Original Article

Extraction. Isoaltion, Purification,Stability Studies And Cytotoxic Studies Of Phycocyanin From Spiruline Culture

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Abstract

The aim of work is focus on Extraction. Isoaltion, putification of phycocynin from spirulina culture. In this method extraction was done by centrifuge process by using the buffer and saturated amonium sulphate, form crude extract purification done by using the ammonium sulfate extract using DEAE-Cellulose was used for anion exchange chromatography. The spectral charrecterzation was done by using IR,NMR and Mass Spectroscopy. Phycocyanin concentration and purity were determined by spectrophotometry using absorbance at 620 nm and 652 nm the purity was foune to be between 08-4.3 for diffent extractions like Crude extract ,Ammonium sulphate precipitation with 25% saturation ,Ammonium sulphate precipitation with 50% saturation, DEAE-cellulose-52. The stabilty studies for phycocyanin was perfomed ar different temperature and pH: 4 °C, 25 °C, and − 20 °C and pH: 5, 6, and 7.the phycocyanin content decreased to 15% at 25 °C, 83% at 4 °C, and 51% at − 20 °C. The most appropriate storage condition was 4 °C at pH 5. It was found that the most appropriate storage temperature at pH 6 was − 20 °C for better preservation of phycocyanin. We found that the most appropriate pH was 7, at − 20 °C. the cytotoxicity studies were performed for crude extract and crude Phycocyanin, pure phycocyanin were evaluated by the MTT assay against the HT-29 (colon cancer), MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines. Of all the three components tested against HT-29 cell lines, the pure Phycocyanin shows 135± 2 than the standard Methotrexate. Of all the three components tested against MCF-7 cell lines, the pure Phycocyanin shows 155± 3 than the standard Methotrexate. Of all the three components tested against DU-145 cell lines, the pure Phycocyanin shows 174 ± 2 than the standard Methotrexate. Based on the above results it concludes that the pure Phycocyanin shoes better cytotoxicity against the HT-29 (colon cancer), MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines.

Key words: *Spirulina,* phycocyanin, stability studies, cytotoxicity, MTT assay, Cell lines

INTRODCUTION

Spirulina is gaining attention due to its high nutritional value and extensive pharmaceutical applications. The correct identification of a certain species is a fundamental requisite for research study and/or developmental applications. The genus *Spirulina* has been incorrectly used to describe two different genera, *Spirulina* and *Arthrospira*. However, Stizenberger (1854) and Gomont (1892–1893) classified the forms with visible septa within the genus *Arthrospira*Stizenberger 1852; while coiled filaments with invisible septa were classified as belonging to the genus *Spirulina* Turpin 1829[1] The view of *Arthrospira*and *Spirulina* as two separate genera has been officially accepted by Bergey's Manual of Systematic Bacteriology [2-3]The separation between these two genera has been repeatedly affirmed on the basis of many other characteristics such as cell wall structure, helicity, trichome size, motility, gas vesicles, thylakoid pattern, GC analysis and phylogenetically using 16S rRNA. *Spirulina* is a photosynthetic autotrophic organism containing the blue pigment phycocyanin as the main photosynthetic pigment in addition to the green pigment chlorophyll *a*, which results in the blue-green color of the cells. *Spirulina* is one of multicellular unbranched non-heterocystous filamentous microalgae which are recognizable by the unique open left-handed helix along the entire length of the filament. the biochemical composition of *Spirulina* is highly dependent on growth conditions and the methods used for harvest and drying. In general, Capelli and Cysewski (2010)[4] reported that the amount of calcium in *Spirulina* is 1.8 times higher than that in whole milk, total protein is 6.7 times that of tofu, iron is 51 times greater than that of spinach and β-carotene is 31 times more abundant than in carrots. Therefore, *Spirulina* was given the label of 'super food' by The World Health Organization (WHO).

