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C-Phycocyanin Inhibits Cell Proliferation and May Induce Apoptosis in Human HepG2 Cells

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C-Phycocyanin (C-PC) is one of the major biliprotein pigments of unicellular cyanbacterium of *Spirulina platenesis*, it has nutritional, medicinal, and hepatoprotectant application. The growth and multiplication of human hepatoma cell lines (HepG2) under the effect of different concentrations of C-PC (0.8, 1.75, 3.5 and 7.0 µg/ml) against untreated cells as control for 24h were investigated. The results showed that the proliferating cells in presence of C-PC reached 70, 51, 44, and 39%, respectively. The results revealed that the greatest reduction in proliferation of cells was recorded at 7.0 µg/ml and LC_{50} at 1.75 µg/ml of C-PC. In parallel, to the previous results HCl-denatured MG-P revealed that in mass of cells there is a pattern of apoptosis because the expanded cytoplasmic area (bluish-green) reduced and appeared faintly red as C-PC concentration increased. Moreover, the cells lost all the nuclear entities then, become fragmented and having no nuclear remnants. The C-PC may be a new potential anti-cancer drug for therapy of human hepatoma cells.

In recent years, there is an increasing awareness that certain naturally occurring compounds in plants and other sources, have protective effects against environmental mutagens/carcinogens and endogenous mutagens (Abraham, 2001). Dietary intake of such chemo preventive compounds has been suggested as an effective strategy for minimizing the deleterious effects of genotoxins and carcinogens. *Spirulina* is one of blue green microalgae, that has been used since ancient times as a source of food because of its high protein and nutritional values and it is gaining attention as a source of potential pharmaceuticals. Recent studies have demonstrated that C-PC, extracted from *Spirulina*, has several effects including antioxidant (Miranda *et al.,* 1998), antimutagenic (Chamorro *et al.,* 1996), antiviral (Ayehunie *et al.,* 1998), anticancer (Chen *et al.,* 1995; Schwartz *et al.,* 1988), anti-allergic (Kim *et al.,* 1998), immune enhancing (Qureshi *et al.,* 1996), hepato-protective (Gonzalez *et al.,* 1993), blood vessel relaxing (Paredes-Carbajal *et al.,* 1997) and blood lipid-lowering effects (Iwata *et al.,* 1990). The biological and pharmacological properties of *Spirulina* were attributed mainly to calcium-spirulan and (C-PC). C-PC, a water-soluble non-toxic biliproteins pigment isolated from *Spirulina platenesis*, has significant antioxidant and radical scavenging properties (Vadiraja *et al.,* 2000). C-PC is one of the major biliproteins of *Spirulina platenesis*, this water soluble protein pigment was shown to be hepatoprotective (Vadiraja *et al.,* 1998), antiarthritic (Remirez *et al.,* 1999), and anti-inflammatory (Romay *et al.,* 2000; Romay *et al.,* 1998) in both *in vitro* and *in vivo* experimental models. However, little is known about its mechanism of action. Earlier, Reddy *et al.,* 2000, showed that C-PC selectively inhibits cyclooxygenase-2 (COX-2), the inducible isoform of cyclooxygenase, implicated in the mediation of inflammation, and arthritis. C-PC is used for the treatment of

diseases such as Alzheimer's and Parkinson's (Rimbau *et al.,* 2000) and prevents experimental oral and skin cancers (Morcos *et al.,* 2004). On the same line, C-PC was evaluated as a putative antioxidant *in vitro* and inhibits liver microsomal lipid peroxidation in rat liver *in vivo*. These studies clearly suggested that the covalently linked chromophore, with phycocyanobilin, is involved in the antioxidant and radical scavenging by C-PC (Bhat *et al.,* 2000). The anti-inflammatory effect of C-PC extract was studied in acetic acid-induced colitis in rats. The histopathological and ultra structural studies showed inhibition in inflammatory cell infiltration and reduction to some extent in colonic damage in rats treated with C-PC (Gonzalez *et al.,* 1999). Moreover, Benedetti *et al.,* 2004 found that, C-PC protects normal human erythrocytes and plasma samples against oxidative damage *in vitro* and has a potent action as anti-oxidant, anti-inflammatory and anti-cancer properties. C-PC also reduced the levels of tumor necrosis factor (TNF-alpha) in the blood serum of mice treated with endotoxin and it showed neuroprotective effects in rat cerebellar granule cell cultures and in kainate-induced brain injury in rats (Romay *et al.,* 2003). Our previous studies showed that C-PC induces apoptosis in human chronic myeloid leukemia cell line-K562 (Subhashini *et al.,* 2004) and also in LPS-stimulated RAW 264.7 cells (Reddy *et al.,* 2003). C-PC was shown to be a peroxyl radical scavenger both *in vivo* and *in vitro* (Bhat & Madyastha, 2000), inhibitor of CCl4 induced lipid peroxidation (Vadiraja *et al.,* 1998) and inhibitor of ONOO-mediated deleterious biological effects (Bhat & Madyastha, 2001).

