CHAPTER 5

CYTOTOXIC, ANTI-TUMOUR

AND APOPTOSIS INDUCING

ACTIVITIES OF MESO-ZEAXANTHIN
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5.4. DISCUSSION
5.1. INTRODUCTION

Remarkable progress in the field of cancer biology in recent years helps us to find several ways to intervene the carcinogenic process. Oxidative stress, inflammation, and evasion of apoptosis are crucial biological mechanisms involved in carcinogenesis. Hence compounds with antioxidant, anti-inflammatory and pro-apoptotic activity represent important candidates for preventing, suppressing, or reversing the development of carcinogenesis. Several phytochemicals (bioactive nonessential nutrients from plants) that can modulate diverse biochemical processes involved in carcinogenesis have been identified. Apoptosis is the mechanism by which the cells undergo programmed cell death. It is now well documented that most of the cytotoxic anticancer agents induce cell death via apoptosis (Roos and Kaina, 2006). The majority of cancer cells have defects in apoptotic pathway paving way for drug resistance. Modulation of apoptotic pathways by phytochemicals would therefore provide more new opportunities for chemoprevention based on specific molecular targets (Martin, 2006).

A number of results 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 (Zhang et al., 2011). Preclinical studies have shown that some carotenoids have potent antitumour effects both in vitro and in vivo, suggesting potential preventive and therapeutic roles of the compounds (Tanaka et al., 2012). In the present, study we have checked cytotoxic and anti-tumour activity of MZ and also the apoptosis inducing property of MZ in tumour cell lines of murine origin.
5.2. MATERIALS AND METHODS

5.2.1. Chemicals

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 was bought from Biological Industries, Kibbutz, Israel. Trypan blue, haematoxylin and eosin were obtained from E-Merck, Germany.

5.2.2. Kit

Cell-to-cDNA™ II Kit (Ambion Inc, Texas, USA).

5.2.3. Drug Preparation

MZ was dissolved in tetrahydrofuran (THF) for cell culture studies.

5.2.4. Cell lines

L929 cells, Dalton’s Lymphoma Ascites (DLA) cells and Ehrlich’s Ascites Carcinoma (EAC) cells were used in the study.

5.2.5. Animals

Swiss albino mice (male, 6-8 weeks old, weighing 22-25g) were used in this study.

5.2.6. Determination of In vitro Cytotoxic Activity of MZ towards DLA and EAC Cells

Short term cytotoxic activity of MZ was analysed by determining the percentage of viability of Daltons Lymphoma Ascites (DLA) and Ehrlich’s Ascites Carcinoma (EAC) cells using trypan blue dye exclusion method (Moldeus et al., 1978). EAC and DLA cells were grown in the peritoneal cavity of Swiss albino mice by injecting a suspension of cells (1x10^6 cells/ml) intra peritoneally. The cells were aspirated from the peritoneal cavity of the mouse on the 15th day and washed with PBS (0.2 M, pH 7.4) and centrifuged for 15 minutes at 1500 rpm. 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 1 x 10^6 cells/ml. 100 µl of this diluted cell suspension was distributed into test tubes and incubated with different concentration of MZ (1, 2.5, 5, 10, 20, 25, 50, 75, 100, 125, 150 and 200 µg/ml) for 3 hours at 37°C. After 3 hours, the trypan blue dye exclusion
test was performed to determine the percentage of viability and IC$_{50}$ value was calculated.

5.2.7. 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. The animals were sacrificed, an incision was made on the left side just below the rib and spleen was removed without any adherent tissue. Spleen was cut into small pieces and teased out over a stainless steel mesh in cold PBS. Clumps were allowed to settle in a centrifuge tube and kept in ice bath for 2 minutes. Supernatant was collected, washed three times with PBS and resuspended in RPMI-1640 medium at required concentrations.

- **Preparation of Bone Marrow Cells**
  
  Bone marrow cells from both femurs of animals were flushed into phosphate buffered saline containing 10% goat serum. Bone marrow cellularity was adjusted to required concentrations as described in chapter 2, section 2.2.8.