Based on the literature revire the present was focused on the extraction and isoaltion and purification of the phycocynin perform the charecterzation using IR,NMR ,Mass spectrosocpy. And perform the purity content in extract using

specctrophotometry. And detreine stabilty of extracts at different temperature and pH conditions. Perform the cytotoxicity using the MTT assay methods

MATERIALS AND METHODS:

Materials:

Spiruline purchased from the local market hyderabad. ammonium suplphate, DEAE-Cellulose, acetate buffer, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide , DMSO ,distilled water, HT-29 (colon cancer), MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines were obtained from National Centre for Cell Science (NCCS), Pune, India. DMEM (Dulbeccos Modified Eagels Medium), MEM (Minimum Essential Media Eagle), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], Trypsin, EDTA were purchased from Sigma chemicals (St.Louis,MO). Fetal bovine serum (FBS) was purchased from Arrow Labs, 96 well flat bottom tissue culture plates were purchased from Tarson.

Methods:

Isoaltion phycocynin from spiruline culture[5]

Take 50 ml of the spiruline culture centrifuge at 8000 rpm for 10 min at 4^0C in 50 ml of centrifuge tube ,these was washes trice with distilled water ,freeze the solution and wash the the biomass with 50 mM sodium phosphate buffer three to four times and cnetrifuge the tubes at at 8000 rpm for 15 min at 4^0C. to this solution add 30 % of saturated ammonium suplphate and keepit for 4 hours at 4^0C. .take the supernate blue colur solution and add 60 % of saturated ammonium sulphate and keepit for 4 hours at 4^0C cnetrifuge the tubes at at 11000 rpm for 15 min at 4^0C. the formed blue colur ppt is collected and add 2.5mM sodium phosphate byuffer 6.8 pH purify agaisnt the same buffer over night at 4^0C. collected precippate and dried.

Purification of phycocynin[6]

The crude extract was subjected to a single step precipitation using 65 % $(NH_4)_2SO_4$ (Bio Xtra, >99 %; Sigma-Aldrich) and kept overnight at 4 °C. The pellet was recovered by centrifugation at 27,000 rpm for 15 min at 4 °C and dissolved in 10 ml of the same extraction buffer and termed as ammonium sulfate extract (ASE). Ten ml of ASE was dialyzed against the extraction buffer using dialyses membrane (Dialyses membrane-70, MWCO; 12–14 kD) procured from Hi-Media. Dialyses was performed twice against 1,000 ml extraction buffer, first at room temperature and again dialysed against 1,000 ml of extraction buffer at 4 °C overnight. The resultant extract was recovered from the dialyses membrane and filtered through 0.45 µm filter.

DEAE-Cellulose was used for anion exchange chromatography. A column (30 × 2 cm) was prepared for purifying the phycocyanin, and equilibrated with 150 ml of acetate buffer (pH-5.10). Dialyzed filtered sample (10 ml) was placed on the column. A linear gradient of acetate buffer with pH ranging from 3.76 to 5.10 was used to developed the column and elutes were collected in 5 ml fractions. Flow rate was kept 20 ml h^{-1}. Absorption spectrum was also determined by scanning the sample in the range of 300–750 nm by using SHIMADZU spectrophotometer

Determination of concentration and purity of phycocyanin[7]

Phycocyanin concentration and purity were determined by spectrophotometry as described by Bennet and Bogard 1973 Concentration was calculated with the formula below using absorbance at 620 nm and 652 nm with a Biotek Eon™ spectrophotometer with a high-performance microplate. For purity, a ratio of A_{620}/A_{280} was used.

Phycocyanin absorbs light at about 620 nm and emits fluorescence at about 640 nm . The purity of C-PC is usually evaluated using an absorbance ratio of A_{620}/A_{280}, and a purity of 0.7 is considered as food grade [Absorbance at 620 nm indicates maximum C-PC absorption, while at 280 nm, it is due to the presence of other proteins in the solution C-PC purity is a ratio regarding the contamination of other proteins if present in the same sample.

C−PC (mg/mL)=(Abs620−0.474(Abs652))/5.34

Yield (mg/g)=(C−PC×Volume)/Biomass

Determination of phycocyanin stability at different storage conditions[8-10]

Phycocyanin stability in the crude extract was evaluated with the following storage conditions in the dark: temperature: 4 °C, 25 °C, and − 20 °C and pH: 5, 6, and 7. The storage time was 5 weeks. The pH, purity, and concentration of phycocyanin were determined at 1 week intervals up to 5 consecutive weeks by spectrophotometer.