The aim of the present study was to evaluate the effects of C-PC on the proliferation and apoptosis of human hepatoma cell lines HepG2.

Materials and Methods

Extraction and Determination of the C-PC

C-PC was extracted from the blue green alga *Spirulina platenesis* (Egyptian isolate) according to the method of Boussiba & Richmond (1979). Approximately 20 g of experimental alga was suspended in 200 ml of 0.1M Sodium phosphate buffer pH 7.2 containing 100 µg/ml lyzozyme and 10 mM EDTA. The enzymatic disintegration of cell wall was brought about by placing the alga in a shaking water bath at 30 ºC for 24 h. The slurry was centrifuged for 10 min at 12.000X g to remove cell debris, yielding a clear blue supernatant of C-PC. The crude of extract C-PC centrifuged for 30 min at 12000Xg at 4 ºC. C-PC extract was precipitated and brought to two steps of ammonium sulfate precipitation (from 50% to 75% $(NH_4)_2SO_4$ (w/v) at pH 7.2 for 6 h). The suspension of each step was centrifuged at 10,000 rpm for 20 min. The precipitated C-PC was dissolved in phosphate buffer (pH 7.2) and dialyzed overnight at 4 C against the same buffer. The concentration (µg/ml) of the partially purified extract was determined according to (Bennett & Bogorad, 1973).

In vitro Application of C-PC Against Human Hepatoma Cell Lines (HepG2)

Stock solution (1%) of C-PC in phosphate buffered saline (PBS); pH 7.2 was prepared and stored refrigerated until used for the treatments. The different concentrations of C-PC were prepared through the dilution of stock solution in PBS at the concentrations of 7, 3.5, 1.75 and 0.8 µg/ml.

Cell Line Culture and Treatment

HepG2 cells were routinely grown at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a confluent humidified atmosphere containing 5% CO_2. The cultured cells were sub-cultured twice each week. HepG2 were plated at 5 x 10^5 cells /cm^2 and placed in a humidified, 5% CO_2: 95% air incubator over night at 37°C. For application of C-PC, the medium was replaced with DMEM enriched with 10% FBS and the C-PC add at concentrations of 7, 3.5, 1.75 and 0.8 µg/ml. In addition to the previous concentrations cells without treatment with Phycocyanin as (negative control) were also tested. Plates were incubated at 37ºC and 5% CO2, and cells were harvested after 24 h.

Biochemical Analysis

Cell viability assay

- Measurement of cytotoxicity to cells by (MTT assay)

Cell proliferation was determined using the MTT assay (Mosmann, 1983). HepG2 (150-200,000cells/well) were incubated in 96-well plates (Nunc- Nunclon) in the presence or absence of C-PC (7, 3.5, 1.75 and 0.8 µg/ml) for 24 h in a final volume of 100 ml. Aliquots of 0.2 ml of MTT (5 mg/ml in PBS) were added to each well and incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 ml of DMSO and the absorbance values at 570 nm were measured on a multi-well plate reader ELISA Micro plate reader (Meter tech *E* 960. USA).

Histopathological Examination

- HCl-Denatured Methyl Green-Pyronin (MG-P) Staining

HCl-denatured-methyl green-pyronin (BDH Chemicals) technique was performed based on the method of Iseki & Mori (1986) and modified by Sen *et al* (1999). In which the post fixed cells in Carnoy's solution (6:3:1 v/v mixture of alcohol, chloroform, and iced acetic acid) were spread on clean glass slides, and then iced in ice box for 10 min. The fixed slides were hydrolyzed with 0.1N HCl in 80 % alcohol for 5 min, then rinsed with distilled water, and stained with freshly prepared MG-P solution (0.5% of methyl green and pyronin in a 100 mM sodium acetate buffer, pH4.8) for 6 min at room temperature. The excess stain was removed with acetone and raised with distilled water.

