblast cells. However, 100 µg/mL of the pyocyanin pigment showed cytotoxic potential on L929 healthy mouse fibroblast cells. This confirms our results.

AChE is an enzyme that is responsible for the rapid termination of a nerve impulse formed by the synaptic release of acetyl coenzyme A, which is known as one of the products of cellular respiration in mitochondria, as well as ACh, a neurotransmitter derived from choline that takes part in lipid metabolism. Increased AChE enzyme activity and the resulting decrease in the amount of ACh cause neuronal loss. This causes many neurodegenerative diseases, particularly AD [50-53]. Thus, agents considered to have a potential for the prevention/treatment of neurodegenerative diseases should importantly show AChE inhibitory (AChE-i) action. There are studies indicating that the available AChE-i drugs are not fully effective in treating neurodegenerative diseases [54-57]. Since the inhibition of AChE enzyme activity is still the current prevailing strategy for the treatment of neurodegenerative diseases, AChE-i activity is an important marker for the discovery of effective substances to treat neurodegenerative diseases [58]. At the same time, there is

mounting evidence that, in a nonneuronal context, AChE is involved in the regulation of cell proliferation, differentiation, apoptosis, and cell cell interaction [3]. An irregular expression and structural alteration of AChE has been found in different types of tumors, such as brain, lung, ovarian, breast, hepatocellular, renal, and colon cancers, suggesting the involvement of AChE in the regulation of tumor development. Having all this in mind, there is a possibility that some AChE-i's could be used as anticancer agents [59,3]. In the context of our study, the inhibitory action of the pyocyanin pigment was shown on *in vitro* and cellular AChE enzyme activity. Various studies have proven that AChE enzyme activity is induced by oxidative stress increased by ROS causing oxidative injury, such as H_2O_2 [60-64]. Furthermore, it has been shown that H_2O_2 can easily penetrate cell membranes and cause cell injury by altering various proteins, as well as it increases intracellular AChE enzyme activity by altering cell membrane structure and activity [65-67,60]. It was determined that the application of 150 µM H_2O_2 increased AChE enzyme activity, translating into an increase in the AChE enzyme activity of SH-SY5Y human neuroblastoma cells by 43% relative to the untreated control group (p<0.05). Our results showed that the pyocyanin pigment's AChE-i activity increased in proportion to its concentration (p<0.05), and the maximum inhibitory action occurred at 100 µM, which was the highest concentration tested. It was even showed that the pyocyanin pigment at this concentration reduced H_2O_2 induced AChE enzyme activity to a greater degree than it reduced AChE activity in the control group. However, according to the ISO 10993-5 standards 75-100 µM pyocyanin pigment was considered to have cytotoxic potential on SH-SY5Y human neuroblastoma cells (Data not shown). Therefore, it is recommended to use pyocyanin pigment concentrations (10-50 µM) that do not have cytotoxic effects. Studies investigating AChE-i activities on different cell models with the Ellman method have reported that genistein inhibited AChE enzyme activity by approximately 4-33% and huperzine-A by approximately 40% [68,69]. According to these data, the pyocyanin pigment used in our study was shown to possess a similar activity potential with the substances already used as AChE-i. The pyocyanin pigment's low molecular weight and zwitterion property facilitate its passage through cell membranes in eukaryotic systems [70]. It is thought that the inhibitory activity of the pyocyanin pigment having 210 Da molecular weight on AChE enzyme activity results from a sufficiently small size to pass through the cell membrane and its redox active compound property [45]. According to our results, and considering previous studies suggesting that increased cellular AChE enzyme activity causes the loss of cholinergic conduction and leads to neurodegenerative processes, it has been predicted that it is possible to use the pyocyanin pigment against neurological injury [51,71,72]. Additionally, as an AChE-i agent, the pyocyanin pigment also has the potential to be used as an anticancer agent. Since no study has yet shown the inhibitory effect of the pyocyanin pigment on AChE activity, this study has a pioneering role for future research efforts in this field.