Stability analysis of phycocyanin at pH 5 at different temperatures

The temperature and pH of the solvent were evaluated for the crude extract of phycocyanin. These are the key factors that influence the stability of phycocyanin during storage. Starting with the same initial concentration at pH 5, after 3 weeks of storage at different temperatures, the phycocyanin content showed variation. After 5 weeks of storage, the phycocyanin content decreased to 15% at 25 °C, 83% at 4 °C, and 51% at − 20 °C. The most appropriate storage condition was 4 °C at pH 5. Citric acid used for acidification may act as a preservative of the phycocyanin solution to inhibit microbial growth.

Stability analysis of phycocyanin at pH 6 at different temperatures

Phycocyanin starting at pH 6 was kept at different storage temperatures and evaluated at 1-week intervals. At room temperature (25 °C), the amount of C-PC was only 56.60%, stable from the initial amount after 5 weeks. At 4 °C, the stability was similar, 93.07% and 92.75% after the third and fifth weeks, respectively. At − 20 °C, the stability was found to be 95.24% and 94.32% after the third and fifth weeks, respectively. It was found that the most appropriate storage temperature at pH 6 was − 20 °C for better preservation of phycocyanin. The most considerable amount was conserved at 4 °C up to 35 days with 93% of C-PC (Fig. 5, Table 1); at − 20 °C the PC quantity was conserved up to 94% after 35 days, while at room temperature only 56% was preserved, an undesirable value. When compared with pH 5, the highest stability was found with pH 6 at − 20 °C.

Phycocyanin stability analysis (pH 7)

Starting with pH 7 at room temperature (25 °C), only 25.64% of the initial amount of PC was found after the fifth week. At 4 °C, the stability was different, approximately 89.56% and 73.09% after the third and fifth weeks, respectively, at pH 7. At − 20 °C, the stability was approximately 98.77% and 98.69% after the third and fifth weeks, respectively (Fig. 3a, b, Table 1). We found that the most appropriate pH was 7, at − 20 °C. Potassium hydroxide was added to raise the pH of the phycocyanin solution to 7 from an initial pH of 6.4. The highest amount of C-PC was 89.5% at 4 °C up to 21 days. However, at − 20 °C, the amount conserved up to 21 days was 98.7%, while at room temperature, only 46% was retained. When stability at pH 5 and 6 was compared, the highest stability obtained was at pH 7 at − 20 °C

Interaction analysis of stability of PC with different pH, temperature, and time

At pH 5 the best stability was at 4 °C between the third and fifth weeks. At pH 6 the best stability was found at 4 °C and − 20 °C. The data obtained were similar. The best stability obtained was at − 20 °C and pH 7 between the third and fifth weeks. Among all pH values, it was observed that room temperature was not viable to account for phycocyanin stability, since the values obtained were very low compared to the other storage temperatures, 4 °C and − 20 °C. Between the two cold storage temperatures, at − 20 °C there was better stability at pH 6 and pH 7, but at pH 5 it was better at 4 °C. There was no significant difference in phycocyanin stability between the third and fifth weeks. It is also clear that pH 7 and − 20 °C was the best storage condition for phycocyanin stability, which was 98.7% for 35 days, although 40 °C was also good for phycocyanin stability at pH 6 and pH 7 (Fig. 3, Table 1). Stability was remarkable at − 20 °C up to 5 weeks, since there was very little loss of phycocyanin and it lasted longer.

CYTOTOXICITY STUDIES:

The *in vitro* cytotoxicity of the test compoundcrude extract and crude Phycocyanin, pure phycocyanin were evaluated by the MTT assay. This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product The cells are then solubilized with DMSO and the released, solubilized formazan reagent is measured spectrophotometrically at 570 nm. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells. When the amount of dark purple formazan produced by the cells is treated with an agent compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced through the production of a dose-response curve.

