INTRODUCTION

Carotenoids are phytocarotenoids belonging to the category of tetraterpenoids, which are naturally occurring in a number of fruits and vegetables. Carotenoids have unique chemical and physical properties that allow them to serve many functions in different organisms. A number of experimental studies have reported that β-carotene and other carotenoids are able to inhibit the growth of several cancer cells, including breast cancer, colon cancer, prostate cancer, lung cancer, melanoma, and leukemia cells [1, 2].

Meso-Zeaxanthin is a xanthophyll carotenoid, having a chemical formula C_{40}H_{56}O_{2}. Meso-Zeaxanthin [(3R,3'S)-zeaxanthin, MZ], (3R,3'S,6'S)-lutein and (3R,3'R)-zeaxanthin are the macular carotenoids present in the fovea centralis of primate retina [3]. These macular carotenoids protect the persons from age-related macular degeneration (AMD) by functioning as antioxidants and/or optical filters. Epidemiological studies indicated that high intake of lutein and zeaxanthin have not been studied well yet. Emerging studies suggest the potential use of lutein and zeaxanthin in the prevention of heart disease and stroke [5]. Recent studies indicated that zeaxanthin is able to induce apoptosis in human uveal melanoma cells through Bcl-2 family proteins and intrinsic apoptosis pathway [6]. Our previous studies have shown that MZ synthesised from marigold flower extract has profound antioxidant activity both in vitro and in vivo [7]. MZ also exhibited significant antimutagenic [8], anticarcinogenic [9], radioprotective [10] and chemoprotective properties [11]. In the present, study we have checked cytotoxic and antitumour activity of MZ as well as the apoptosis-inducing property of MZ in tumour cell lines of murine origin and its possible mechanism of action.

MATERIALS AND METHODS

Chemicals

Meso-zeaxanthin used in this study was supplied by Omni Active Health Technologies, Mumbai, Rose well Park Memorial Institute medium (RPMI-1640), Dulbecco’s Modified Eagle’s Medium (DMEM), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), ethidium bromide and oligonucleotide primer sequences for mouse Bcl-2, Caspase-3, Caspase-9, p53 and GAPDH were purchased from Sigma-Aldrich Inc. Foetal Calf Serum (FCS) was bought from Biological Industries, Kibbutz, Israel. Trypan blue, haematoxylin and eosin were obtained from E-Merck, Germany. Cell-to-cDNA™ II Kit was purchased from Ambion Inc, Texas, and USA.

Drug preparation

MZ was dissolved in tetrahydrofuran (THF) for cell culture studies. For animal studies, 5% suspension of MZ in sunflower oil was used. The drug was stored in dark bottles at 4°C to prevent oxidation. THF was found to be non-toxic to cells at the concentrations studied.

Cell lines

Dalton’s Lymphoma Ascites (DLA) and Ehrlich Ascites Carcinoma (EAC) cell lines were initially procured from Adyar Cancer Institute, Chennai and propagated as transplantable tumours in Swiss albino mice by injecting a suspension of cells (1x10^6 cells/ml) intraperitoneally. The cells were aspirated from the peritoneal cavity of the mouse on the 15th day for further studies. L929 (mouse lung fibroblast) cell line and Vero cells (monkey kidney cells) were purchased from National Centre for Cell Sciences (NCFS), Pune.

Animals

Swiss albino mice (male, 6-8 w old, weighing 22-25 g) were purchased from Small Animal Breeding Station, Kerala Agricultural University, Thrissur, Kerala, India. They were housed in well-ventilated cages with controlled conditions of light and humidity. They were provided with normal mouse chow (Sai Durga Food and Feeds, Bangalore, India) and water ad libitum. All the animal experiments were performed as per the instructions prescribed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and implemented through the Institutional Animal Ethical Committee of the Research Centre (No. ACR/IAEC-7/01-(1) on 17/5/2011).
Determination of in vitro cytotoxic activity of MZ towards DLA and EAC cells

Short term cytotoxic activity of MZ was done by determining the percentage of viability of Daltons Lymphoma Ascites (DLA) and Ehrlich’s Ascites Carcinoma (EAC) cells using trypan blue dye exclusion method [12]. EAC and DLA cells aspirated from the peritoneal cavity of mouse and washed with PBS (0.2 M, pH 7.4) and centrifuged at 3000 RPM for 15 min.

