Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.04.033

Evolution of cytotoxicity of the phytopigments Isolated from Spirulina platen sis using MTT assay

Salma shaik[1,2*], N. Harikrishnan[3]

[1]Research Scholar, Faculty of Pharmacy, Dr.M.G.R. Educational and Research Institute, Deemed to be University, Chennai, Tamil Nadu-600 077, India.

[2]Department of Pharmaceutical chemistry, narayana pharmacy college, chinthareddy palem, Nellore-524002

[3]Department of Pharmaceutical Analysis, Faculty of Pharmacy, Dr.M.G.R. Educational and Research Institute, Deemed to be University, Chennai, Tamil Nadu-600 077, India.

***Corresponding author:** Salma shaik, Research Scholar, Faculty of Pharmacy, Dr.M.G.R. Educational and Research Institute, Deemed to be University, Chennai, Tamil Nadu-600 077, India, Email: salma.mpharm@gmail.com

Submitted: 14 January 2023; Accepted: 15 February 2023; Published: 11 March 2023

ABSTRACT

Phytopigments of Spirulina platen sis extract has anticancer activity against various types of cancer cell cultures. However, study about effect of phytopigments on Monkey Kidney Epithelial Cells (Vero Line), Human breast cancer (MCF-7) and Human Colon Cancer-(HT-29)cell lines. This study aimed to reveal the anticancer activity of phytopigments from Spirulina platensis extract on Vero line, MCF-7 and HT-29 cells. The research was an in vitro experimental study, with the investigation on cytotoxicity as the anticancer parameters. cytotoxicity test was conducted through MTT assay to observe the visualization and inhibition of proliferation of different concentrations of phytopigments like Zeaxanthin, Phycocyanin, β-Carotene, Phycoerythrin, Chlorophyll-a, and Chlorophyll-b in several incubation times on the cancer cell line. The obtained data were then processed statistically with the Two-Way ANOVA test at a significance value of $p < 0.05$. Based on the results, it could be postulated that in all pigments the phycocyanin shows better activity compare with other at IC50on Vero at152.2 ± 0.20, MCF-7 at 22.60 ± 0.30, HT-29 at 23.30 ± 0.32 compare with standard doxorubicin.

Keywords: *Anticancer; Monkey Kidney Epithelial Cells; Human breast cancer; Human colon cancer; MTT assay*

INTRODUCTION

"More than 8.8 million people died from cancer in 2015, making it the second-leading cause of death globally and accounting for 1 in 6 fatalities worldwide"1 (Dewi et al.2018). "A normal cell can change into cells that express the malignant phenotype through a multistep process called carcinogenesis. As a result of the process, which involves frequent feedback loops as well as the full collapse or failure of controlled stages such cell differentiation, proliferation, and programmed cell death (apoptosis), cancer cells proliferate quickly and begin to metastasize"2 (Nazih & Bard et al.,2018).

