

Anti-leukemic Effect of β -Cryptoxanthin against Leukemia Cell Line Via Attenuating Inflammatory Pathway

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ABSTRACT

The aim of the current experimental study to estimate the cytotoxic effect of β -Cryptoxanthin on acute and chronic cell lines (Kasumi-1 and K-562) and *in vivo* scrutinized the anti-leukemia potential. Different concentrations of β -Cryptoxanthin treat to the cells Kasumi-1 and K-562 for 72 h. Cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and trypan blue solution were performed. Intravenous injection of benzene was used for induction leukemia and oral suspension of β -Cryptoxanthin was administered in a dose-dependent manner. Hematological parameters, leukopoiesis and phagocytic assay were performed. β -Cryptoxanthin exhibited the cytotoxicity effect against the Kasumi-1 and K-562 cells. β -Cryptoxanthin demonstrated the cytotoxicity effect against the Kasumi-1 and K-562 cells. Leukemia control rats exhibited the alteration of hematological parameters and suggesting the anemia and leukocytosis and dose-dependently treatment of β -Cryptoxanthin significantly ($P < 0.001$) altered the hematological parameters, increased leukopoiesis and showed the phagocytic effect. Based on the result, β -Cryptoxanthin exhibited the anticancer effect against the acute leukemia cell lines Kasumi-1 and chronic cell line K-562 and *in vivo* it showed the antileukemia effect.

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Authors' Contribution

YY performed the study. LY and KW interpreted the experimental data and provided the necessary chemicals and reagents. LZ designed the experiments. All the authors equally contributed in drafting the manuscript and proof reading.

Key words

Leukemia, β -Cryptoxanthin, Kasumi-1, K-562, hematological parameters

INTRODUCTION

Cancer is a genetic word used for the set of diseases defined by the presence of abnormal cells that increase quickly beyond their normal ranges, thus they may conquer adjacent organs and tissues and induces metastasis (Bhagat *et al.*, 2012; Bhalla *et al.*, 2013). As per the report of the World Health Organization (WHO) 14 million new cases of cancer registered in 2012 and 8.2 million cancer-related patient deaths occurred and this figure is expected to increase by about 70% over the next 2 decades (Facts, 2014). Cancer can be categorized into 2 main categories such as hematological and solid tumors (Deschler and Lübbert, 2006). Hematological cancer is those initiated in a lymph node or bone marrow and is divided into myelomas, leukemias and lymphomas. Solid tumor cancer derived from numerous tissues and is distributed into carcinoma, comprising about 80% of sarcomas and all solid tumors, which are devised from connective tissue viz., fat, bone and muscle

(Xie *et al.*, 2014). Among all the new cases of malignancy diagnosed every year, hematologic malignancies such as acute leukemias epitomize the 5th most prevalent (Gilliland, 2001; Dirnhofer, 2018). Recent time, cancer is one of the major health threat to the human in all countries in the world and its get 2nd rank in all disease spread worldwide (Gilliland, 2001; Avery, 2009; Fong *et al.*, 2014). Among all cancer, leukemia is the major cause of cancer patient death worldwide (Gilliland, 2001). Leukemia mostly affected the adults and infants. Leukemia is the bone marrow or blood-related cancer, which is categorized by deposition of malignant white blood cells in the peripheral blood and bone marrow, unusual enhance in blood cells (leukocytes), which results of somatic mutation in deoxyribonucleic acid (DNA) that activates oncogene or deactivates the disorder of division or differentiation and regulation of cell

Abbreviations

MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; WHO, World Health Organization; DNA, deoxyribonucleic acid; EDTA, Ethylenediaminetetraacetic acid; WBC, White blood cells; RES, Reticuloendothelial system; TEM, Transmission electron microscope.

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tumor suppressor genes, resultant ultimately inducing the death (Gilliland, 2001; Aleem and Arceci, 2015; Abel and Klepin, 2018). These mutations may arise unexpectedly or after the radiation exposure or carcinogenic substance and are mostly to be influenced by genetic factors (Lightfoot *et al.*, 2016; Arber, 2017).

