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Research Paper

Apoptotic Effect of Phycocyanin on HT-29 Colon Cancer through Activation of Caspase Enzymes and P53 Cell Signaling Pathway

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ABSTRACT

Background: C-phycocyanin, a biliprotein from Spirulina platensis, is a future candidate for cancer management. This agent is originated from edible blue-green algae, and numerous in vivo and in vitro research have reported on its anti-cancer properties. The effects of C-phycocyanin have been investigated on caspases 3, 8, 9, and p53 pathways in the human colorectal adenocarcinoma cell line (HT-29) and human umbilical vein endothelial cells (HUVECs).

Methods: In the current study, we investigated the effect of C-phycocyanin on caspase 3, 8, 9, and p53-mediated apoptosis pathways in two cell lines (HT-29 & HUVEC), using quantitative real-time PCR and flow cytometry. The cytotoxicity of phycocyanin on HT-29 cells was compared with HUVEC normal cells via colorimetric assays.

Results: Based on our findings at molecular level, the expression of caspases 3, 8, 9, and p53 genes were increased in colorectal cancer cells treated with C-phycocyanin.The results were confirmed by an increase in the number of colorectal cancer cells in the early and late stages of apoptosis as compared to the control, untreated cells. In addition, the results of colorimetric assay showed that C-phycocyanin has no cytotoxic effects on normal HUVECs cells.

Conclusion: Based on our experimental data, it is evident that C-phycocyanin has measurable effects on cell apoptosis. Since tumorigenesis is halted by apoptosis, C-phycocyanin can be a hopeful candidate for the treatment of human colorectal cancer in the future.

Keywords: Apoptosis; Caspases; Colorectal Cancer; Human Umbilical Vein Endothelial Cells; Phycocyanin; Tumor Suppressor Protein p53.

Introduction

Colorectal cancer cells (CRC) have been responsible for 900,000 deaths in 2020 [1, 2]. New natural products for the treatment of CRC are necessary to lower the death rate in the long-term administration [3]. As a member of the phycobiliprotein (PBP) family, C-phycocyanin (C-PC) is a remarkable photosynthetic associate protein obtained from cyanobacterial and other algal species [4]. There are two available mechanisms for algal pigments to prevent the development of human tumor cells: G0/G1 cell cycle arrest and activation of the apoptotic pathway [5]. Recent studies have assessed the anti-cancer properties of C-phycocyaninin cancer cells. This compound shows good antineoplastic effects on various types of cancer cells *in-vitro* including lung cancer [6], ovarian cancer [7], and melanoma cancer [8]. Further, utilization of C-phycocyanin to enhance radiation therapy in colon cancer model has been evaluated previously [9]. However, the regulatory effects of C-phycocyanin on pathways associated

with cancer cells, such as apoptosis, have not yet been investigated.

One of the modifications in cell physiology is partial suppression and bypassing the apoptosis that leads to the growth of malignant cells, and tumor progression [10]. Most chemotherapeutic drugs activate the apoptotic pathway that play strategic roles in their cytotoxic activities. Moreover, dysregulation of cell cycle is strongly involved in tumor growth [11]. In this context, caspases 3, 7 amd 9 have key roles in the apoptotic pathways [12]. As an important tumor suppressor gene, p53 activates apoptosis and its mutation is effective in the late stages of colon cancer. Colorectoal cancer patients with p53 mutations have been reported to have the worst prognosis and short survival time [13]. As a transcription factor, p53 protein causes cell cycle arrest and apoptosis under cellular stress. It has been found that 40-50% of colorectoal cancer patients have p53 mutations, which is related to the progression and poor clinical outcomes [14].

In recent years, our knowledge of molecular mechanisms and the effect of gene mutations in colon cancer development has improved. Further, chemotherapy has adverse effects on normal body cells, thus it is nessesary to investigate the potential natural products that induce apoptosis with low or no side effects. Therefore, the anti-cancer effects of C-phycocyanin in human colorectal cell line (HT-29) were investigated *in -vitro* through molecular and cellular asssements.

Materials and Methods

Materials

The frozen cells of HT-29 colerectal cancer cell line and HUVEC origin were obtained from the National Cells Bank of Iran at the Pasteur Institute, and Stem Cell Technology Research Center of Iran (Tehran, Iran), respectively. Also, the following materials were used in our experiments during this research:

Dulbecco's Modified Eagle Medium (DMEM) from Capricorn (Germany) (Cat. #: DMEM-HA).