When compared to conventional treatment methods, "cancer treatment with plants and plant-based products is a revolutionary method, aside from being simple, safer, environmentally friendly, quick, more selective following its function, and potentially acting specifically on tumour cells without affecting healthy cells"3. (Iqbal et al., 2017). Because it is known to be able to influence some cellular mechanisms including cellular cytotoxics, limit tumor cell invasion, and increase apoptosis of cancer cells, one possible phytochemical is obtained from several marine sources, primarily algae"4 (El-hack et al., 2019). "It has been demonstrated in numerous studies that microalgae activity can combat malignancies (breast, ovarian, skin, lung, kidney, and stomach) as well as suppress cell proliferation and lessen the ability of various cancer cells to form colonies. Because Spirulina platensis satisfies the phylogenetic and cytological requirements, it was placed in the genus Arthrospira (Oscillatoriales). Blue-green, multicellular, filamentous microalgae called spirulina work in symbiosis with bacteria to bind nitrogen from the atmosphere. Algae that uses photosynthetic energy to grow is spirulina. Chlorophyll and carotenoids are also found in phycocyanin, the main pigment produced during photosynthesis"6 (Vo, et al., 2015). "70% Spirulina ethanolic extract demonstrated notable cytotoxicity in K562 and Kasumi-1 cell cultures. Blue trypan solution yielded IC50 values of 4.64 mg/mL for K-562 and 3.68 mg/mL for Kusumi-1 cell lines. Using the blue trypan method, aqueous extract from spirulina also demonstrated cytotoxicity at a higher dose, with an IC50 value of 12.68 mg/mL for K-562 and 2.13 mg/mL for Kusumi-1 cell lines. With the MTT test technique, the IC50 value for 70% ethanolic extract Spirulina was 0.40 mg/mL for K-562 cell lines and 0.31 mg/mL for Kusumi-1 cell lines. Although the IC50 value for Spirulina water extract using the MTT test technique was slightly higher at 15.77 mg/mL for K-562 and 9.44 mg/mL for Kusumi-1 cell lines" 7(Yohana, et al., 2016). "Other research, particularly those focusing on Spirulina's chemopreventive characteristics, show that commercial Spirulina products have an anti-cancer effect against lung cancer cells. The cytotoxic effects of ethanolic Spirulina extract are quite comparable to those of cyclophosphamide, an anticancer chemical substance. Carotenoids, chlorophyll, and phycocyanin, as well as the polysaccharides that make up these extracts, may have a role in the cytotoxicity that Spirulina extracts exhibit against cancer cell types"7 (Yohana, et al., 2016). "Other research findings indicate that phycocyanin and all-trans-retinoic acid (ATRA) may work together to restrict the growth of HeLa cells by causing cell death, decreasing cell cycle progression, and boosting complement-mediated cytolysis"5 (Yang et al., 2014). Additionally, "Spirulina platensis ethanolic extract test results for phytochemicals revealed the presence of tannins, flavonoids, steroids, glycosides, saponins, and alkaloids"9 (Fithriani, et al., 2015). "The ethanolic extract of Spirulina sp. had antioxidant activity (IC50), antioxidant capacity (FRAP test), and total phenolic content, with successive values of 518.94 ppm, 49.95 2.02 (mol Fe2 + eq.g-1 DW), and 0.32 0.0025 mg GAE g-1 DW 8 (Fithriani, et al., 2015). It is possible to draw the conclusion that Spirulina has the potential to be one source that can be turned into an anticancer medication based on the findings of these earlier investigations. Given the high frequency of colon cancer in Indonesia, patients must receive the proper care, particularly medicines with minimal side effects and derived from natural ingredients. Therefore, it is essential to create herbal-based medicines with the ability to eradicate cancer cells in vitro. Spirulina platensis extract contains phycocyanin, which has anticancer properties against several cancer cell cultures".

Experimental work

Extraction and isolation of marine secondary metabolites

Phycocyanine

Spirulina platensis, marine microalgae, was collected from East Godavari, Andhra Pradesh, India and identified by microscopic examination using morphological key features.For pigment extraction, mature Spirulina platensis was gathered and processed Spirulina platensis culture was homogenised and centrifuged at 4000 rpm to get apellet in a clean tube. To the pellet following buffer solution of 100 ml added.

Buffer composition

20 mM acetate 50 mM sodium chloride (NaCl2) and 0.002 M sodium azide (NaN3) was added to thepellet (pH -5.10).The C-Phycocyanin was extracted by freezing at 20°C along with the

buffer solution and then thawing at room temperature until the blue color appeared in the buffer solution supernatant part of the tube which indicates saturation of phycocyanin. The cell debris was removed apart from the buffer was removed by centrifugation at 5000 rpm for 10 minutes, and the resulting extract was dubbed as crude phycocyanin extract.

Crude extract was subjected to precipitation by using 65% (NH4)2SO4 and kept overnight at 4 °C. The pellet was obtained by centrifugation at 27,000 rpm for 15 min at 4 °C and dissolved in 10 ml of the same extraction buffer solution and named as ammonium sulfate extract (ASE).The 10 ml of ASE was packed in dialysis tube dialysesmembrane (HiMedia, Mumbai, 12–14 kDa). Dialyzed against the extraction buffer solution by Dialyses was carried out twice against 1000 ml extraction buffer, first at room temperature and again dialyzed against 1000 ml of extraction buffer at 4°C overnight. The resultant extract was recovered from the dialyses membrane and filtered through 0.45 mm filter membrane.The dialyzed phycocyanin was passed through Sephadex-LH-20 column (2.5′20 cm) pre-equilibrated and eluted with 0.005 M Na-phosphate buffer (pH 7) at1 ml/min−1.

Phycoerythrin

The obtained crude extract was left at 4°C for an overnight 65 percent ammonium sulphate precipitation. Centrifugation at 5000 rpm for 15 minutes at 4°C was used to recover the pellet, which was then dissolved in 10 mL of the same extraction buffer. Then, it was dialyzed using Dialysis membranes (Dialysis membrane-70, MWCO- 12-14 kDa) obtained from HiMedia against the extraction buffer (India). Two times against a 1000 mL extraction buffer, once at ambient temperature and once overnight at 4 °C. After recovering from the dialysis membrane, the resulting extract was filtered through a 0.45 m filter (Sartorious).