Studies suggest that about 50% of all chemotherapeutics agents used to treat cancer, nowadays were directly or indirectly derived from the natural sources (Landau *et al.*, 2014; Tomizawa and Kiyokawa, 2017). The use of the natural source obtaining drugs as a prototype for the investigation of new semi-synthetic and synthetic phytoconstituents has donated to the development of new drugs (Jang *et al.*, 1997; Cragg *et al.*, 2006). Previously published literature suggests that 65 new anti-cancer drugs registered between 1981 and 2002 against cancer, 48 drugs obtained from the natural products (Jang *et al.*, 1997; Cragg *et al.*, 2006; Rezvani *et al.*, 2017).

Still a lot of medicinal plants already scrutinized at preclinical and clinical stages. Few of the plant-derived natural product such as taxol, camptothecin, vincristine, vinblastine, etoposide, topotecan and irinotecan, etc (Fingrut and Flescher, 2002; Cragg *et al.*, 2006; Kuldeep *et al.*, 2013). Now a day, researcher focuses their research to identify the novel anticancer agent from the plant-derived natural product. Many researchers focus on the herbal medicine to modify the body's immune response against cancer cells where direct selective destruction of cancer cells is impossible (Kuldeep *et al.*, 2013; Senthilkumar *et al.*, 2014). Understanding the complex synergistic interaction of numerous phyto-constituents of anticancer herbal formulation, herbs can be designed to attack the cancerous cells without showing the effect on the normal cells.

β -Cryptoxanthin, an important carotenoid, present in tangerines, pumpkin, sweet red peppers, tangerine juice, papayas, oranges, carrots, watermelon, corn, peaches, plums and many more. β -Cryptoxanthin having the 8 isoprene units along with the β -rings, significant carotenoid found in the serum of human. In the rodent and human, the intake of β -Cryptoxanthin reflects the quantity of β -Cryptoxanthin rich foods. Previous research suggests that β -Cryptoxanthin use as chemotherapeutic agent against lung cancer. according to the studies that large intake of β -Cryptoxanthin reduce the risk to development of lung cancer. According to the studies carried out in Europe and North America showed that the 3,155 incident of lung cancer identified among the 399,765 patients and the incidence of lung cancer reduced almost 24%, those patient regular intakes the β -Cryptoxanthin. β -Cryptoxanthin is a potent antioxidant and having property to scavenge the free radicals. Due to the nature to scavenge the free radicals, in this experimental study, we made attempt to scrutinize the

anti-leukemic effect of β -Cryptoxanthin against the blood cancer cell lines.

MATERIALS AND METHODS

Cell culture

The cell lines such as K-562 (CCL-243) and Kasumi-1 (CRL-2724) used in the current experimental study were purchased from the American Type Culture Collection, USA. Dulbecco's medium supplemented with fetal serum (10%) was used for the growing acute leukemia Kasumi-1 and K-562 cells. The cells were grown in RPMI-1640 supplemented with 20% fetal bovine serum and the cells were maintained in 95% humidity, temperature 37°C and CO₂ (5%).

Cell viability assay

Ninety-six well plates were used for the estimation of cell viability assay (Flores *et al.* 2017). Briefly, the 96 well plates inoculated at 100 μ L per well of cell suspension of leukemia cell lines (K-562 and Kasumi-1) in a concentration (1×10^5 cells/mL) and various concentration of β -Cryptoxanthin were treated. The plates were incubated at 37°C with CO₂ (5%) for the 72 h after that each cell suspension (200 μ L) was removed and mixed with the equal volume of trypan (0.4%) and incubated at room temperature for 5 min the hemocytometer was used for counting the stained (non-viable) and unstained (viable) cells.

Cell viability assay (V) was estimated by using the following formula:

$$V = \frac{C_v}{C_t} \times 100$$

Where Ct is the total number of cells and Cv is the viable cell number.