Fetal bovine serum (FBS) from Gibco Life Technology, (USA) (Cat. #: 11573397).

[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) from Sigma-Aldrich (Germany) (Cat. #: M5655).

Total RNA extraction Kit of Pars Tous (Iran) (Cat. #: A101231), TRIzol® Reagent from Thermo Fisher Scientific (Waltham, USA) (Cat. #: 15596026).

Dimethyl sulfoxide (DMSO) from Merck (Germany) (Cat. #: 67-68-5).

Phycocyanin powder from Bio Green (USA) (Cat. #: FSSC22000),

Flow cytometry kit from ApoFlowEx FITC Kit, Exbico company (USA) (Cat. #: ED7044). Primers from Pishgam Co. (Tehran, Iran).

Cell Culture Conditions

The human HT-29 colerectal cancer cells were cultured at a seeding density of 10^4 viable cells/cm^2 in DMEM culture medium, containing 10% FBS and 1% penicillin/streptomycin. They were incubated at 37°C, 5% CO_2 and humidity, and passaged 15 times. The endothelial cell line, HUVEC, was isolated from an umbilical cord vein, and cultured in DMEM medium, and was passaged six times under the same condition as that of HT-29.

Phycocyanin Treatment and MTT Assay

The human HT-29 colerectal cancer cells and HUVEC cell line were seeded at 10^4 cells in 200µL DMEM medium per well in 96-well plates a day prior to the treatment. After incubation for 24hr, the cell line was treated in the absence or presence of phycocyanin at 12.5, 25, 50, 75, or 100 mg/ml for 3hr. Then, 10µL/well tetrazolium salt solution (MTT colorometric assay) was added to the cells and incubated for another 3hr at 37°C. Finally,the medium was discarded and DMSO was added to the wells. The absorbance of the solution from each well was measured spectrophotometrically at 540 and 570 nm, respectively [15, 16].

Gene Expresion Analyses

Using total RNA extraction Kit (Pars Tous; Tehran, Iran) based on Trizol method, the total RNA contents from both cell lines were extracted. The quality and quantity of the isolated RNA were assessed on a nanodrop spectrophotometer. One µg of normalized RNA was utilized for cDNA synthesis based on the manufacturer's protocol for Norgen's TruScript™ First Strand cDNA Synthesis Kit (Canada). Using gene specific primers and SYBR green, the gene expressions in both untreated and Phycocyanin-treated cell lines were evaluated. The final reaction volume was 20µL per well. Each well contained Power SYBR Green PCR Master Mix (10 µL), cDNA (1 µL), and both forward and reverse primers (each 0.25 µM). The sequences of the primers are listed in Table 1.

Table 1. Primer sequences in real-time PCR

Gene Title	Primer Sequence
Beta actin	F: GCCAACAGAGAGAAGATGACAC R: GTAACACCATCACCAGAGTCCA
p53	F: TGCGTGTGGAGTATTTGGATGA R: TGGTACAGTCAGAGCCAACCTCG
Caspase-3	F: CCTCAGAGAGACATTCATGGG R: GCAGTAGTCGCCTCTGAAGAT
Caspase-8	F: CAGGCAGGGCTCAAATTTCTG R: TCTGCTCACTTCTTCTGAAATC
Caspase-9	F: AGTTCCCGGGTGCTGTCTAT R: GCCATGGTCTTTCTGCTCAC

The experimental steps for PCR cycling were as follows: once incubated at 95°C for 10 minutes, the forty cycles were run over the following three steps:

Denaturation step (10 seconds at 95°C),

Annealing step (20 seconds at 52°C), and

Extension step (20 seconds at 72°C) .

Beta actin was selected as the house-keeping gene. Finally, using the Paddle method $2^{-\Delta\Delta CT}$, the acquired cycle threshold (Ct) was analyzed [17, 18].

Flow Cytometry

To evaluate the apoptotic cells, the Annexin V-FITC test was conducted on HT-29 colorectal cancer cell lines. The cells were cultured in the presence or absence of C-PC. Briefly, after washing the cells twice with phosphate buffer saline (PBS), the cells were detached by trypsin-EDTA, then were centrifuged at 3000 rpm at 4ºC for 5-min, and the supernatant was collected. The detached cells were resuspended in binding buffer and labeled with Annexin V-FITC for 15-min. After incubation in the dark at room temperature, the cells were washed twice with Annexin-V binding buffer and fixed with cold 70% ethanol (4°C for 30-min). Then, 200µl of the same buffer was added to the cell pellets followed by adding 10µg/mL RNase, the cells were incubated at room temperature for 40-min. Finally, the cells were resuspended in propidium iodide (PI) binding buffer and the apoptosis rate was estimated.