- **Isolation of Peripheral Blood Lymphocytes**
  
  Peripheral blood lymphocytes were isolated from human blood by Ficol-Hyapaque gradient centrifugation method (Boyum, 1968). For this study, human blood was collected from vein and diluted with PBS (1:3). 3ml of Ficol-Hyapaque solution was taken in a clean centrifuge tube and the diluted blood was slowly layered over the Ficol-Hyapaque solution. It was then centrifuged at 1500rpm for 20 minutes 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 Ficol-Hyapaque solution.

  100 µl of one million normal cells-spleen cells, bone marrow cells and human peripheral blood lymphocytes were incubated with and without different concentrations of MZ (1, 2.5, 5, 10, 20, 50, 75, 100, 125, 150 and 200 µg/ml) for 3 hours at 37°C. After 3 hours, the cytotoxicity of MZ to these normal cells were analysed by trypan blue dye exclusion method.

5.2.8. Determination of *in vitro* Cytotoxicity of MZ towards L929 Cells in Culture

Long term cytotoxicity of MZ was determined by MTT assay. L929 cells (5000 cells/ well) were seeded in 96 well flat-bottom titre plates. After 24 hours of
incubation at 37°C in 5% CO₂ atmosphere, different concentrations of MZ (1, 2.5, 5, 10, 25, 50, 75, 100, 110 and 125 µg/ml) were added and further incubated for 48 hours. 20 µl of MTT (5 mg/ml) was added 4 hours before the completion of incubation (Cole, 1986; Campling et al., 1991). The plates were centrifuged and the supernatant was removed. Then 100 µl of DMSO was added and the intensity of the blue colour formed was read at 570 nm using ELISA plate reader.

5.2.9. Determination of Anti-tumour Effect of MZ on Ascites Tumour Bearing Animals

Ascites tumour was induced by injecting EAC cells (1x10⁶ cells/animal) in the peritoneal cavity of Swiss albino mice. Thirty six animals were divided into six groups (6 animals/group) as follows:
Group I: EAC cells alone.
Group II: EAC cells + sunflower oil (vehicle control).
Group III: EAC cells + MZ (50 mg/kg b.wt)
Group IV: EAC cells + MZ (100 mg/kg b.wt)
Group V: EAC cells + MZ (250 mg/kg b.wt)
Group VI: Cyclophosphamide (10 mg/kg b.wt)

MZ administration was started 24 hours after tumour inoculation and continued for 10 consecutive days. The death of the animals due to tumour burden was noted everyday and the percentage of increase in lifespan (% ILS) was calculated using the formula [(T-C)/C] x 100, where ‘T’ and ‘C’ are the mean survival days of treated and control animals respectively (Kuttan et al., 1985).

5.2.10. Determination of Anti-tumour effect of MZ on Solid Tumour Development

Thirty six animals were divided into six groups (6 animals/group) as follows:
Group I: DLA cells alone.
Group II: DLA cells + sunflower oil (vehicle control).
Group III: DLA cells + MZ (50 mg/kg b.wt)
Group IV: DLA cells + MZ (100 mg/kg b.wt)
Group V: DLA cells + MZ (250 mg/kg b.wt)
Group VI: Cyclophosphamide (10 mg/kg b. wt)

Dalton’s Lymphoma Ascites (DLA) cells (1x10⁶ 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 calipers. The tumour diameter was measured from 7th day and continued on every 3rd day. It was recorded up to 31 days. The tumour volume was calculated using the formula \( V = \frac{4}{3} \pi (r_1^2 \times r_2) \), where \( r_1 \) and \( r_2 \) represent the radii of the tumour at two different planes (Atia and Weiss, 1966).