The pellet was resuspended in PBS and the process was repeated 3 times. Finally, the cells were suspended in a known quantity of PBS and the cell count was adjusted to 1x10^6 cells/ml. MZ was distributed into test tubes and incubated with different concentration of MZ (1 to 200 μg/ml) for 3 h at 37 °C. After 3 h the trypan blue dye exclusion test was performed to determine the percentage of viable cells and IC50 value was calculated.

Analysis of the effect of MZ on normal spleen cells, bone marrow cells and peripheral blood lymphocytes by trypan blue dye exclusion method.

Preparation of spleen cells

All the procedures were done under sterile condition. Mice were sacrificed and an incision was made on the left side just below the rib and spleen was removed without any adherent tissue. The spleen was cut into small pieces and teased out over a stainless steel mesh. All the procedures were done under sterile condition. Mice were sacrificed and an incision was made on the left side just below the rib and spleen was removed without any adherent tissue. The spleen was cut into small pieces and teased out over a stainless steel mesh. All the procedures were done under sterile condition. Mice were sacrificed and an incision was made on the left side just below the rib and spleen was removed without any adherent tissue. The spleen was cut into small pieces and teased out over a stainless steel mesh.

Preparation of bone marrow cells

All the procedures were done under sterile condition. Mice were sacrificed by cervical dislocation and fixed on a board stretching forelegs and hind legs. Skin and flesh are overlying the limbs were removed and femur was exposed. The shaft of the femur was separated from both ends and bone marrow was flushed out of the cavity by passing a jet of PBS with 10% FCS through the ends of the shaft using a 26G needle and syringe. The flushed bone marrow was made into a single cell suspension by repeated pipetting. It was then centrifuged and suspended at required cell concentrations (1x10^6 cells) in PBS with 10% FCS.

Isolation of peripheral blood lymphocytes

Peripheral blood lymphocytes were isolated from human blood by Ficoll-Hypaque gradient centrifugation method [13]. For this study, human blood was collected from the vein and diluted with PBS (1:3). 3 ml of Ficoll-Hypaque solution was taken in a clean centrifuge tube and the diluted blood was slowly layered over the Ficoll-Hypaque solution. It was then centrifuged at 1500 rpm for 20 min at room temperature. The slight yellow coloured solution (which contains the lymphocyte) found in the middle of the centrifuge tube was carefully collected using a Pasteur pipette and washed three times using PBS to remove the Ficoll-Hypaque solution.

One million normal cells-spleen cells, bone marrow cells and human peripheral blood lymphocytes in 100 μl were incubated with and without different concentrations of MZ (1 to 200 μg/ml) for 3 h at 37 °C. After 3 h, the cytotoxicity of MZ to these normal cells was analysed by trypan blue dye exclusion method.

Determination of in vitro cytotoxicity of MZ towards L929 cells by MTT assay

Cytotoxicity of MZ to L929 and Vero cells were determined by MTT assay. L929 and Vero cells (5000 cells/well) were seeded in 96 well flat-bottomed plates. After 24 h of incubation at 37 °C in 5% CO2 atmosphere, different concentrations of MZ were added and further incubated for 48 h, 20 μl of MTT (5 mg/ml) was added 4 h before the completion of incubation. The plates were centrifuged and the supernatant was removed and then 100 μl of DMSO was added and the intensity of the blue colour formed was read at 570 nm using ELISA plate reader.

Determination of anti-tumour activity of MZ on ascites tumour bearing animals

Ascites tumour was induced by injecting EAC cells (1x10^6 cells/animal) in the peritoneal cavity of Swiss albino mice. Thirty-six animals were divided into six groups (6 animals/group). Animals in Group I was treated with EAC cells alone. Animals in Group II were treated with sunflower oil along with EAC cells (vehicle control). Animals in Group III, IV and V received MZ 50, 100 and 250 mg/kg b. wt respectively along with EAC cells. Animals in Group VI received cyclophosphamide (10 mg/kg b. wt) along with EAC cells. MZ administration was started 24 h after tumour inoculation and continued for 10 consecutive days. The death of the animals due to tumour burden was noted every day and the percentage of increase in lifespan (% ILS) was calculated using the formula (T−C)/C × 100, where ‘T’ and ‘C’ are the mean survival days of treated and control animals respectively [16].