- Analysis of DNA Fragmentation

DNA laddering was detected by isolating fragmented DNA using SDS\ proteinase K\RNasa A extraction method. Which allows the isolation of only fragmented DNA without contaminating genomic DNA. Briefly, cells were washed in cold PBS and lysed in a buffer containing 50 Mm Tris-HCL (PH 8.0), 1 mM EDTA, 0.2% Triton X-100 for 20 min. at 4 C. After centrifugation at 14,000x g for 15 min, the supernatant was treated with proteinase K (0.5 mg \ml)and 1% SDS for 1 hour at 50 C. DNA was extracted twice with buffered phenol and precipitated with 140 mM NaCL and 2 vol. of ethanol at -20 C. overnight. DNA precipitates were washed twice in 70% ethanol, dissolved in TE buffer, and treated for 1 hour at 37 C. with RNase A.

Finally, DNA preparations were electrophoresed in 1% agarose gels, stained with ethidium bromide and visualized under UV light (Fig. 1).

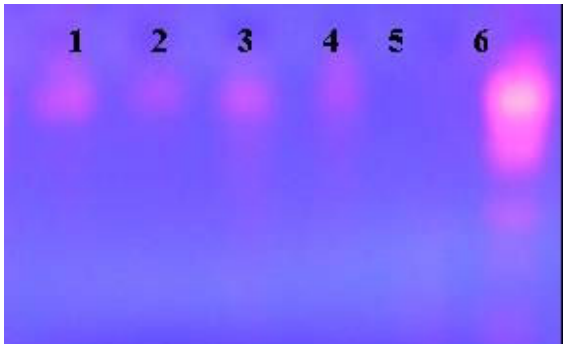

Figure 1. Agarose gel electrophoresis of DNA extracted from HepG2 cells treated with phycocyanin.
Lane 1: HepG2 cells treated with 7 µg/ml phycocyanin; lane 2: HepG2 cells treated with 3.5 µg/ml phycocyanin; lane 3: HepG2 cells treated with 1.75 µg/ml phycocyanin; lane 4: HepG2 cells treated with 0.8 µg/ml phycocyanin; Lane 5: HepG2 control cells; Lane 6: 500 bp DNA ladder.

Statistical Analysis

Statistical analysis of variance (ANOVA) and Student–Newman–Keul multiple comparison test were applied to determine the significant differences among the groups (Bailey, 1994). A confidence level of 95% (P>0.05) was considered significant.

Results

Cell Viability Assay

Cultured human (HepG2) cell lines were treated with C-PC at different concentrations for 24 h. Data from the MTT reduction assay showed a direct proportional relation between proliferating cells and the dye formation. The MTT reduction was dose dependent as the viability of cells was clearly decreased as the concentrations of C-PC increased and, reached maximum reductions at a concentration of 7 µg /ml (Fig. 2). The half maximal inhibitory concentration (IC_{50}) values were estimated as 1.75 µg /ml for C-PC. The following order of decreasing potency on HepG2 cell viability was found to be 7 > 3.5 > 1.75 > 0.8 µg/ml of C-PC.

HCl-Denatured Methyl Green-Pyronin (MG-P) Staining

In cytochemistry the DNA has bluish-green color following stained with methyl green, while, RNA has red color when it stained by

pyronine. As the intranuclear DNA of the nonproliferating cells is more susceptible to acid denaturation than that of the proliferating cells, the proliferating cells are mainly stained in bluish-green color, whereas the nonproliferating cells appeared reddish colored. The proliferating cells being affected with Phycocyanin concentration showed that the bluish green color was gradually disappeared with apoptotic signs which include smaller area of cytoplasm; lost all nuclear entities as well small mass in the cell periphery without nuclear remnants and cytoplasmic area of cells became fragmented especially at the highest concentration of phycocyanin (Fig. 3a-e).