**5.2.11. Determination of Apoptosis Inducing Property of MZ in DLA Cells**

**5.2.11.1. Morphological Analysis**

To detect the morphological changes during apoptosis, 2\( \times 10^6 \) DLA cells were incubated in DMEM with 10% FCS in the presence and absence of different concentrations of MZ (10, 20 and 25 \( \mu \)g/ml) at 37°C in 5% CO\(_2\) atmosphere for 48 hours. After incubation, the cells were washed twice with PBS (pH 7.4) and centrifuged at 1000rpm for 15 minutes. The cell button was collected and smears were made. The smears were then stained using haematoxylin and eosin. The cells were observed under microscope (100X) and photographs were taken.

**5.2.11.2. 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 as described in Chapter-2 (Section 2.2.13). DNA samples were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide. The resulting DNA fragmentation was visualized and recorded using the gel-documentation system (Vilber Lourmat, France).

**5.2.11.3. Determination of the Effect of MZ on Gene Expression**

Gene expression study was carried out by reverse transcription polymerase chain reaction (RT-PCR) method. Cells to cDNA \(^\text{TM} \) II kit was used for producing cDNA from DLA cells without isolating mRNA. DLA cells (1\( \times 10^6 \) cells/well) were seeded in 96 well ‘U’ bottom titre plate using DMEM with and without MZ (25 \( \mu \)g/ml) and incubated for 4 hours at 37°C in 5% CO\(_2\) atmosphere. After incubation, medium was removed and the cells were washed with ice cold PBS. Ice cold cell lysis buffer (100\( \mu \)l) was added to the cells and immediately transferred to a water bath. It was incubated for 15 minutes at 75°C and transferred to nuclease free micro centrifuge tubes. To this 2\( \mu \)l of Dnase-1 was added and incubated for 15 minutes at 37°C. Dnase was inactivated by heating at 75°C for 5 minutes. cDNA was prepared from the cell lysate using cells to cDNA \(^\text{TM} \) II kit as described in chapter 2 (section
2.2.14.1. The mouse Bcl-2, Caspase-3, Caspase-9 and p53 genes were amplified against housekeeping gene GAPDH. Amplified PCR products were subjected to electrophoresis on 1.8% agarose gel and analyzed using gel documentation system (chapter 2, section 2.2.14.1.1, 2.2.14.3 and 2.2.14.4).

5.3. RESULTS

5.3.1. Cytotoxicity of MZ towards DLA and EAC Cells

MZ was found to be cytotoxic towards DLA and EAC cells. Maximum cytotoxicity (100%) was attained at a concentration of 125μg/ml for DLA cells and 150μg/ml for EAC cells. IC₅₀ values were 46 and 51 μg/ml for DLA and EAC cells respectively (Table 5.1).

5.3.2. Effect of MZ on Normal Cells

MZ did not show cytotoxicity towards normal bone marrow cells, spleen cells and human peripheral lymphocytes up to 200 μg/ml.

5.3.3. Cytotoxicity of MZ towards L929 Cells in Culture

MZ showed a dose dependent cytotoxic effect towards L929 cells in long term culture. It showed 100% cytotoxicity at the concentration of 110 μg/ml (Table 5.2). IC₅₀ value was found to be 37μg/ml.

5.3.4. Effect of MZ on Ascites Tumour Development

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 lifespan 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 days respectively (Table 5.3). Standard anti-neoplastic drug (cyclophosphamide) treated group of animals survived 26.63±1.6 days. There was 83% increase in lifespan of ascites tumour bearing mice by 250 mg/kg b.wt MZ treatment.

5.3.5. Effect of MZ on Solid Tumour Induced by DLA Cells

A significant reduction of 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 3.83mm³ on 31st day while that of 250 mg/kg b.wt MZ treated animals was only 0.593mm³ on the same day. As shown in Figure 5.1, there were significant decrease in tumour volume in 50, 100 and 250 mg/kg b.wt MZ treated groups on other days as well.
Table 5.1. *In vitro* cytotoxicity of MZ Using DLA and EAC Cells by Trypan Blue Exclusion Method

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% of cell death (DLA)</th>
<th>% of cell death (EAC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
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</tr>
<tr>
<td>200</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 5.2. Cytotoxicity of MZ towards L929 Cells by MTT Assay