Oxidative stress leads to the development of a diverse spectrum of diseases, including neurodegenerative diseases [73-75]. ROS, including H_2O_2, hydroxyl radical, superoxide anion, and nitric oxide reacts with cellular DNA, carbohydrates, lipids, proteins, and other molecular groups, affecting their metabolism. ROS that are produced during the metabolism of organisms tend to trigger chain reactions that cause irreversible chemical alterations in lipid and protein structure. These chain reactions may also lead to cellular dysfunction and cytotoxicity [73,74]. ROS also starts lipid peroxidation by leading to the oxidation of polyunsaturated fatty acids found in biological membranes. Lipid peroxides that are produced by lipid peroxidation reactions (lipid peroxide, cyclic peroxide, and cyclic endoperoxide) are converted into MDA, 4-hydroxynonenal and hexagonal aldehydes [76]. If everything works in balance in the body, these activities are neutralized by antioxidant enzymes. When their production surpasses the body's antioxidant capacity, tissue damage begins [77]. Antioxidant enzymes preventing such damage are the first line defense system directly involved in ROS detoxification. SOD, CAT, and glutathione peroxidase enzymes perform ROS detoxification and create a cellular antioxidant defense mechanism [73]. The suppression of oxidative stress known to be related to neurodegenerative diseases by antioxidant activity can be used to prevent/treat neurodegenerative diseases [78,79]. The present study investigated the protective activities of the pyocyanin pigment against oxidative injury (SH-SY5Y human neuroblastoma cells). The oxidative injury was created by H_2O_2 in SH-SY5Y human neuroblastoma cells and antioxidant enzyme activities were inhibited (p<0,05). The activities of the SOD and CAT antioxidant enzymes were increased in the pyocyanin pigment applied groups compared with the H_2O_2 group where the oxidative injury was created (p<0.05). The pyocyanin pigment applied to SH-SY5Y human neuroblastoma cells inhibited the increase in the MDA levels formed by the creation of an oxidative injury with H_2O_2 (p<0.05). These results support the view that the pyocyanin pigment shows a protective action against oxidative injury and its consequences by increasing the activities of the SOD and CAT antioxidant enzyme and by inhibiting the increase of the lipid peroxidation product (MDA). In line with this notion, it has been thought that the pyocyanin pigment equipped with antioxidant activity can prevent neurodegenerative diseases by promoting cellular antioxidant mechanisms. Our results showed that

the pyocyanin pigment, which was shown to have antioxidant activity, can be used as a protective agent against neural injury and as an anticancer drug through its inhibitory activity on *in vitro* and cellular AChE enzyme activity. Studies in the literature have established the association between AChE-i activity and antioxidant activity [78-81]. Mounting evidence suggesting that neuroprotection and anticancer activity can be achieved by inhibiting AChE enzyme activity has transformed AChE enzyme activity into a guide for researchers who aim to develop novel effective agents against neurodegenerative diseases and cancer [82]. Preventing or delaying neurodegenerative diseases and cancer is important for slowing down the disease course [83]. Therefore, it is an important finding that it has been shown that the pyocyanin pigment can be effective in eliminating pathologies that may cause disease before the disease occurs.