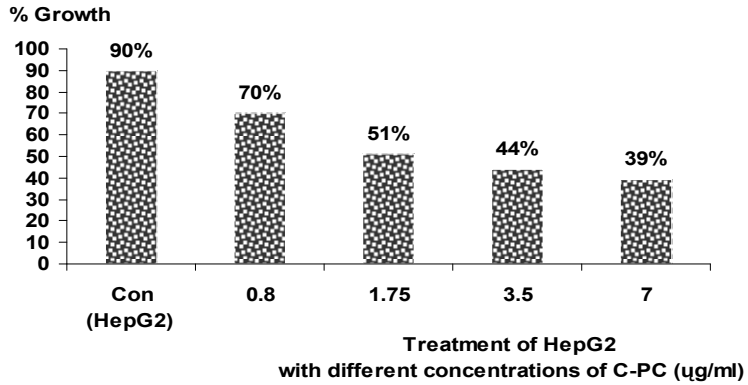

Figure 2. The effect of different concentrations of C-PC on HepG2 cell viability after 24 h.

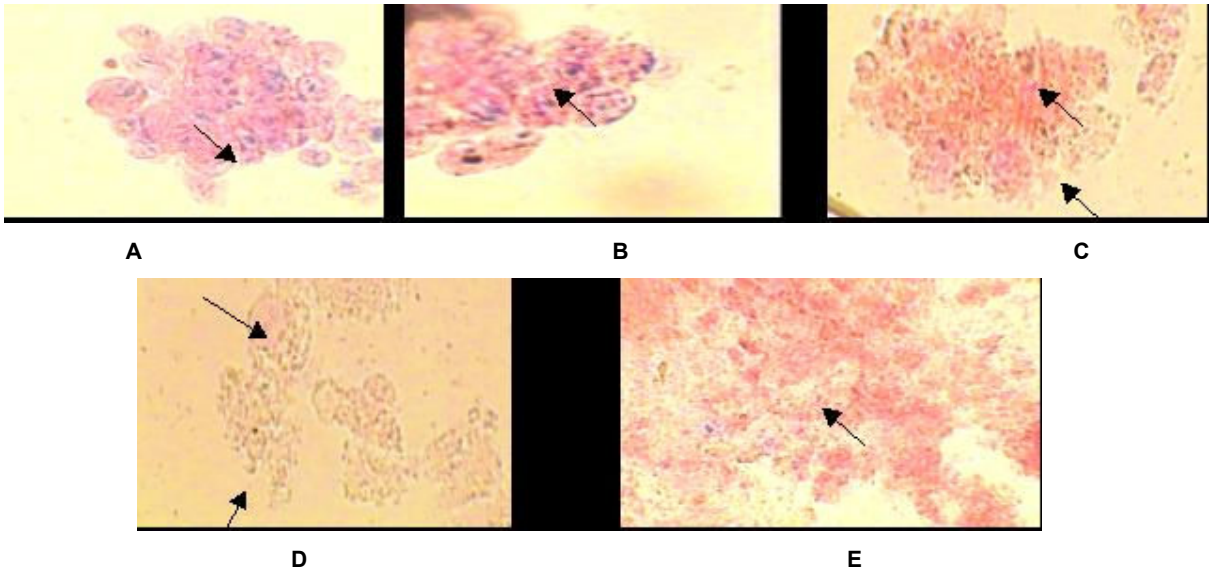

Figure 3. Light microscopic images (x 1000 magnification) of HCl-denatured methyl green-pyronin staining of HepG2 cells following 24h exposure to different concentrations of C-PC: (a) Control group (no treatment with C-PC): cells in mass having an expanded cytoplasmic area with many new nuclei (bluish-green); (b) 0.8 µg/ml of C-PC: cells in mass showed smaller cytoplasmic area (in red) with smaller nuclei due to division, but still having the degree of bluish-green as control cancer cells; (c) 1.75 µg/ml of C-PC: in mass cells some cells still having few dividing small nuclei, whereas other cells lost all the nuclear entities and the cytoplasmic area appear faintly red staining; (d) 3.5 µg/ml of C-PC: in mass cells area few cells were slightly reddish in cytoplasm and having very small remnants of divided nuclei, which were deviated to the periphery of the cells. Moreover, some cells became fragmented as apoptotic cells; (E) 7 µg/ml of C-PC: in cell mass area all of the cells appeared strongly red in cytoplasm and having no nuclear remnants and the cytoplasmic area of the cells became fragmented as process of apoptosis.