METHOD:

a) Maintainence of cell lines:

HT-29 and DU-145cell lines were grown as adherent in DMEM media, whereas MCF-7 was grown in MEM media supplemented with 10% fetal bovine serum. The cultured was maintained in a humidified atmosphere with 5% CO_2.

b) Preparation of samples for cytotoxicity:

Stock solutions of extract and crude and pure phycocyanin were prepared (10 mg/mL) in DMSO and from them various dilutions were made with sterile water to get the final drug concentrations of 10, 50, 100 and 200 µg/mL.

c) Cytotoxicity evaluation:

The cells were seeded in 96 well plates at a density of $1x10^4$ (counted by Tryphan blue exclusion dye method)per well and were incubated for 24 h to recover. After incubation, the medium was replaced with fresh media containing different dilutions of the test compounds. Then the plated were incubated for additional 48 h at 37^0C in DMEM/MEM with 10% FBS medium. Following incubation, the medium was removed and replaced with 90 µl of fresh DMEM without FBS. To the above wells, 10 µl of MTT reagent (5 mg/mL of stock solution in DMEM without FBS) was added and incubated at 37^0C for 3-4 h, there after the above media was replaced by adding 200 µl of DMSO to each well (to dissolve the blue formazan crystals) and incubated at 37^0C for 10 min. The absorbance at 570 nm was measured on a spectrophotometer.

Methotrexate was used as reference drug for comparison. Assay was performed in triplicate for three independent determinations. The cytotoxicity was expressed as IC_{50} (µg/mL) which is the concentration of the compound that

inhibited proliferation rate of the tumor cells by 50% as compared to the control untreated cells. IC_{50} values were determined from the plot: percentage inhibition versus concentration.

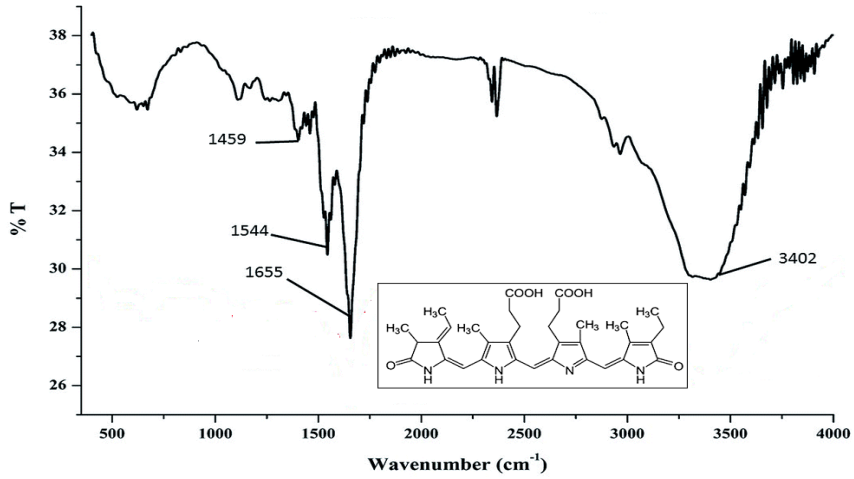
% inhibition at the given concentration = 1- (Absorbance average)

---------- X 100

(Control absorbance average)

IC_{50}=Inv.log (50-c) / m; c and m derived from y=mx+c of plot of percentage inhibition Vs log C.