Statistical Analyses

The statistical differences were determined by one-way analysis of variance (ANOVA) and the results were considered statistically significant at *P* values less than 0.05* and 0.01**. Graphs were plotted by GraphPad Prism software, version 9.4. All of the experiments were run in duplicates.

Results

Inhibition of HT-29 Cell Line

Using the MTT assay, the impact of C-PC was evaluated on the growth of HT-29 and HUVEC cells. The number of HT-29 cells gradually decreased with the rise in C-PC concentration (Figure 1). The data for the HUVEC cell line are presented in Figure 2.

The calculated value of IC_{50} for C-PC in HT-29 cells was 55.47 mg/mL. Moreover, the results showed a marked decrease in the colony formation in HT-29 cells after treatment with C-PC compared to that of HUVEC cells; i.e., there was no toxic effect evident on HUVEC cells. This finding points out to the effective inhibition of growth in colon cancer cells by phycocyanin.

Effects on Caspase Enzymes

In the next step, we evaluated the signaling pathways involved in apoptosis induction in caspases and p53 genes on HT-29 colon cancer cells. The result as presented in Figure 3, demonstrated that C-PC-mediated apoptosis in HT-29 cells is dependent on caspase and p53 signaling pathways. Briefly, phycocyanin can increase the expression of p53 gene and activate caspases 3, 8 and 9. The concentration of C-PC at 55.47 mg/mL prevented the growth of HT-29 cells up to 50%. This compound increases apoptosis in HT-29 cells by activating caspases and p53. The quantities of caspases and p53, as expressed in HT-29 cell line, were higher than that of the control HT-29 cells (P<0.05).

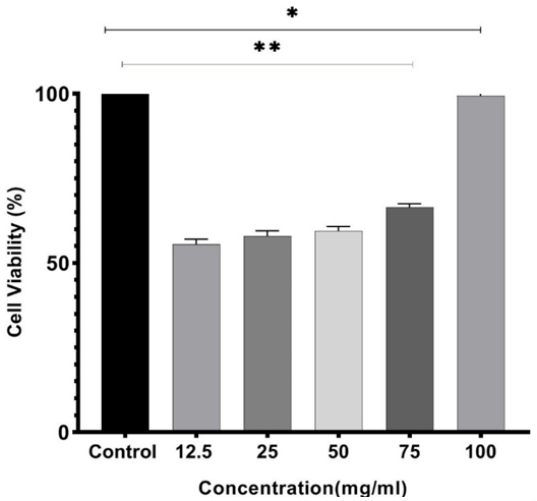

Figure 1. Assessment of HT-29 cell viability by MTT assay after 3 hr of incubation with C-phycocyanin (12.5, 25, 50, 75 & 100 mg/ml). The cell viability of treated cells was compared with the untreated group (control) through ANOVA. * = Significant difference (P<0.05), ** = Highly significant (P<0.01**).

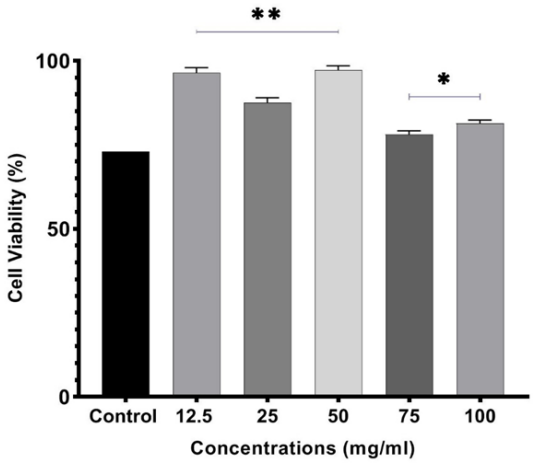

Figure 2. Assessments of HUVEC cell viability by MTT assay after incubation with C-phycocyanin (12.5, 25, 50, 75, and 100 mg/ml). The cell viability of treated cells was compared with the untreated group (control) through ANOVA. * = Significant difference (P<0.05), ** = Highly significant (P<0.01**).