MTT cytotoxicity assay

The current model (MTT assay) based on the fact that metabolically active cells can decrease the MTT via generating the insoluble purple formazan crystal by mitochondrial enzyme succinate dehydrogenase that is solubilized subsequently, and consequently the metabolic activity of cells was estimated via using spectrophotometry (Flores *et al.*, 2017). The cytotoxicity effect of β -Cryptoxanthin was measured in Kasumi-1 and K-563 leukemia cell lines. 96 well plates were incubated at 37°C overnight. After the 24 h incubation, the β -Cryptoxanthin was added from a stock diluted to various concentrations ranging from 1–25 mg/mL. β -Cryptoxanthin (50 μ L) solution was added in each well. The current protocol was performed in the presence of blank for each dilution which contains the medium supplemented with fetal bovine serum

(1%) and β -Cryptoxanthin (50 ml); for the negative control group contain the cell suspension (50 ml) without any treatment spiked with culture medium supplemented with fetal bovine serum (1%) and positive control contain the various concentration of standard drug cyclophosphamide (anticancer drug) and the cells again incubated for 24 h. After that the MTT reagent was added in each well and again incubated at 37°C in a humidified atmosphere for 4 h. after the incubation, the solubilizing reagent sodium dodecyl sulphate was mixed in each well and mixed gently at room temperature and the absorbance of the sample was estimated at 570 nm via using the spectrophotometer and calculated the viability.

Experimental animal

The Wistar rats (125-150 g; either both sex) were used for the current experimental study. The rats were kept in the single polyethylene cage with standard laboratory conditions (22 $\pm$ 5°C; 12/12h dark and light cycle). The rats have received the standard food diet and water *ad libitum*. The whole experimental study was approved by the institutional ethical committee.

Leukemia induction

A diluted solution of benzene (Chromasolv, in water/2-propanol [50/50] v/v) intravenously was used for the induction of leukemia. The solution of benzene was induced every 2 days for consecutive weeks (3). Various doses of β -Cryptoxanthin (1.25, 2.5 and 5 mg/kg, body weight) were orally given via using the gavage before and after during the leukemia induction. For the estimation of leukemia burden via comparing the hematological parameters at baseline and after leukemia induced in a various group of experimental animal groups (Saha *et al.*, 2012).

Collection of blood samples

After successfully induction the leukemia via using the benzene injection and orally administration the tested drug (as discussed in the preclinical section), the blood sample was collected via puncturing the retro-orbital plexus and the blood samples collected into the ethylenediaminetetraacetic acid (EDTA) containing vials, gently mixed and used for the biochemical and hematological parameters.

Determination of hematological parameter

Flow cytometry was used for the estimation of hematological parameters and indices via using hr suitable cell packs via using the manufacture's instruction for the desired population of cells in the autoanalyzer.

Estimation of WBC count

For the estimation of the white blood cells (WBC) count, Leishman staining method was used via a previously used method with minor modification. The prepared slides were stained and allowed for the dry and scrutinized via using the battlement model with minor modification. 100 consecutive leukocytes indicating different types of encountered were counted and recorded.

Estimation of immunomodulatory function

Previous research suggests that the leukocytes comprise of macrophages and NK cells play a significant role in the cancer surveillance in the human body and subsequently excrete of cancerous cells (Saha *et al.*, 2012) for the estimation of the immunomodulatory function of β -Cryptoxanthin, their protective effect increases the bone marrow leukopoiesis, which was estimated based on phagocytic function and total leukocyte of WBCs as determined via using the carbon clearance test as given below. The rats were randomly divided into 4 groups and each group contains the 8 rats as follows: Gp I: normal control received normal saline; Gp II-IV received the β -Cryptoxanthin (1.25, 2.5 and 5 mg/kg, body weight), respectively. All group rats received the above-discussed treatment orally once a day for 8 days.

Leukopoiesis count

A previously published method was used for the estimation of leukocytes count. Briefly, the blood samples of all group rats were collected via puncturing the retro-orbital plexus and collecting into the EDTA containing vials (Saha *et al.*, 2012). Further, the 20 μ L of blood was diluted into the Turk's solution (0.38 mL) and the suspension was transferred into the Neubauer counting chamber and counted the leukocytes microscopically.

Phagocytic activity

The carbon clearance model was used for the estimation of phagocytic activity of the reticuloendothelial system (RES) with minor modification (Saha *et al.*, 2012). Briefly, Ink (1 mL) was intravenously administration of all group rats after the administration of β -Cryptoxanthin and the blood sample of all group rats were collected after the regular time interval (every 3 min) and blood samples transferred into the centrifuged tube and centrifuged at 5000 rpm for 10 min at room temperature. After that, the clear supernatant was collected and transferred into the volumetric flask and made the volume up to 25 mL using the distill water and calculate the absorbance at 650 nm using the spectrophotometer.