Using a DEAE-Cellulose column measuring 25 cm x 2 cm, the phycoerythrin protein was purified. Acetate buffer in the amount of 150 mL was used to equilibrate the column (pH 5.10). 10 mL of the dialyzed and filtered material was injected into the top of the DEAE-Cellulose column using a syringe. 50 mL of acetate buffer (pH 5.10) was then added to the column to eliminate any unbound protein. The bound proteins and phycobilliproteins were eluted using a linear gradient of acetate buffer with a pH range of 3.76 to 5.10, and 5 ml fractions were collected at a flow rate of 20 ml/hr.

β-Carotene

In order to make the extraction procedure more effective, carotenoids are extracted from biological samples such as microalgae that contain a lot of water using dried samples (dry biomass). Microalgae dry biomass totaling 1000 gm is first weighed and placed three times into a test tube. 500 mL of 0.9 percent NaCl, 100 mL of 1N KOH, and 500 mL of ethanol were added to the test tube. 0.9 gm of NaCl are dissolved in 100 mL of distilled water to create a 0.9 percent NaCl solution. While 2.8 gm of KOH are dissolved in 50 mL of distilled water to create 1N KOH solution. This process, known as saponification, involves the reaction of lye and fats or oils. Because fats are not anticipated in microalgae and saponification attempts to remove chlorophyll, it has no impact on the measurement of carotenoids. The samples were then sonicated for 4 minutes with a Branson Sonifier 250 at a frequency of 40 kHz. Sonication aims to break down the cell walls of microalgae using ultrasonic waves. The samples were then homogenised using powerful divorcts. The sample was then heated for five minutes over a water bath at a temperature between 40ºC and 50ºC. After the samples had reached room temperature, they were centrifuged using a Hitachi CT6900 GEL at 6000 revolutions per minute for five minutes. The filtrate and precipitate are the two stages that follow the centrifugation process. 100 mL of diethyl ether were added to the filtrate for further separation, resulting in the formation of two layers: a top layer with a yellow and green undercoat. The bottom layer (green) is the chlorophyll removed using a solvent, while the top layer (yellow) is an extract of beta carotenoids.

Zeaxanthin

To 10 ml of methanol containing 0.57 (w/v) magnesium carbonate, 0.5 g of spirulina platensispowder was added. The samples were then homogenized in a vortex for 30 seconds. It was then followed by the addition of 10 ml of trichloromethane, which contained 0.005 (w/v) butylated hydroxyl toluene. The samples were

then homogenized in the vortex blender for an additional 30 seconds. After 15 minutes, 10 milliliters of distilled water were added. The samples were spun using a Hettich Universal 32R centrifuge at 2000 g for 10 minutes (Hettich, Tuttlingen, Germany). The samples' lower stages were gathered, and over the course of three rounds, the top phases were repeatedly extracted. The lower layer's extracts were then collected, dried by evaporation, and then re-dissolved in 8 ml of acetonitrile/dichloromethane (50:50; v/v). Then, the extract was filtered with Whatman polytetrafluoroethylene (PTFE) 0.22 µm syringe filter.

Chlorophyll

Way of extracting chlorophyll utilising Becker's technique. 100 mL of samples for the examination of chlorophyll were divided again into 33 mL three times as replicates, namely A, B, and C. Each sample (33 mL) is placed into the Corning 50 mL centrifuge vial, where it is filtered to produce biomass. Additionally, this biomass is weighed using the Precisa 40SM-200A analytical balance. Acetone is the solvent employed in the extraction of chlorophyll.

Compared to chlorophyll b, chlorophyll a is more polar. Acetone is thus employed as the extraction solvent for chlorophyll. Chlorophyll can be extracted in the following steps. first, add 5 ml of acetone to the Corning 50-mL bottle that has the biomass in it. After that, it was strongly homogenised for about a minute. Each sample was then placed in a test tube and heated for 5 minutes at a temperature of 400ºC over a water bath. Then, the sample was cooled to room temperature, as the heat will affect the next process. After that, the entire sample was centrifuged for five minutes at a speed of 6000 rpm to separate it into two layers, the filtrate and the precipitate. Using a pipette, the filtrate is then separated and placed in a Corning 15 mL bottle for the spectrophotometric procedure. The filtrate is green because it contains an acetone-based chlorophyll extract. In their purest form, polar and non-polar pigments each have a distinct colour. The bluish-green pigment chlorophyll an also contains blue phycocyanin, orange carotenoids, and turquoise. Once more adding acetone to the residual precipitate, extraction was performed to acquire the extract's light green chlorophyll. The Corning 15 mL vial that had previously held the extract chlorophyll from the previous procedure was filled with the acquired extracts.