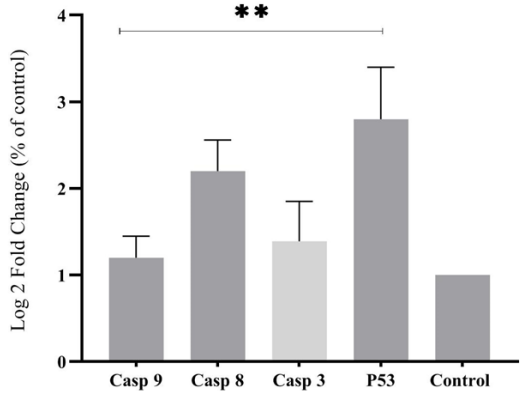

Figure 3. Assessments of pre-apoptotic effects of C-phycocyanin in the HT-29 human colorectal cell line with qRT- PCR method. The reactions showed increases in the expression of caspases 9, 8, 3 and p53 genes after treatment with C-phycocyanin (IC50 value). The viability of treated cells was compared with the untreated group (control) through ANOVA. ** = Highly significant difference (P<0.01).

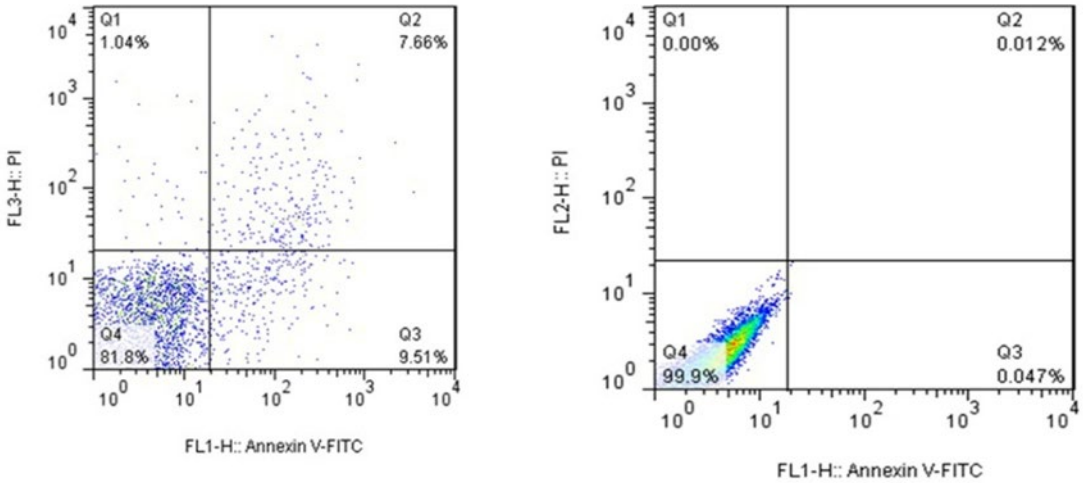

Figure 4. HT-29 cell line treated with C-phycocyanin and its pre-apoptotic effects was analyzed by flow cytometry method. Left Panel: HT-29 cells treated with C-phycocyanin (IC50 value). Right Panel: Control or untreated cells. Q1: Necrosis, Q2: Late apoptosis, Q3: Early apoptosis, Q4: Live cells.

The treated cells demonstrated higher levels of caspases and p53 genes. This finding indicated the up-regulation of the genes in the treated cells.

Flow Cytometry

Although C-PC has been proven to induce apoptosis in cancer cells in numerous studies [16-18], we specifically examined the effect of C-PC on the apoptosis in HT-29 cells by Annexin V. The induction of both early and late apoptosis was indicated based on the results from the C-PC-treated HT-29 cells (Figure 4).

Discussion

Colorectal cancer represents a global health challenge as the third most common malignancy. Therefore, novel therapeutic methods are urgently warranted to control this cancer. In the past decades, exploiting natural resources for cancer inhibition and treatment has become critical, and cyanobacteria hold a great potential among the essential products found in *S. platensis* [19].

Recently, more studies have explored the pharmacological and immunological effects of C-PC, such as photo-induced cytotoxicity [20], activation of the immune system [21], and anti-oxidative [22] and anti-inflammatory properties [23]. Besides, there is a potent anti-tumor role for C-PC in a number of *in vitro* and *in vivo* cancer cells from the blood, liver, breast, colon, and lung tissues [24-28].