Statistical analysis

The current result expressed as mean value $\pm$ SD.

Statistical significance of the presented data expressed using the independent t-test. $P < 0.05$ was considered as significant. Data were analyzed using the one-way ANOVA and Dunnett test was performed. $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered as significant, more significant and extremely significant.

RESULT

Cytotoxic effect

Trypan blue method was used for the estimation of cell viability effect of β -Cryptoxanthin on the leukemia cell lines. Figure 1a showed the cell viability effect of β -Cryptoxanthin against the k-562 and kusumi-1. Trypan blue method showed the dose-dependent response on the cell viability of β -Cryptoxanthin when exposed to different time intervals.

Figure 1b showed that the effect of β -Cryptoxanthin on cell death against the k-562 and kusumi-1 cell lines. Dose-dependent treatment of β -Cryptoxanthin increased the cell death against both cell lines.

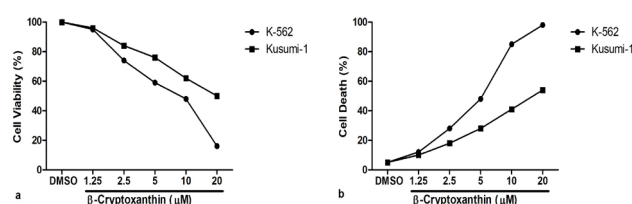


Fig. 1. effect of β -cryptoxanthin on the K-562 and Kusumi-1 cell lines. a) showed the effect on the cell viability and b) showed the effect on cell death.

Table I. cell viability percentage by using the MTT assay.

S. No	Concentration	Cell lines	
		K-562	Kusumi-1
1	DMSO	100±2.45	100±3.76
2	1.25	80.34±4.34	57.65±3.47
3	2.5	62.45±3.76	43.45±5.43
4	5	51.34±2.56	32.34±4.23
5	10	38.45±3.45	17.54±2.34
6	20	14.34±4.34	0.45±1.57

Cytotoxic effect by MTT method

MTT assay method was used for the estimation of the cytotoxic effect of β -Cryptoxanthin against the k-562 and kusumi-1 cell lines. MTT assay showed that the k-562 and kusumi-1 cell lines were exposed to the β -Cryptoxanthin exhibited the dose-response on the cell viability after 72 h. Table I exhibited the cell viability effect of β -Cryptoxanthin against the k-562 and kusumi-1 cell lines.

Cell cycle arrest

The effect of β -Cryptoxanthin on the cell cycle progression against the K-562 and Kusumi-1 cell lines. Table II showed the separation of cell cycle phase via setting adjacent cursors without deconvolution of overlapping S, G0/G1 and G2/M phases. The control group (without drug treatment) exhibited a considerable boost in the proportion of cells in the sub G0/G1 phase, recommending the up-regulation of apoptosis rate. β -Cryptoxanthin treated cells induces the cell cycle arrest in both the cell lines (K-562 and Kusumi-1), suggesting the considerable enhancement of the percentage of cells in all phases in which the cell cycle blocking occurred.

Hematological parameters

Hematological parameters such as WBC, RBC, PCV, HGB and platelet were estimated in the normal and leukemia group rats. Figure 2 exhibited the effect of the β -Cryptoxanthin on the hematological parameters of a treated group of rats. Normal control group rats demonstrated the normal level of hematological parameters. Leukemia control group rats demonstrated the increased level of WBC, platelets and reduced level of PCV, RBC and HGB and dose-dependent treatment of β -Cryptoxanthin significantly reduced the level of WBC, platelets and increased the level of PCV, RBC and HGB.

Effect on β -Cryptoxanthin leukopoiesis

During the blood cancer, the leukocytes count decrease and a similar result was found in this experimental study. The control group showed a reduced level of leukocytes and dose-dependent treatment of β -Cryptoxanthin increased the leukocytes count (Fig. 3).

Phagocytic activity

For the estimation of phagocytic activity, carbon clearance assay was performed. Table III exhibited the carbon clearance assay for β -Cryptoxanthin at a different time interval.