Materials

The cell lines (Monkey Kidney Epithelial Cells (Vero Line), Human breast cancer (MCF-7) and Human Colon Cancer-(HT-29)) were procured from National Centre for Cell Sciences (NCCS), Pune, India. The cell lines were sub-cultured in Dulbecco`s Modified Eagle Media (DMEM) media containing 10% Fetal bovine serum (FBS) and 1% Antibiotic – Antimycotic. The cells were incubated in a CO2incubator (New Brunswick galaxy 170 R, Eppendorf IndiaPrivate Ltd., Chennai, India) at 37 °C in a humidified environmentof 5% CO2. Trypsinization was done to detach the cells prior to MTT assay.

METHODOLOGY

The cytotoxic effect of Zeaxanthin, Phycocyanin, Beta-Carotene, Phycoerythrin, Chlorophyll-A, and Chlorophyll-B on Vero, MCF-7 and HT-29 cells were measured by the MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (1). Approximately 5 x 103cells per well were seeded in a 96-well flat-bottom microplate (Wuxi Nest Biotechnology, Jiangsu, China)) and maintained at 37 ºC in a 5% CO2incubator overnight. The cells were treated with different concentrations (100, 50, 25, 12.5, 6.25, 3.125 µg/ml) of pigments and incubated for 48 h. After completion of incubation, the wells were washed twice with DPBS (Dulbecco`s phosphate buffer saline) and 20 µL of the MTT staining solution was added to each well and the plates was incubated at 37 °C.After 4h, 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and absorbance was recorded at 570 nm using a microplate reader (Lisa Plus, Rapid Diagnostic Pvt. Ltd. Mumbai, India). The untreated cells with culture media were used as negative control and doxorubicin was used as positive control. The percentage cell viability was calculated by using the following formula.

Surviving cells (%) = Mean OD of test compound /Mean OD of Negative control ×100

Half-maximal inhibitory concentrations (IC50) of the pigments were calculated by using Graph Pad Prism Version 6.01 software.

RESULTS AND DISCUSSION

It is crucial to assess the cytotoxicity of the pigments isolated from Spirulina platensisbefore considering their potential application as anticancer agents. The cytotoxic activity of the pigments was evaluated by using the MTT assay, which enables a direct quantitative assessment of anticancer capabilities of the test compounds. This test relies on the mitochondrial dehydrogenase activity of live cells to convert the yellow water-soluble tetrazolium dye MTT to purplecoloured formazan crystals. It is impossible for dead cells to carry out this metabolic reduction. Therefore, this assay is a reliable indicator of cell viability. Figure 1, 2 and 3 showed the percentage viability of the Vero, MCF-7 and HT-29 cells after incubating with the pigments for 48 h. The viability of Vero cells after treatment with all the pigments at concentration ranges of 3.125 µg/ml to 50 µg/ml remained at about 80%.These findings showed that all the pigments have no cytotoxic effects on normal Vero cell lines demonstrating their biocompatibility and possible use as a therapeutic agent. According to the literature, substances with cell viability levels above 80% are frequently regarded as biocompatible. In contrast, MCF-7 and HT-29 cells treated with the all the pigments displayed concentration dependent cytotoxicity. Compared to other pigments, Phycocyanin pigment reduced the cell viability of MCF-7 and HT-29 followed by Phycoerythrin and zeaxanthin. Phycocyanin, a biologically active compound isolated from the Spirulina platensis possess a wide range of pharmacological activities such as antioxidant, anti-cancer, anti-inflammatory, photo-cytotoxicity and immune stimulant. The higher cytotoxicity of phycocyanin on both the cell lines compared to other pigments is accredited due to its ability to stop the progression of cancer cell cycle leading to its arrest. It was reported in the literature that phycocyanin causes cell cycle arrest in G0/G1 phase in breast cancer MDA-MB-231, colon cancer HT29 andadenocarcinoma A549 cells (2).

The IC50 values of all the pigments against the all the cell lines were shown in the Table 1. IC50 is defined as concentration of test compounds required to reduce cell viability by 50%. Lower the IC50 value, higher will be the cytotoxicity. TheIC50 values for all the pigments were found to be in range of 152.2 ± 0.20 to 307.0 ± 0.45µg/ml against Vero cell lines. The IC50 values for all the pigments were found to be in range of 22.6 ± 0.3 to 193.97±3.71 µg/ml against MCF-7 cell lines. Among all pigments, phycocyanin pigment showed the lowest IC50 values and Chlorophyll-A showed the highest IC50 values. Similarly, The IC50 values of pigments against HT-29 cells were found to be in the range of 23.3 ± 0.32 to 202.77 ± 3.80 µg/ml. The phycocyanin showed the lowest IC50 values whereas Chlorophyll-B showed the highest IC50.