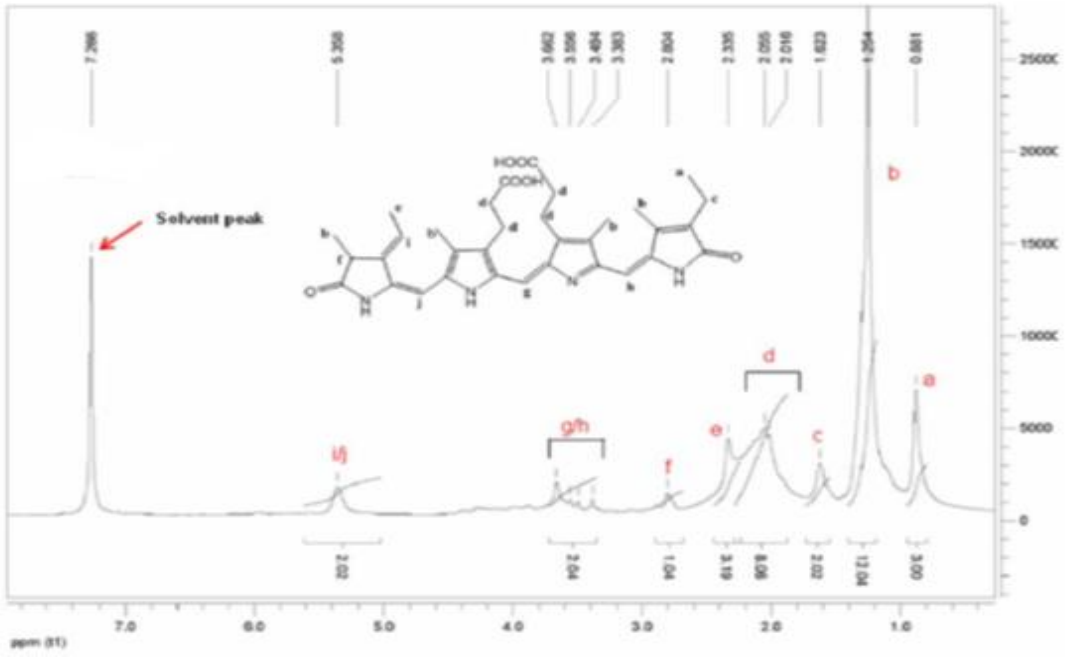
RESULTS AND DISCUSSION



IR spectrum of phycocynin

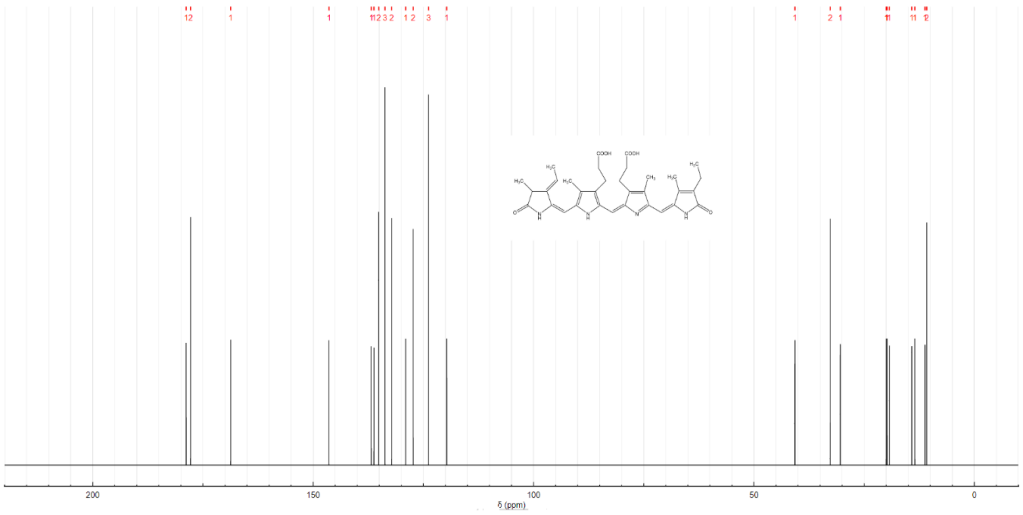
Interpretation:

Characteristic band (2100–3700 cm-1) that is indicated mainly from -COO, -CO and conjugated double bond. These bonds showed spectral bands peak 2985 cm-1 and 2860 cm-1, 2986 cm-1.