Despite the encouraging results, the pharmaceutical market still lacks phycocyanin-based chemotherapy, largely due to the poorly known mechanisms of its action. In the current study, we investigated the effects of C-PC on some cellular targets and mechanisms. C-PC appears to have a key role in decreasing the expression of transcription factors, signal transducers, and pro-inflammatory cytokines. Also, this biliprotein is able to increase the expression of IL-4, a potent anti-inflammatory cytokine [29]. Further, C-PC can improve immune function by enhancing lymphocytic activities, thus increasing the body's defense against diseases. This compound is likely to become a new candidate for cancer therapy [30]. This is mainly because of the high expression of scavenger receptor-A (SR-A) expressed on the surface of tumor-associated macrophages (TAMs) and due to its affinity toward this receptor [30].

The antiproliferative and cytotoxic properties of C-PC have been shown by earlier *in vitro* and *in vivo* studies. Thangam, *et al*. have demonstrated the antioxidant and antiproliferative potential of C-PC against A549 and HT-29 cell lines through G0/G1 phase arrest and DNA fragmentation [5]. Also, C-PC prevents cancer cell proliferation by recruiting GAPDH from the nucleus to the plasma membrane, arresting the cell cycle at the G0/G1 phase [31, 32]. Further research has demonstrated that C-PC stops G1-phase in myeloid leukemia cells (K562), allowing them to follow the apoptotic pathway [33]. Moreover, C-PC can arrest the cells at the G2-phase and induce apoptosis in human hepatoma cell line (HepG2) [20] and human ovarian cancer cell line (SKOV-3) [7].

Phycocyanin is able to cleave polyADP-ribose polymerase 1(PARP1) and change the Bcl-2/Bax ratio by activating caspases in both apoptotic pathways. Subhashini, *et al.* have shown that there is a significant decrease (49%) in proliferation, elevated apoptosis and down regulation of Bcl-2 after treatment of human chronic myelogenous leukemia cells (K562) by C-PC [24]. In another research, Ravi, *et al.* suggested the increased expression of p21 and decreased expression of cyclin-E in human breast cancer cell line [34]. Liao, *et al.* have shown that C-PC causes a stop in human pancreatic cancer cell line (PANC-1) at the G2 phase checkpoint, after which the cells undergo apoptosis. Further, these authors have demonstrated that

apoptosis can be activated by phycocyanin through several pathways, such as NF-κB, PI3K/Akt/mTOR and MAPK [35].

High levels of vascular endothelial growth factor (VEGF-A), matrix metallopeptidase enzymes (MMP2 & MMP9), are the basis for metastasis, which are down regulated by C-PC through binding to VEGFR1 [29]. In addition, C-PC alters the cellular redox state and inhibits cell proliferation by targeting enzymatic and non-enzymatic antioxidants. This compound also alters the mitochondrial membrane potential by releasing cytochrome C from mitochondria during the early stages of apoptosis. The release of cytochrome C increases the production of reactive oxygen species (ROS) and ultimately activates the signaling pathways of pro-apoptosis in cancer cell lines [36, 37]. Another role for phycocyanin in cells is scavenging free radicals that prevents DNA oxidative damage in nerve cells [38].

The p53 as a tumor suppressor gene has a key role in the regulation of cell cycle, and is strictly related to tumor cell proliferation [39]. The p53 pathway plays an important role in arresting cells at the G1 and G2 checkpoints after DNA damage, thus forcing them to apoptosis [40]. Saini, *et al*. have shown that C-PC can stimulate the cell cycle arrest and mediate apoptosis by activating p53 gene [41]. Overall, p53 and caspase-mediated apoptosis pathways are involved in anti-cancer effects of C-PC against colon cancer HT-29 cell line. These effects were established via cellular and molecular assessments in the current study (Figures 3 & 4).

Our study showed that Phycocyanin inhibited the proliferation of tumor cells in HT-29 colerectal cancer cell line and promoted tumor cell apoptosis through p53 and caspase-mediated apoptosis pathways. Our data suggest that activation of apoptosis-p53 pathway by Phycocyanin can be a novel therapeutic candidate to overcome resistance to chemotherapy. Considering the fact that phycocyanin has not shown any cytotoxic effects on endothelial cells *in-vitro*, it is likely to be a promising, natural therapeutic agent for clinical use in the management of human colorectal cancer. However, further well-designed studies are warranted on Phycocyanin.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethical Approval

The survey was confirmed by the Ethics Committee, the University of Medical Science, Iran (Code: IR-UMS.REC.1399.545). This study did not conduct any experiments on humans or animals.

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Authors' Contributions

HP and AP designed the study; MS, AAF, and MM performed laboratory tests and data collection. HP and IS carried out the data analyses. MM, RN and AA wrote the final draft of the manuscript. All authors reviewed and approved the final manuscript.

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