Determination of anti-tumour activity of MZ on solid tumour

Thirty-six Swiss albino mice were divided into six groups (6 animals/group). Animals in Group I received DLA cells alone. Group II animals were treated with DLA cells and sunflower oil (vehicle control). Animals in Groups III, IV and V received MZ 50, 100 and 250 mg/kg b. wt respectively along with DLA cells. Group VI animals received Cyclophosphamide (10 mg/kg b. wt) and DLA cells. Dalton’s Lymphoma Ascites (DLA) cells (1x10^6 cells/animal) were injected subcutaneously on the right hind limb of mice. Twenty-four hours after tumour inoculation, different doses of MZ were given orally and continued for 10 consecutive days. Initial diameter of the hind limb was measured using vernier callipers. The tumour diameter was measured from 7th day and continued on every 3rd day. It was recorded up to 31 d. The tumour volume was calculated using the formula V = 4/3πr12r2, where r1 and r2 represent the radii of the tumour at two different planes.

Determination of apoptosis inducing property of MZ in DLA cells

Morphological analysis

To detect the morphological changes during apoptosis, 2x10^4/DLA cells were incubated in DMEM with 10% FCS in the presence and absence of different concentrations of MZ (10, 20 and 25 μg/ml) at 37 °C in 5% CO2 atmosphere for 48 h. After incubation, the cells were washed twice with PBS (pH 7.4) and centrifuged at 1000rpm for 15 min. The cell button was collected and smeared were made. The smears were then stained using haematoxylin and eosin. The cells were observed under a microscope (100X) and photographs were taken.

DNA fragmentation assay

Two million DLA cells were treated with different concentrations of MZ as described in the previous experiment. After incubation, DNA was extracted using phenol:chloroform:isoamyl alcohol method. DNA samples were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide. DNA fragmentation was visualised and recorded using the gel documentation system (Vilber Lourmat, France).

Determination of the effect of MZ on gene expression

Gene expression study was carried out by the reverse transcription-polymerase chain reaction (RT-PCR) method. Cells to cDNA Kit were used for producing cDNA from DLA cells without isolating mRNA. DLA cells (1x10^6 cells/well) were seeded in 96 well U bottom titre plate using DMEM with and without MZ (25 μg/ml) and incubated for 4 h at 37 °C in 5% CO2 atmosphere. After incubation, the medium was removed and the cells were washed with ice-cold PBS. Ice cold cell lysis buffer (100 μl) was added to the cells and immediately transferred to a water bath. It was incubated for 15 min at 75 °C and transferred to nuclease-free microcentrifuge tubes. To this 2 μl of Dnase-1 was added and incubated for 15 min at 37°C. Dnase was inactivated by heating at 75 °C for 5 min. cDNA was prepared from the cell lysate using cells to cDNA Kit II kit. Anti-apoptotic Bel-2 gene and pro-apoptotic genes like caspase-3, caspase-9 and p53 were amplified by using specific primers by RT-PCR.

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Cytotoxicity of MZ towards DLA and EAC cells

MZ was found to be cytotoxic towards DLA and EAC cells. Cytotoxicity (100%) was attained at a concentration of 125µg/ml for DLA cells and 150µg/ml for EAC cells. IC50 value of MZ for DLA cells was 46µg/ml and for EAC cells was 51µg/ml (fig. 1). The internal control GAPDH was found to be significantly downregulated by 25µg/ml of MZ treatment when compared to that in the untreated control. But the expression of pro-apoptotic genes such as p53, Caspase-3 and Caspase-9 were significantly up-regulated by the treatment of DLA cells with 25µg/ml of MZ (fig. 4). The internal control GAPDH was found to be expressed in all the samples. The results indicated that MZ-induced apoptosis in DLA cells via p53 dependent Caspase-9 mediated intrinsic (mitochondrial) pathway.

**Effect of MZ on normal cells**

MZ did not show cytotoxicity towards normal cells like bone marrow cells, spleen cells, human peripheral lymphocytes and Vero cells up to a concentration 200 µg/ml (fig. 1).

**Cytotoxicity of MZ towards L929 cells in culture**

MZ showed a dose-dependent cytotoxic effect towards L929 cells during MTT assay. It showed 100% cytotoxicity to L929 cells at concentration 110 µg/ml (fig. 1). IC50 value was found to be 37µg/ml.

**Anti-tumour activity of MZ**

**a) Ascites tumour**

Animals of the ascites tumour control group survived only for a period of 15.8±0.81days. Treatment of MZ at different concentrations increased the average life span of tumour bearing animals. MZ treatment at concentrations 50, 100 and 250 mg/kg b. wt increased the survival rate of animals to 24.88±3.5, 26.75±2.2 and 28.88±1.2 d respectively (table 1). The increase in the lifespan of ascites tumour bearing mice was 83% after 250 mg/kg b. wt of MZ treatment. Standard anti-neoplastic drug (cyclophosphamide) treated a group of animals survived 26.63±1.6 d.