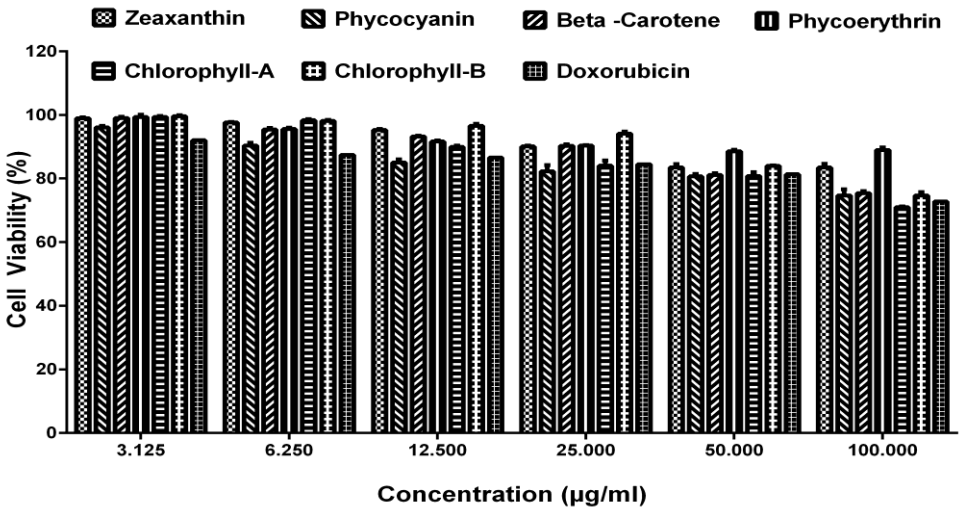

FIGURE 1: The in vitro cytotoxicity effects of zeaxanthin, phycocyanin, β-carotene, phycoerythrin, chlorophyll-a, chlorophyll-b and doxorubicin on Vero cell lines. Data are mean ± SD of three independent experiments.

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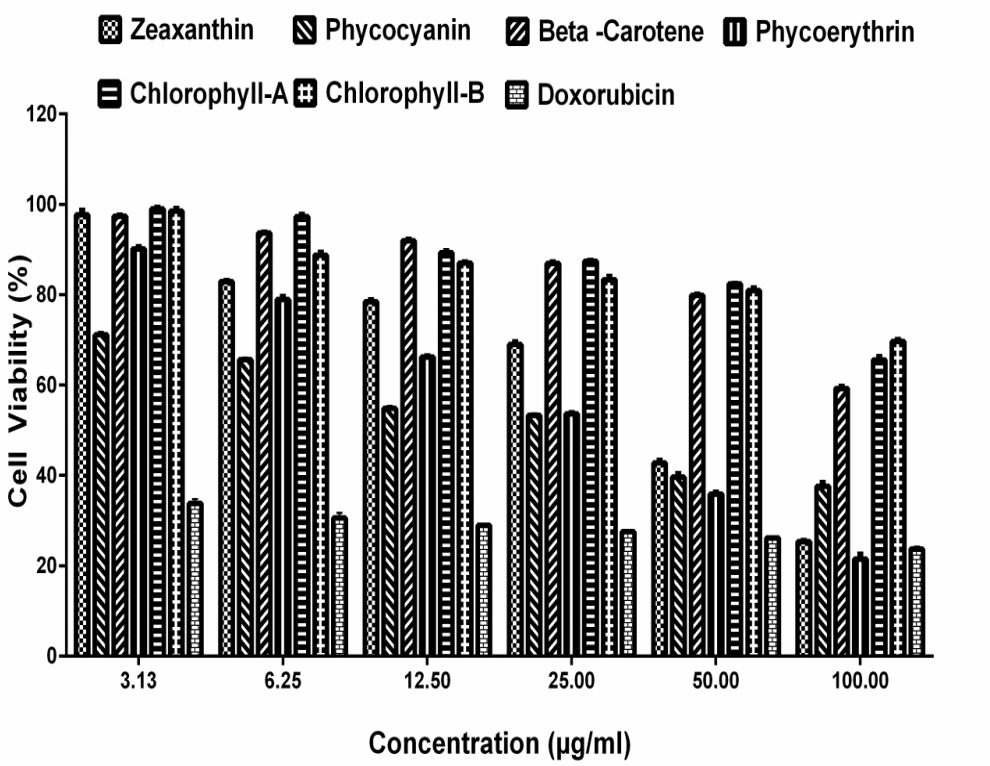

FIGURE 2: The in vitro cytotoxicity effects of zeaxanthin, phycocyanin, β-carotene, phycoerythrin, chlorophyll-a, chlorophyll-b and doxorubicin on MCF-7 cell lines. Data are mean ± SD of three independent experiments.