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Cell viability</th>
<th>% Cell death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>81.4</td>
<td>18.6</td>
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<td>25</td>
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<tr>
<td>110</td>
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<td>100</td>
</tr>
<tr>
<td>125</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>
Table 5.3. Effect of MZ Administration on the Survival Rate and Life Span of Ascites Tumour Bearing Animals

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean survival days</th>
<th>% increase in life span</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15.8±0.81</td>
<td>-</td>
</tr>
<tr>
<td>Vehicle Control</td>
<td>16±0.72&lt;sup&gt;ns&lt;/sup&gt;</td>
<td>1.6</td>
</tr>
<tr>
<td>MZ (50 mg/kg b.wt)</td>
<td>24.88±3.5&lt;sup&gt;***&lt;/sup&gt;</td>
<td>58</td>
</tr>
<tr>
<td>MZ (100 mg/kg b.wt)</td>
<td>26.75±2.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>69.9</td>
</tr>
<tr>
<td>MZ (250 mg/kg b.wt)</td>
<td>28.88±1.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>83.4</td>
</tr>
<tr>
<td>Standard (Cyclophosphamide-10mg/kg b.wt)</td>
<td>26.63±1.6&lt;sup&gt;***&lt;/sup&gt;</td>
<td>69.08</td>
</tr>
</tbody>
</table>

The values were expressed as mean ± standard deviation (SD); n=6. The mean values were statistically analyzed by one way analysis of variance (ANOVA) followed by appropriate post hoc test (Dunnett’s multiple comparison test) using Graph pad Instat 3 Software.

<sup>ns</sup> - Not significant (p>0.05), <sup>***</sup> p<0.001 significant.
Figure 5.1. Anti-tumor effect of MZ on solid tumor induced by DLA cells
5.3.6. 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) (Evan and Littlewood, 1998) were observed in 10, 20 and 25 μg/ml of MZ treated DLA by nuclear staining [Figure 5.2 (a)]. Untreated DLA cells were characterized by less eosinophilic cytoplasm, nucleus with uniform distribution of chromatin material and absence of any morphological modification. MZ did not produce any characteristic apoptotic features at lower concentrations like 1, 2, 2.5 and 5μg/ml but treatment with 10 μg/ml of MZ displayed the stages of apoptosis.

Result of DNA fragmentation assay showed that MZ treated cells had extensive DNA strand breaks, thereby appearing in a ladder pattern. DNA isolated from untreated DLA cells did not show any DNA strand break (lane 2). The DNA isolated from DLA cells treated with 25μg/ml of MZ showed more fragmentation (lane 3) than that isolated from DLA cells treated with 20 μg/ml of MZ [Figure 5.2(b)].

5.3.7. Effect of MZ on the Expression of Apoptotic Genes

Expression of anti-apoptotic gene Bcl-2 in DLA cells was found to be significantly down regulated by 25μg/ml of MZ treatment when compared to that in untreated control [Figure 5.3(A)]. But the expression of pro-apoptotic genes such as p53 [Figure 5.3 (B)], Caspase-3 and Caspase-9 were significantly up-regulated by the treatment with 25 μg/ml of MZ [Figure 5.4 (a) and (b)]. 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.
Figure 5.2. Apoptosis inducing property of MZ on DLA cells

(a) Morphological Analysis

![Images showing untreated and treated DLA cells]

Untreated DLA cells  DLA cells+ 20 μg/ml MZ  DLA cells+ 25 μg/ml MZ

(b) DNA ladder formation on DLA cells

Lane 1: Molecular weight marker. Lane 2: Untreated DLA cells. Lane 3: DLA cells + 10 μg/ml MZ. Lane 4: DLA cells + 20 μg/ml MZ. Lane 5: DLA cells + 25 μg/ml MZ.
Figure 5.3. Effect of MZ on the expressions of Bcl-2 and p53

(A) Bcl-2

Lane 1: Molecular weight markers. Lane 2: Bcl-2 expression in DLA cells.
Lane 3: No Bcl-2 expression in MZ treated DLA cells.
Lane 4: Expression of GAPDH