**b) Solid tumour**

A significant reduction of the solid tumour was found in MZ-treated groups when compared with the control group animals from 16th day of tumour inoculation. Tumour volume of control animals was 3.83 cm³ on 31st day while that of 250 mg/kg b. wt MZ-treated animals was only 0.593 cm³ on the same day. There were also significant decreases in tumour volume of animals treated with 50 and 100 mg/kg b. wt of MZ (fig. 2).

**Induction of apoptosis by MZ-morphology and laddering**

Morphological analysis demonstrated that MZ was able to induce programmed cell death or apoptosis in DLA cells. Morphological changes indicating apoptosis (membrane blebbing, chromatin condensation, vacuole formation, DNA fragmentation and appearance of apoptotic bodies) [17] were observed in 10, 20 and 25 µg/ml of MZ-treated DLA by nuclear staining [fig. 3(a)]. Untreated DLA cells were characterised by less eosinophilic cytoplasm, a nucleus with a uniform distribution of chromatin material and absence of any morphological modification. MZ did not produce any characteristic apoptotic features at lower concentrations such as 1, 2, 2.5 and 5µg/ml. The result of DNA fragmentation assay showed that MZ-treated cells had extensive DNA strand breaks, thereby appeared in a ladder pattern. The DNA isolated from DLA cells treated with 20 µg and 25µg/ml of MZ showed more fragmentation [lane 4 & 5] [fig. 3(b)].

**Effect of MZ on the expression of apoptotic genes**

Expression of anti-apoptotic gene Bcl-2 in DLA cells was found to be significantly downregulated by 25µg/ml of MZ treatment when compared to that in the untreated control. But the expression of pro-apoptotic genes such as p53, Caspase-3 and Caspase-9 were significantly up-regulated by the treatment of DLA cells with 25 µg/ml of MZ (fig. 4). The internal control GAPDH was found to be expressed in all the samples. The results indicated that MZ-induced apoptosis in DLA cells via p53 dependent Caspase-9 mediated intrinsic (mitochondrial) pathway.

**DISCUSSION**

Cytotoxicity towards transformed cells is a fundamental property required for an anticancer agent [18]. In the present study, short-term cytotoxicity of MZ was evaluated by trypan blue exclusion method. Results showed that MZ had profound cytotoxicity towards both DLA and EAC cells and IC50 values were 46 and 51µg/ml respectively.
MZ also showed cytotoxicity towards transformed mouse lung fibroblasts cells (L929) and IC50 value was 37µg/ml. Interestingly MZ did not show cytotoxicity towards normal cells like bone marrow, spleen and human peripheral blood lymphocytes. This indicated that MZ was selectively cytotoxic towards transformed cells.

Carotenoids are well known for their cytotoxic and antitumour effects. In vivo tumoricidal activity of MZ was evaluated by using Ehrlich Ascites Carcinoma (EAC) induced ascites tumour model and Dalton’s Lymphoma Ascites (DLA) cells induced solid tumour model. EAC is referred to as an undifferentiated carcinoma. It has the high transplantable capability, rapid proliferation, shorter life span and 100% malignancy. EAC resembles human tumours and are most sensitive to chemotherapy due to the fact that they are undifferentiated and that they have a rapid growth rate. Many plant extracts were found to be effective against EAC [19]. The present study revealed that MZ possessed considerable antitumour activity against EAC cells induced ascites tumour. MZ treatment significantly increased the lifespan of ascites tumour bearing animals. DLA is transplantable, a poorly differentiated malignant tumour cell. It grows in both solid and ascitic forms [20]. DLA tumorigenesis model in Swiss albino mice provides a convenient model system to study anti-tumour activity within a short time. MZ was also found to reduce the solid tumour induced by DLA cells.

Fig. 2: Effect of meso-zeaxanthin administration on solid tumour reduction in mice

The anti-tumour activity of MZ was found to be concentration dependent. The dose of MZ needed for tumour reduction was higher compared to other standard drug used for cancer chemotherapy. Equivalent human dosage of MZ is approximately 5-25 mg/kg i.e., for a 60 kg body weight person it will be 300-1500 mg/day. Dose of o xo-carotenoids useful to prevent macular degeneration is only 10 mg/day. Present study indicates that much higher concentration of oxycarotenoids is needed in order to produce tumour reduction. Similar results are also seen in the case of lutein [21]. MZ was found to be highly non-toxic to animals at this dosage.