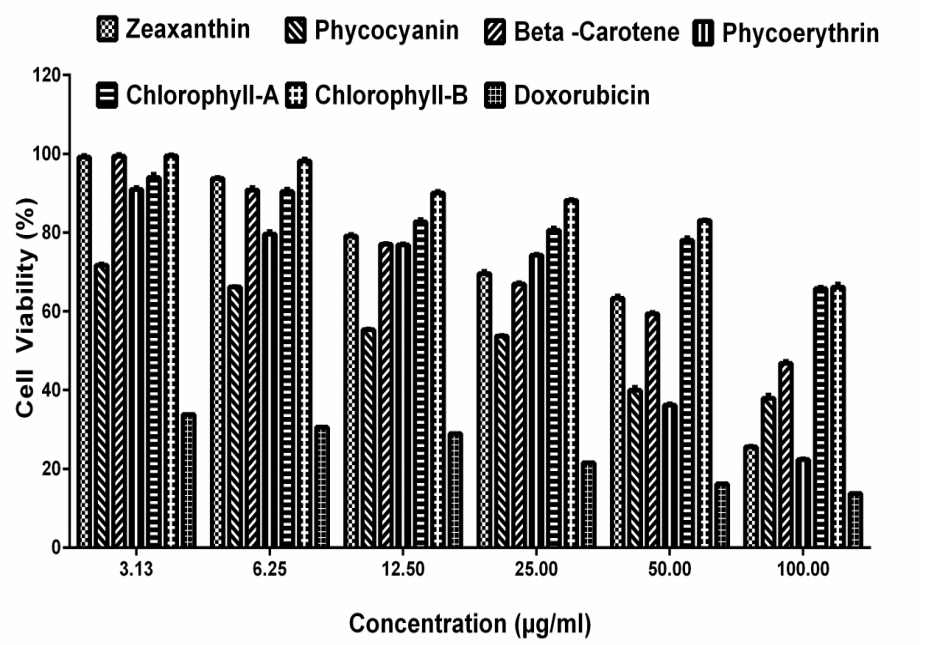

FIGURE 3: The in vitro cytotoxicity effects of zeaxanthin, phycocyanin, β-carotene, phycoerythrin, chlorophyll-a, chlorophyll-b and doxorubicin on HT-29 cell lines. Data are mean ± SD of three independent experiments.

TABLE 1: IC50 values of pigments against Vero, MCF-7 and HT-29 cell lines

Name of the Pigment	IC50 (µg/ml)		
	Vero	MCF-7	HT-29
	Mean ± SD	Mean ± SD	Mean ± SD
Zeaxanthin	246 ± 0.46	41.88 ± 0.58	57.37 ± 0.92
Phycocyanin	152.2 ± 0.20	22.60 ± 0.30	23.30 ± 0.32
Beta -Carotene	214.6 ± 0.15	160.07 ± 1.14	67.41 ± 0.65
Phycoerythrin	307.0 ± 0.45	26.83 ± 0.37	38.01 ± 0.37
Chlorophyll-A	172.9 ± 0.47	193.97 ± 3.71	155.17 ± 3.51
Chlorophyll-B	292.0 ± 2.34	191.03 ± 4.94	202.77 ± 3.80
Doxorubicin	165.5 ± 1.25	3.16 ± 0.10	2.15 ± 0.52

The cytotoxic effects of zeaxanthin, phycocyanin, β-carotene, phycoerythrin, chlorophyll-a, and chlorophyll-bon Vero, MCF-7 and HT-29 cells were furthersupported by optical microscopic images (Fig. 4, 5 and 6). In case of Vero cell lines, the cells in the untreated and pigment treated group showed spindle shaped morphology, adherence to neighbouring cells and had greater confluency of cells. The negligible cytotoxicity on Vero cells confirms the biocompatible nature of the pigments. In case of MCF-7 and HT-29, thecells in the untreated control group were spindle-shaped,adhered to neighbouring cells, and had higher confluency of monolayer cells. In the doxorubicin treated group, mostof the cells were spherical shaped, detached from adjacent cells and less in number. In case of pigment treated cells, most of the cells lost their typical morphologyand became rounded up and detached from adjacent cells indicatingthat cells were undergoing apoptosis. These results confirmed the cytotoxicity of pigments on MCF-7 and HT-29 cells.