DISCUSSION

Leukemia is a major hematological cancer that results from a reduction of distinction of hematopoietic stem cells that arise due to a range of epigenetic faults. The pathological condition is characterized through hysterical propagation of myeloid blasts (Lightfoot *et al.*, 2016; Arber, 2017). Now a day available treatment for cancer patient such as immunotherapy, radiotherapy and surgical interventions, successful treatment with anticancer drugs remain a challenge due to the non-selective toxic, cytotoxic effect of available drugs

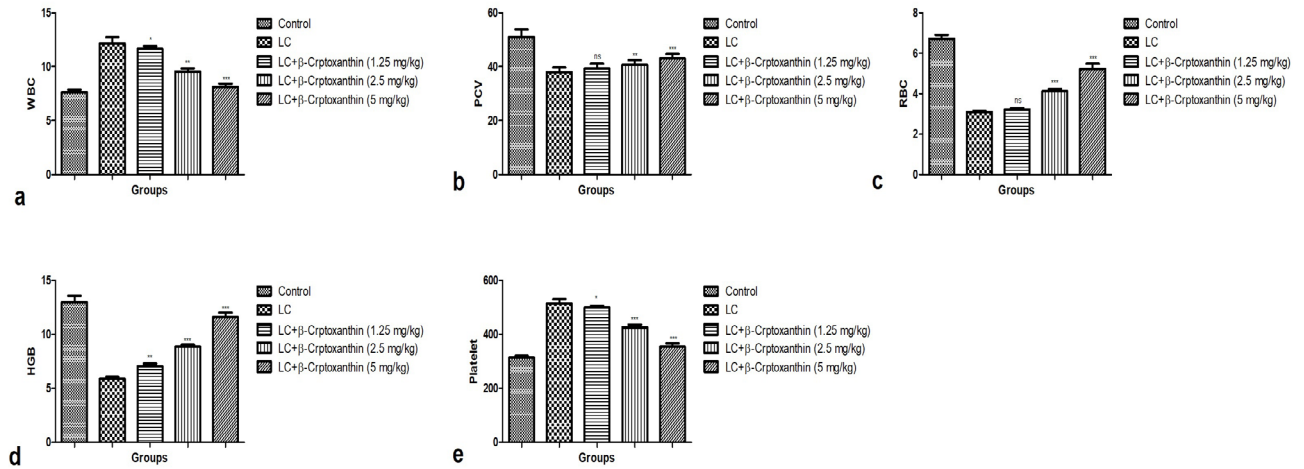


Fig. 2. Effect of β -cryptoxanthin on the haematological parameters of Benzene induced leukemia rats. a, WBC; b, PCV; c, RBC; d, HGB and e, platelets. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control groups, using ANOVA followed by the Dennett test.

Table II. Effect of β -Cryptoxanthin on the cell cycle of acute leukemia cell lines.

Cell		G0/G1 (%)	S (%)	G2/M (%)	Sub G0/G1 % (apoptosis %)
K562	Control	38.45 $\pm$ 1.23	35.21 $\pm$ 1.45	22.34 $\pm$ 0.93	6.12 $\pm$ 0.34
	β -Cryptoxanthin	29.34 $\pm$ 2.12	34.32 $\pm$ 1.34	30.23 $\pm$ 1.93	13.23 $\pm$ 0.32*
Kasumi-1	Control	68.87 $\pm$ 3.23	24.34 $\pm$ 1.23	12.53 $\pm$ 1.23	8.34 $\pm$ 0.23
	β -Cryptoxanthin	58.34 $\pm$ 2.34	20.34 $\pm$ 4.56	19.23 $\pm$ 1.54*	15.45 $\pm$ 0.73*

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control groups, using ANOVA followed by the Dennett test.

Table III. Comparison of phagocytic activity of β -Cryptoxanthin using the carbon clearance assay.

Concentration	Time (mins)	β -Cryptoxanthin
1.25 mg/kg	3	53.34 $\pm$ 1.03
	6	57.86 $\pm$ 1.43
	9	60.12 $\pm$ 1.93
	12	63.26 $\pm$ 2.03
2.5 mg/kg	3	45.45 $\pm$ 1.23
	6	46.78 $\pm$ 0.94
	9	48.76 $\pm$ 1.04
	12	51.04 $\pm$ 1.03
5 mg/kg	3	44.34 $\pm$ 1.06
	6	45.85 $\pm$ 1.34
	9	47.54 $\pm$ 1.04
	12	50.23 $\pm$ 0.84

(Daaboul *et al.*, 2018). For the leukemia patient, chemotherapy with cytostatic drug and cytotoxic drugs remain the primary treatment option. But the discuss

treatment having the high remission rates in the initial stage of cancer, diagnosis is often dismal given the occurrence of relapses and hard to treat the refractory disease (Lightfoot *et al.*, 2016; Arber, 2017). Therefore, a long term survival in the disease is very low, resultant generate a series of serious adverse effects.