This study investigated the antiproliferative activity of the pyocyanin pigment on SK-MEL-30 human melanoma and HT-29 human colon cancer cells. A differential cytotoxic response between cancer cells were observed, with HT-29 human colon cancer cells being resistant and SKMEL-30 human melanoma cells being susceptible to pyocyanin pigment-induced cytotoxicity. This is thought to be due to the characteristic features of the cells. The IC_{50} value of the pyocyanin pigment for SK-MEL-30 human melanoma cells was determined as 72 µM. It was observed that the pyocyanin pigment had negligible antiproliferative activity on HT-29 human colon cancer cells. It was determined that the concentration corresponding to the IC_{50} value of the pyocyanin pigment, whose antiproliferative activity was determined against SK-MEL-30 human melanoma cells, also had no cytotoxic activity on L929 healthy mouse fibroblast cells (except 50-100 µM concentrations). Hence, it is reasonable to propose the use of the pyocyanin pigment with antiproliferative action as an active anticancer substance in a safe manner. It is thought that the antiproliferative activity of the pyocyanin pigment stems from its zwitterion property enabling it to easily cross cell membrane [84]. It has been demonstrated that the biological properties of the pyocyanin pigment, such as DNA intercalation, topoisomerase inhibition, and cell permeability induction, can be used to inhibit cancer cells [70]. Patil and coauthors [85] determined that the pyocyanin pigment (10-80 µg/ml) exhibited antiproliferative action against SK-MEL-2 human melanoma cells at a low concentration (GI_{50} value<10 µg/mL). Zhao and coauthors [84] detected the antiproliferative activities of the pyocyanin pigment on HepG2 human hepatoma cells. The results supported the view that acute ROS production followed by oxidative stress mediated the pyocyanin pigmen induced cytotoxicity in HepG2 human hepatoma cells. Moayedi and coauthors [49] determined that the pyocyanin pigment showed cytotoxic action by inducing apoptosis and necrosis in Pan-1 human pancreatic cancer cells. Sengupta and Bhowal [86] determined that the pyocyanin pigment showed cytotoxic activity against MG-63 human osteosarcoma cells. Literature data support the antiproliferative activity of the pyocyanin pigment against cancer cells.

Our study results suggest that the pyocyanin pigment is neither mutagenic nor carcinogenic in healthy cells, and additionally, it exerts no toxic activities in acute oral, fish aquatic, and *Tetrahymena pyriformis* (Table 3). After *in silico* predictions are confirmed by experimental-based assays, it is thought that pyocyanin pigment can be used safely as a drug candidate in the treatment of cancer and neurodegenerative diseases.

A rigorous analysis based on structure activity relationship was used to film the pyocyanin pigment *in silico*. Table 4 lists the most likely biological activities of the pyocyanin pigment as determined by a computer based PASS online program. The obtained “Antineoplastic (alkaloid, brain cancer, cervical cancer, colorectal cancer, lymphocytic leukemia, lung cancer, melanoma, pancreatic cancer, renal cancer, small cell lung cancer)”, “Preneoplastic condition treatment”, “Dementia treatment” and “Nootropic” activities suggested that pyocyanin pigment can be used as a drug candidate in cancer and neurodejenerative diseases treatment. Many chemotherapeutic agents cause DNA damage and activate the caspase 3, caspase 8, and p53 genes to induce growth arrest and apoptosis [87,88]. The activity of pyocyanin pigment as caspase 3 and caspase 8 stimulator and TP53 expression enhancer was thought to lead cancer cells to apoptosis. Aberrant expression of JAK2, MAP kinase kinase 4, mitochondrial processing peptidase (MMP), and MMP9 have been shown to be involved in many cancers [89-91]. The activity of pyocyanin pigment as inhibitors of expression of JAK2, MAP kinase kinase 4, MMP and MMP9 suggested that it may have a potential use in cancer therapy. However, for the pigment to be used as a drug candidate in the treatment of cancer and neurodegenerative diseases, it is essential to confirm the *in silico* predictions with experimental-based analyzes.

CONCLUSION

A general review of our results indicates that the maximum production of the pyocyanin pigment obtained from *P. aeruginosa* MB713 strain occurred in the MFDM growth medium and under shaking conditions. Pyocyanin pigment was confirmed by UV-VIS spectroscopy, GC-MS, and FT-IR analysis. It was determined that pyocyanin pigment did not exert no cytotoxic effect on L929 healthy mouse fibroblast cells (except 50-100 µM concentrations) while it showed an antiproliferative effect on SK-MEL-30 human melanoma cells.

Based on its *in vitro* and cellular AChE inhibitory and antioxidant effects shown by the present study, it is thought that pyocyanin pigment may be recommended as a food additive or as a neuroprotective agent, an active drug substance, or an anticancer agent. This situation can be clarified by conducting advanced molecular studies.

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