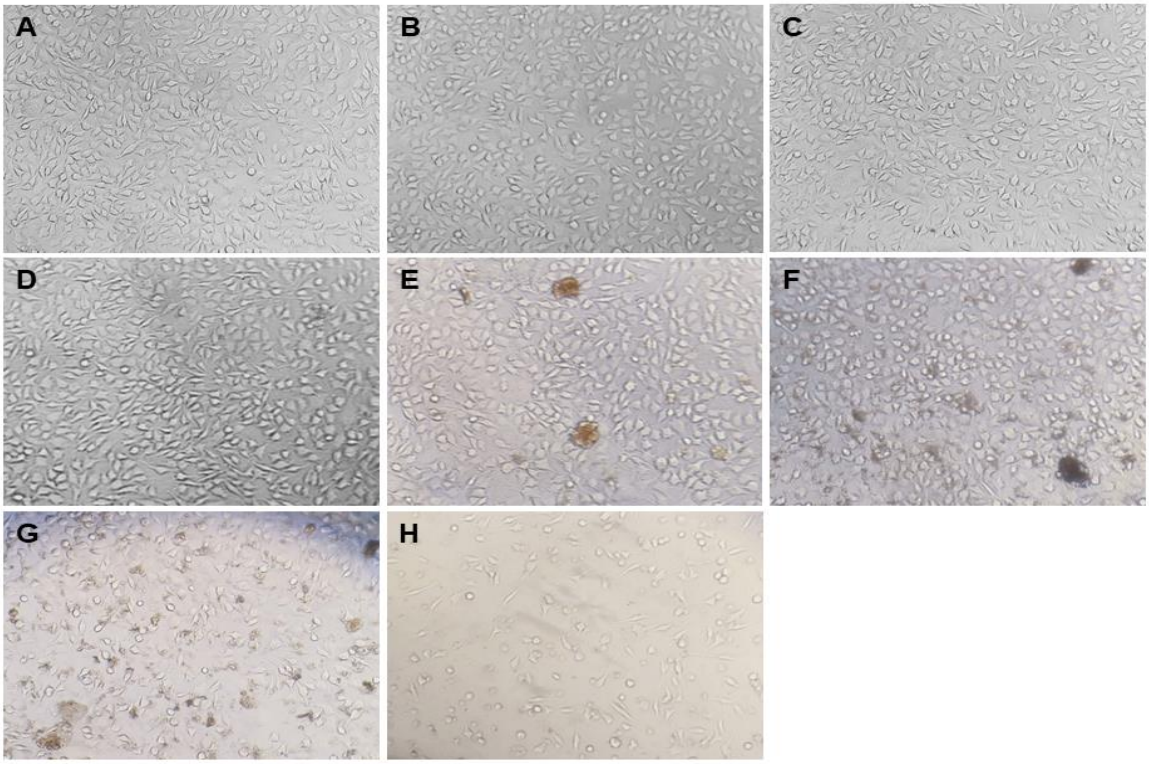

FIGURE 4: The optical microscopic images showing the morphological changes in Vero cells after treatment with (A) untreated control cells, (B) zeaxanthin, (C) phycocyanin, (D) β-carotene, (E) phycoerythrin, (F) chlorophyll-a, (G) chlorophyll-b and (H) doxorubicin, for 48 h.