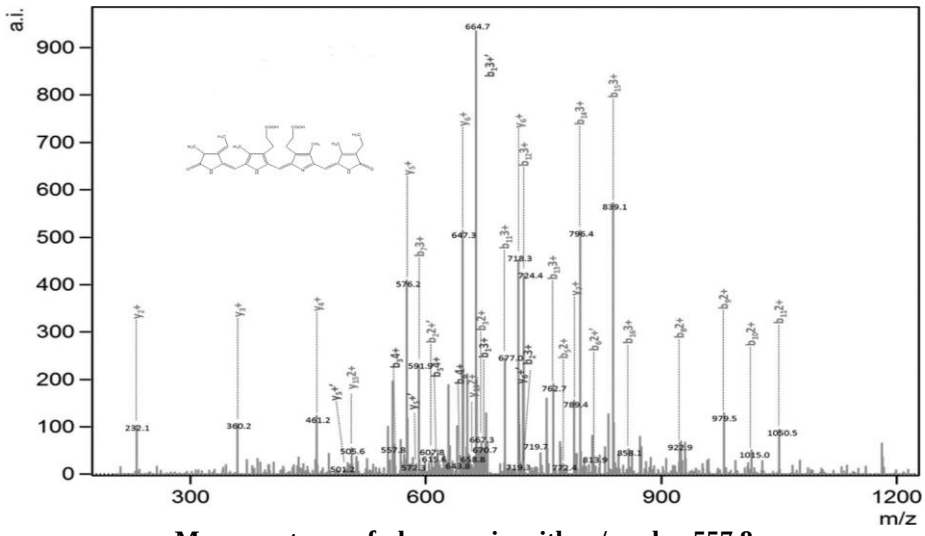
^{1}HNMR spectrum of phycocynin

Interpretation^{1}H NMR: δ 1.06-1.26 (6H, 1.12 (t, *J* = 7.0 Hz), 1.20 (d, *J* = 6.6 Hz)), 1.65 (3H, d, *J* = 7.2 Hz), 2.03 (3H, s), 2.14-2.35 (8H, 2.19 (s), 2.26 (q, *J* = 7.0 Hz), 2.26 (q, *J* = 7.0 Hz), 2.30 (s)), 2.50-2.77 (6H, 2.56 (t, *J* = 7.4 Hz), 2.56 (t, *J* = 7.4 Hz), 2.67 (t, *J* = 7.4 Hz), 2.67 (t, *J* = 7.4 Hz), 2.71 (t, *J* = 7.4 Hz), 2.71 (t, *J* = 7.4 Hz)), 2.83-2.95 (2H, 2.89 (t, *J* = 7.4 Hz), 2.89 (t, *J* = 7.4 Hz)), 3.41 (1H, q, *J* = 6.6 Hz), 5.76 (1H, q, *J* = 7.2 Hz), 6.01 (1H, s), 6.44 (1H, s), 6.68 (1H, s)



C^{13} NMR spectrum of phycocynin

^{13}C NMR: δ 10.8-10.9 (2C, 10.8 (s), 10.8 (s)), 11.2 (1C, s), 13.5 (1C, s), 14.2 (1C, s), 19.3 (1C, s), 19.8 (1C, s), 20.0 (1C, s), 30.4 (1C, s), 32.7-32.8 (2C, 32.7 (s), 32.7 (s)), 40.7 (1C, s), 119.7 (1C, s), 123.8-123.8 (3C, 123.8 (s), 123.8 (s), 123.8 (s)), 127.3-127.3 (2C, 127.3 (s), 127.3 (s)), 129.0 (1C, s), 132.1-132.3 (2C, 132.2 (s), 132.2 (s)), 133.6-133.8 (3C, 133.7 (s), 133.7 (s), 133.7 (s)), 135.0-135.2 (2C, 135.1 (s), 135.1 (s)), 136.2 (1C, s), 136.8 (1C, s), 146.4 (1C, s), 168.7 (1C, s), 177.8-177.9 (2C, 177.8 (s), 177.8 (s)), 178.8 (1C, s).