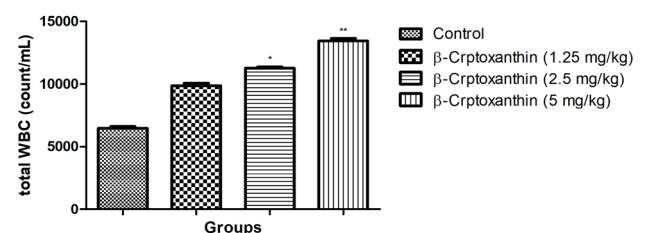


Fig. 3. Effect of β -cryptoxanthin on total WBC count. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ compared to control groups; using ANOVA followed by the Dennett test.

Studies suggest that morphological characteristics considered as the key parameters for the estimation the apoptosis, and its respect in a transmission electron microscope (TEM) measured and confirming the apoptotic

state in the cells (Nakase *et al.*, 2012). Moreover, the limitation of this method identified the apoptosis in the cells only late stage (Nakase *et al.*, 2012). In the current experimental study, the cells have a smaller size as compared to control cells after the 24-72h and can be seen in a symmetrical fragmentation of nucleus and with different apoptotic bodies. On the basis of the result, conclude that β -Cryptoxanthin showed the growth inhibitory effect against the leukemia cell lines.

Research suggest that the KI-67 is the essential protein for cell proliferation and the percentage of cells positively stained for this protein has been used for the diagnostic and prognostic indicator of various types of cancer (Alkhateeb, 2013). Clinical studies suggested that uncontrolled cell proliferation is the main biological mechanism involved in oncogenesis (Daniluk, 2012; Jones, 2012). Previous studies suggest that the few Phyto-constituents showed the cytostatic effect on the Myeloid leukemia derived cell lines (K-562) (Lightfoot *et al.*, 2016; Arber, 2017). Conspicuously, arsenic trioxide (potent pro-oxidant) used in the myeloid leukemia therapy, also induced the cell cycle arrest at the G2/M phase in K-562 cells, whereas leukemia derived cell lines lacking induced the cell cycle arrest at G1 phase (Flores *et al.*, 2017) in the current experimental study, β -Cryptoxanthin induces the cell cycle arrest at the G1 phase and G2/M phase due to its antioxidant property. Based on previous research, we can conclude that β -Cryptoxanthin induced the cell cycle arrest in both cell lines due to its anti-oxidant nature and presence of BCR-ABL fusion. In the current experimental study, we observed that the differential effects of β -Cryptoxanthin on cell death between both cell lines. In the case of CRL-2724 cells treated with the β -Cryptoxanthin exhibited the induction of caspase-dependent apoptosis. On the other hand, K-562 cell, β -Cryptoxanthin induced the cell death at the G2/M transition phase due to activation of caspase-3 and caspase-9. This effect may be due to the presence of an alteration in the regulation of apoptosis in K-562 cells that escape the caspase full activation and thereby activates an alternative form of cell death (Bhattacharya *et al.*, 2010; Chandramohan *et al.*, 2012). Based on the result, say that β -Cryptoxanthin activate the caspase 3 and 9 followed by induced apoptosis. Researcher suggests that the modulation of tubulin metabolism and reactive oxygen species, both are the possible mechanism on the regulation of cell cycle expansion and cell death (Amarante-Mendes *et al.*, 1998; Donato *et al.*, 2003; Sztiller-Sikorska *et al.*, 2009; Chandramohan Reddy *et al.*, 2012). In this experimental study, β -Cryptoxanthin induce the cell cycle arrest at the G1 phase, due to its antioxidant nature. It also induces apoptosis, with full activation of caspases. β -Cryptoxanthin arrests the cell at the G2/M phase in K-562 cells may be due to disrupting the tubulin metabolism.

Statement of conflict of interest

The authors have declared no conflict of interest.

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