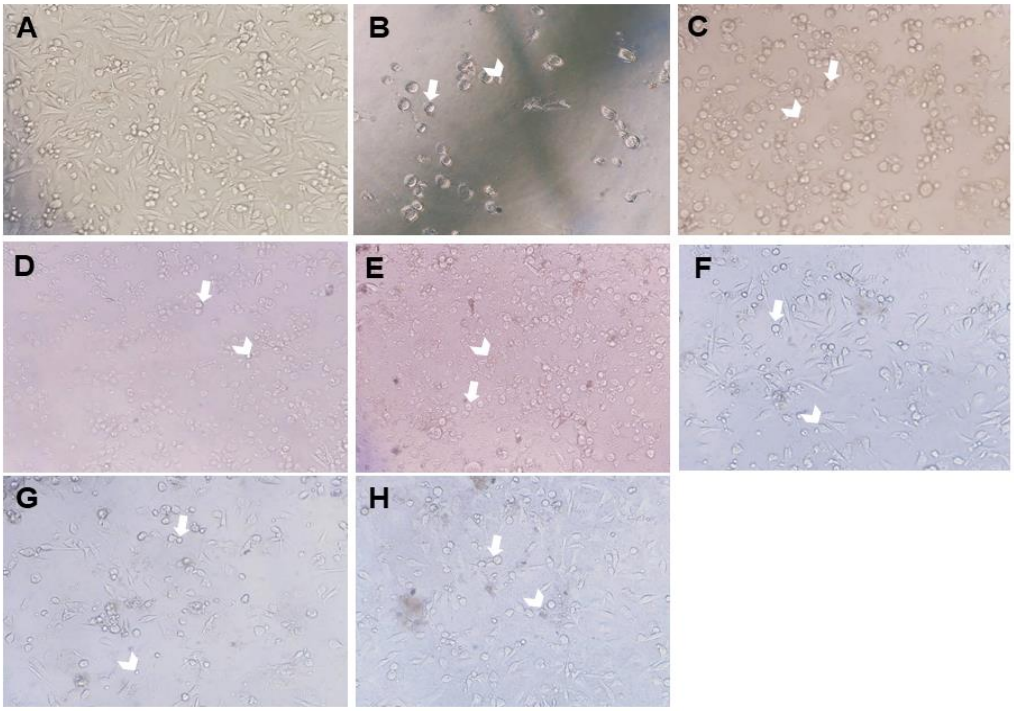

FIGURE 5: The optical microscopic images showing the morphological changes in MCF-7 cells after treatment with (A) untreated control cells, (B) doxorubicin, (C) zeaxanthin, (D) phycocyanin, (E) β-carotene, (F) phycoerythrin, (G) chlorophyll-a, and (H) chlorophyll-b for 48 h. Arrows indicate the rounded up cells and arrow heads denote shrinkage of cells.

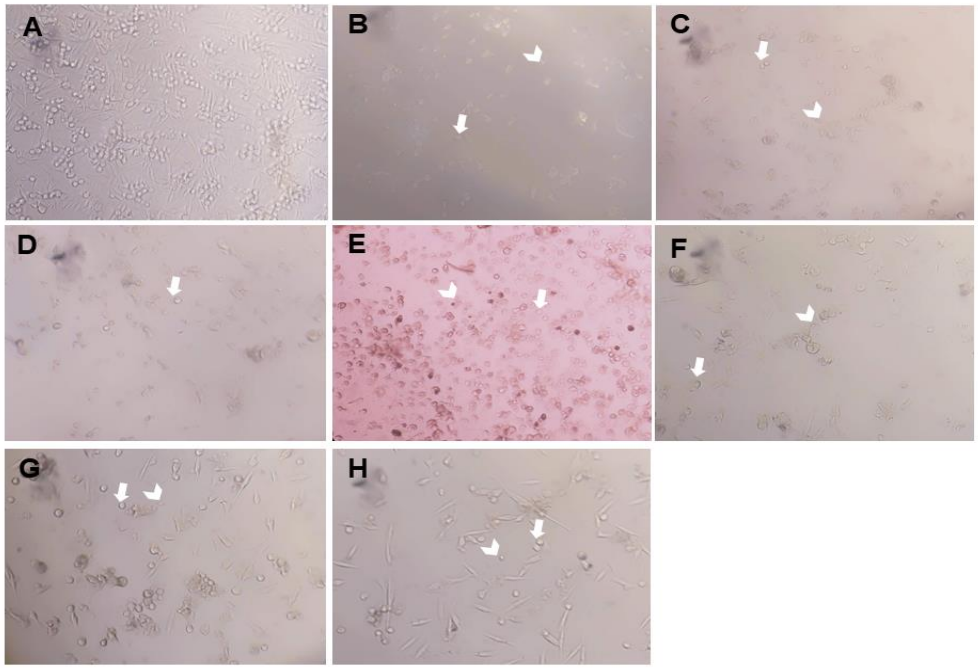

FIGURE 6: The optical microscopic images showing the morphological changes in HT-29 cells after treatment with (A) untreated control cells, (B) doxorubicin, (C) zeaxanthin, (D) phycocyanin, (E) β-carotene,(F) phycoerythrin, (G) chlorophyll-a, and (H) chlorophyll-b for 48 h. Arrows indicate the rounded-up cells and arrow heads denote shrinkage of cells.

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CONCLUSION

Based on the results it concludes that the, cytotoxicity effects of zeaxanthin, phycocyanin, β-carotene, phycoerythrin, chlorophyll-a, chlorophyll-b on the Vero, MCF-7, HT-29 cell lines. the phycocyanin shows the least IC 50 values against the cell lines concentration of on Vero at152.2 ± 0.20, MCF-7 at 22.60 ± 0.30, HT-29 at 23.30 ± 0.32 compare with standard doxorubicin.

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