Mass spectrum of phycocynin with m/z value 557.8

Table: 1 Determination of concentration and purity of phycocyanin Using Uv

Purification step	Purity ratio A_{620}/A_{280}	Separation factor A_{620}/A_{652}	C-PC (mg ml^{-1})	Recovery of C-PC (%)
Crude extract	0.8	2.4	0.2	100
Ammonium sulphate precipitation with 25% saturation	0.9	2.4	0.3	93
Ammonium sulphate precipitation with 50% saturation	2.4	2.9	0.3	83
DEAE-cellulose-52	4.3	4.6	0.4	48

Table2: Phycocyanin stability at different storing temperatures and pH

	p^H-5	p^H-6	p^H-7
4 °C	83.04 ± 0.36	92.75 ± 0.69	73.09 ± 0.32
25 °C	14.55 ± 0.03	56.60 ± 0.17	25.64 ± 0.04
− 20 °C	51.03 ± 2.41	94.32 ± 0.05	98.69 ± 0.32

Table3: Cytotoxicity results of Crude extract, Crude Phycocyanin, Pure phycocyanin

S.No	Components	Cell line		
		HT-29	MCF-7	DU-145
1	Crude extract	NA	33 ± 1	78 ± 2
2	Crude Phycocyanin	126 ± 2	137 ± 2	82 ± 2
3	Pure phycocyanin	135± 2	155± 3	174 ± 2
4	Methotrexate	12 ± 1	9 ± 1	5 ± 1
Data presented as mean ± SD (n=3). All the compounds and the standard dissolved in DMSO, diluted with culture medium containing 0.1% DMSO. The control cells were treated with culture medium containing 0.1% DMSO. NA- No Activity (i.e $IC_{50} > 200$ µg/mL)				

DISCUSSION

In this method extraction was done by centrifuge process by using the buffer and saturated amonium sulphate, form crude extract purification done by using the ammonium sulfate extract using DEAE-Cellulose was used for anion exchange chromatography. The spectral charrecterzation was done by using IR,NMR and Mass Spectroscopy. Phycocyanin concentration and purity were determined by spectrophotometry using absorbance at 620 nm and 652 nm the purity was foune to be between 08-4.3 for diffent extractions like Crude extract ,Ammonium sulphate precipitation with 25% saturation ,Ammonium sulphate precipitation with 50% saturation, DEAE-cellulose-52. The stabilty studies for phycocyanin was perfomed ar different temperature and pH: 4 °C, 25 °C, and − 20 °C and pH: 5, 6, and 7.the phycocyanin content decreased to 15% at 25 °C, 83% at 4 °C, and 51% at − 20 °C. The most appropriate storage condition was 4 °C at pH 5. It was found that the most appropriate storage temperature at pH 6 was − 20 °C for better preservation of phycocyanin. We found that the most appropriate pH was 7, at − 20 °C. the cytotoxicity studies were performed for crude extract and crude Phycocyanin, pure phycocyanin were evaluated by the MTT assay against the HT-29 (colon cancer), MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines. Of all the three components tested against HT-29 cell lines, the pure Phycocyanin shows 135± 2 than the standard Methotrexate. Of all the three components tested against MCF-7 cell lines, the pure Phycocyanin shows 155± 3 than the standard Methotrexate. Of all the three components tested against DU-145 cell lines, the pure Phycocyanin shows 174 ± 2 than the standard Methotrexate. Based on the above results it concludes that the pure Phycocyanin shoes better cytotoxicity against the HT-29 (colon cancer), MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines.

CONCLUSION

The extraction of phycocynin was done by centrifuge process by using the buffer and saturated amonium sulphate, form crude extract purification done by using the ammonium sulfate extract using DEAE-Cellulose was used for anion exchange chromatography. The spectral charrecterzation was done by using IR,NMR and Mass Spectroscopy. The stabilty resulsts shows thatAt pH 5 the best stability was at 4 °C between the third and fifth weeks. At pH 6 the best stability was found at 4 °C and − 20 °C. The data obtained were similar. The best stability obtained was at − 20 °C and pH 7 between the third and fifth weeks. Among all pH values, it was observed that room .temperature was not viable to account for phycocyanin stability. Based on the above MTT results it concludes that the pure Phycocyanin shoes better cytotoxicity against the HT-29 (colon cancer), MCF-7 (breast cancer) and DU-145 (prostate cancer) cell lines.

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