557 bp (GAPDH)
235 bp (Bcl-2)

(B) p53

Lane 1: Molecular weight markers. Lane 2: p53 expression in DLA cells.
Lane 3: Upregulated p53 expression in MZ treated DLA cells.
Lane 4: Expression of GAPDH

557 bp (GAPDH)
205 bp (p53)
Figure 5.4 Effect of MZ on the expressions of Caspase 3 and Caspase 9

(a) Caspase 3

Lane 1: Molecular weight marker. Lane 2: Untreated DLA cells
Lane 3: 25 μg/ml MZ treated DLA cells. Lane 4: GAPDH

(b) Caspase 9

Lane 1: Molecular weight marker. Lane 2: Untreated DLA cells
Lane 3: 25 μg/ml MZ treated DLA cells. Lane 4: GAPDH
5.4. DISCUSSION

Cytotoxicity towards transformed cells is a fundamental property required for an anticancer agent (Flescher, 2007). 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 long term cytotoxicity towards transformed mouse lung fibroblasts cells (L929) and IC₅₀ value was 37µg/ml. MZ did not show cytotoxicity towards normal cells like bone marrow, spleen and human peripheral lymphocytes. This indicated that MZ was specifically cytotoxic towards transformed cells.

Carotenoids are well known for their cytotoxic and anti-tumour effects. In vivo tumouricidal 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 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 (Ahmed et al., 1988). Present study revealed that MZ possessed considerable anti-tumour activity against EAC cells induced ascites tumour. MZ treatment significantly increased the lifespan of ascites tumour bearing animals. DLA is transplantable, poorly differentiated malignant tumour cell. It grows in both solid and ascitic forms (Kleinsmith, 2007). DLA tumourigenesis 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. The anti-tumour activity of MZ was found to be concentration dependent.

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 characterized by too little 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 may have occurred and
that prevent cells from undergoing apoptosis. In this case, there is no check on the cellular proliferation and it consequently leads to tumour formation. Therefore, induction of apoptosis or cell cycle arrest by chemo preventive 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 (Khan et al., 2007).

Apoptosis is characterised 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. Then rapid fragmentation of the nuclear DNA occurs (Inoue et al., 2005). DNA isolated from DLA cells treated with MZ appeared in ladder pattern, confirming apoptosis induction by MZ.

The mechanisms of apoptosis are highly complex. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. Extrinsic, intrinsic and granzyme B pathways converge on the same terminal or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Martinvalet et al., 2005).

Intrinsic pathway is activated by several internal stimuli such as irreparable genetic damage, hypoxia, and extremely high concentrations of cytosolic Ca\(^{2+}\) and lack of growth factors. As a result, activated pro-apoptotic proteins permeabilize the outer mitochondrial membrane to trigger the release of Smac/DIABLO and cytochrome c to cytosol (Elmore, 2007). Smac/DIABLO directly binds to cytosolic
IAP (inhibitor of apoptosis protein) and removes it from active caspases, and thus allows the caspases to cleave their substrates. Cytochrome c released into the cytosol promotes formation of apoptosome. In the apoptosome complex, cytochrome c binds apoptotic protease activating factor-1 (Apaf-1) using the energy provided by ATP and procaspase 9 is activated into its active form i.e. caspase 9 (Hill et al., 2003). Activated caspase 9 activates executioner caspases including caspase-3 as the downstream effector caspase.

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. As the apoptotic function of p53 is critical for tumour suppression, induction of apoptotic pathways through p53-induced apoptotic targets may 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 leukemia/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. This anti-apoptotic subfamily of proteins is now a major target in the development of new methods to improve treatment outcomes for cancer patients. 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 (Weyhenmeyer et al., 2012). In this study, expression of Bcl-2, an anti-apoptotic gene, occurred in transformed DLA cells. But the expression of Bcl-2 was significantly down regulated by MZ treatment. Hence the cytotoxic and anti-tumour effect of MZ is mediated through induction of apoptosis.