Discussion

Dietary intake of such chemo preventive compounds, as C-PC, has been suggested as an effective strategy for minimizing the deleterious effects of genotoxins and carcinogens (Miranda et al., 1998). Aqueous extracts of green, brown and red algae were shown to possess bioactivity against murine immunocytes. *Spirulina* is blue green microalga, has been used since ancient times as a source of food because of its high protein and nutritional value (Sadnori *et al.,* 1993). C-PC is one of the major biliproteins of *Spirulina platenesis*, it has blue color, water soluble protein. This pigment was shown to be hepatoprotective (Vadiraja *et al.,* 1998), antioxidant, radical scavenger (Bhat & Madyastha, 2000), ant arthritic (Remirez *et al.,* 1999), and anti-inflammatory (Romay *et al.,* 2000; Romay *et al.,* 1998) in both *in vitro* and *in vivo* experimental models. Morcos *et al.* (1988) have shown its photodynamic properties and its use in cancer treatment. They have shown that, C-PC specifically binds to cancer cells, and thus can be used for anatomical imaging of tumors *in vivo* (Morcos *et al.,* 1988).

In the present study, effects of C-PC on HepG2 cells viability were evaluated after 24 h incubation of the cells with different doses of C-PC. Our data clearly showed the inhibition in the growth of HepG2 cells in a dose dependent manner, when compared to the control group. A dose dependent decrease in HepG2 cell proliferation was observed within 24h under different concentrations of C-PC 7, 3.5, 1.75 and 0.8 µg /ml. The maximum decrease in cell proliferation being at 7, 3.5 and 1.75 µg/ml and the maximum percent of inhibition was 61, 56, 49 and 30%, respectively. This reduction in the growth of HepG2 cells in the presence of C-PC could be due to apoptosis. In order to test the factors responsible for reduced growth of HepG2 cells, further studies were undertaken on the characteristic markers of apoptosis. Generally, any reduction in MTT absorbance could be attributed to mitochondrial dysfunction that lead to cell proliferation inhibition (Mosmann, 1983). Recent accumulating evidences suggest that defects in the process of apoptosis may be closely associated with carcinogenesis and that many cancer cells have defective machinery for self-destruction (Yano *et al.,* 1994). It is suggested that the susceptibility to apoptosis-inducing effects of chemotherapeutic drugs may depend on the intrinsic ability of tumor cells to respond to apoptosis (Yano *et al.,* 1994; Tseng *et al.,* 2002). Our results are in consistence with those of Karnati *et al.* (2007) who found that *Spirulina platenesis* C Phycocyanin induce apoptosis in the doxorubicin-resistant human hepatocellular-carcinoma cell line HepG2 through alteration of the mitochondrial membrane potential.

The HCl-denatured methyl-green and pyronin staining (MG-P) could identify the proliferating cells (bluish-green) from the non proliferating cells (red colored staining). The proliferating cells with nuclear dsDNA more tolerable to acid denaturation at a certain extent. HCl-denatured MG-P staining has proved to be useful for a well-characterized cell differentiation (Iseki & Mori, 1986; Sen *et al.,* 1999). Herein the red colored cells stained with pyronin could be evaluated as the nonproliferating cells. It has been reported that HCl-denatured MG-P is markedly expressed in proliferating cells and apoptosis tumor cells (Cohen *et al.,* 1992; Ueda *et al.,* 2000). In the present study, HCl-denatured methyl green-pyronin staining in HepG2 cells following 24h incubation with C-PC revealed that HepG2 cells possess an expanded cytoplasmic area with many new nuclei (bluish-green), whereas, treated HepG2 cells with different concentrations of C-PC have slightly reddish in cytoplasm and very small

remnants of divided nuclei, which were deviated to the periphery of the cells. Moreover, the cells became fragmented as process of apoptosis. In addition to morphological evaluation, apoptosis induction by C-PC was ascertained by using an assay to measure DNA fragmentation, a biochemical hallmark of apoptosis .As illustrated in (Fig 1), agarose gel electrophoresis of DNA extracted from HepG2 cells treated with Phycocyanin revealed a progressive increase in the non-random fragmentation into a ladder. The degree of nuclear fragmentation was directly proportional to the concentration of C-PC. Such a pattern corresponds to the endonuclease activity characteristic of apoptosis. Control cells did not show any fragmentation (lane 5).

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