Fig. 3: a) Morphological analysis of the cells after treatment with meso-zeaxanthin indicating membrane rupture and apoptotic bodies, b) Formation of DNA ladder by treatment of DLA cells with meso-zeaxanthin

Apoptosis (programmed cell death) is a process in which cells play an active role in their own death. Apoptosis is a normal component of the development and health of multicellular organisms. Problems with the regulation of apoptosis have been implicated in a number of diseases. Cancer is a disease that is often characterised by a deficiency in apoptosis. Cancer cells typically possess a number of mutations that have allowed them to ignore normal cellular signals regulating their growth and become more proliferative than normal cells. Under normal circumstances, damaged cells will undergo apoptosis. But in the case of cancer cells, mutations occurred and that prevent cells from undergoing apoptosis. Induction of apoptosis or cell cycle arrest by chemopreventive compounds can be an excellent approach to inhibit the promotion and progression of carcinogenesis and to remove genetically damaged, pre-initiated or neoplastic cells from the body [22].

Apoptosis is characterized by membrane blebbing, chromosome condensation, vacuole formation and formation of apoptotic bodies. These features can be seen in DLA cells treated with MZ. One of the hallmarks of apoptosis is the cleavage of chromosomal DNA into nucleosomal units. The fragmentation of DNA into nucleosomal units (multiples of 180-200 bps) is caused by an enzyme known as CAD (Caspase Activated DNase). Normally CAD exists as an inactive complex with ICAD (Inhibitor of CAD). During apoptosis, ICAD is cleaved by caspases such as caspase 3 to release CAD leads to rapid fragmentation of the nuclear DNA (Inoue et al., 2005). DNA isolated
from DLA cells treated with MZ appeared in ladder pattern, confirming apoptosis induction by MZ.

p53 is a tumour suppressor gene that acts to integrate multiple stress signals into a series of diverse antiproliferative responses. One of the most important functions of p53 is its ability to activate apoptosis, and disruption of this process can promote tumour progression and chemoresistance. Induction of p53-dependent apoptotic pathway found to be an attractive strategy for anti-cancer treatment. In the present study, MZ up-regulated the expressions of p53, caspase 9 and caspase 3 genes indicating that MZ can induce p53 dependent intrinsic apoptotic pathway.

Apoptosis regulator Bcl-2 (B-cell leukaemia/lymphoma 2 protein) is a family of evolutionarily related proteins. These proteins govern mitochondrial outer membrane permeabilization (MOMP) and can be either pro-apoptotic (Bax, BAD, Bak and Bok among others) or anti-apoptotic (including Bcl-2 proper, Bcl-xl, and Bcl-w among an assortment of others). Most cells express a variety of both anti-apoptotic and pro-apoptotic Bcl-2 proteins and the interaction within this family dictates whether a cell survives or dies. The dysregulation of the anti-apoptotic Bcl-2 family members is one of the defining features of cancer cells in comparison to normal cells, and this significantly contributes to the resistance of cancer cells to current treatment modalities. Several drugs that can inhibit Bcl-2 and related anti-apoptotic proteins have been developed, and some of them show considerable promise in the clinic [23]. In this study, expression of Bcl-2, an anti-apoptotic gene, occurred in transformed DLA cells. But the expression of Bcl-2 was significantly downregulated by MZ treatment. The present study revealed that the cytotoxic and antitumour effect of MZ on transformed cells is mediated through p53 dependent mitochondrial apoptotic pathway.

CONCLUSION
Xanthophyll carotenoid, meso-zeaxanthin was found to be cytotoxic to tumour cells such as Dalton’s lymphoma ascites tumour cells (DLA) and Ehrlich ascites tumour (EAC) cells as well as L929 fibroblasts. It was found to be nontoxic to normal cells. Meso-zeaxanthin increased the life span of EAC tumour bearing animals to tumour cells such as Dalton’s lymphoma ascites tumour cells and Ehrlich ascites tumour cells (EAC) cells as well as L929 fibroblasts. It was found to be nontoxic to normal cells. Meso-zeaxanthin increased the life span of EAC tumour bearing animals and this significantly contributes to the resistance of cancer cells to current treatment modalities. Several drugs that can inhibit Bcl-2 and related anti-apoptotic proteins have been developed, and some of them show considerable promise in the clinic [23]. In this study, expression of Bcl-2, an anti-apoptotic gene, occurred in transformed DLA cells. But the expression of Bcl-2 was significantly downregulated by MZ treatment. The present study revealed that the cytotoxic and antitumour effect of MZ on transformed cells is mediated through p53 dependent mitochondrial apoptotic pathway.

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CONFLICT OF INTERESTS
No conflict of interest

REFERENCES