Chapter 3

Evaluation of chemopreventive activity of the potential fraction against Ehrlich's Ascites Carcinoma cells in mice
Introduction

Ehrlich Ascites Carcinoma (EAC) is one of the commonest tumors. The use of EAC as a model in anti-cancer research was proven by many authors to give accurate and reliable results (Kuttan et al., 1990; Ramnath et al., 2002; Gupta et al., 2004). The reliability of such test lies in its ability to determine the value of any anticancer drug through prolongation of experimental animal lifespan in addition to the changes in number and viability of the cell line itself in addition to the volume of the liquid generated by the tumor inside the peritoneal cavity (Maity et al., 1999). The tumor is characterized by moderately rapid growth, which kill mice in 16 to 18 days due to accumulation of ascitic fluid and seldom shows distal metastasis or spontaneous regression.

It is now well recognized that apoptosis is a genetically regulated mode of cell suicide to eradicate cells in diverse physiological and pathological settings maintaining tissue homeostasis in multicellular organisms (Webb et al., 1997; Wyllie, 1997). Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischaemic diseases are caused or enhanced by excessive apoptosis (Fadeel et al., 1999). Cancer is one of the scenarios, imparting neoplastic cells with a selective growth advantage because of either accelerated rates of cell proliferation, decreased rates of cell demise, or both. Apoptosis, although being implicated as the cause of various pathological problems, plays an important role in the treatment of cancer as it is a popular target of many treatment strategies. The abundance of literature suggests that targeting apoptosis in cancer is feasible. In this context, it is noteworthy that apoptosis-inducing ability seems to have become a primary factor in considering the efficacy of anti-tumor agents (Ramnath et al., 2002; Huo et al., 2004). The mechanism of apoptosis is complex and involves many pathways. Understanding the mechanisms of apoptosis is crucial and helps in the understanding of the pathogenesis of conditions as a result of disordered apoptosis. Since release of mitochondrial proteins are of central importance in mediating and enhancing apoptotic pathways, those mitochondrial events must be kept under strict control of regulatory mechanisms which are in many ways dependent on members of the Bcl-2 family. It has been demonstrated that release of cytochrome c is inhibited by the anti-apoptotic Bcl-2 or Bcl-xL but is induced by the pro-apoptotic Bax (Kim et al., 2000).
Bax, one of the important pro-apoptotic members of Bcl-2 family, has been reported to translocate from the cytoplasm to mitochondria during induction of apoptosis. It has been proposed that such interaction results in permeability transition (PT) pore opening and cytochrome c release (Marzo et al., 1998; Narita et al., 1998; Shimizu et al., 1999), resulting in apoptosis (Zamzami et al., 1995; Marchetti et al., 1996; Karpinich et al., 2002). On the other hand, abnormalities of p53 are some of the most frequent molecular events in carcinogenesis, and represent one of the most studied areas in contemporary tumor biology. The tumor-suppressor p53 is a central effector of the response of cells to genotoxic stress. It has been demonstrated that following DNA damage, p53 is rapidly activated and binds specifically to DNA, which activates the transcription of target genes involved in negative control of the cell cycle and initiation of apoptosis (Levine, 1997). p53-mediated growth arrest involves p21 as a major effector and thus up-regulation of p21 results in blocking of cell cycle progression (Choi et al., 2001). It is suggested that the activation of p53 blocks the cell cycle progression either in G1/S or G2/M checkpoints and induces apoptosis by tilting the ratio of Bax and Bcl-2 (Yee and Vousden, 2005).

Pro-oxidant states have been considered to be contributing factors for tumorigenesis (Cenni, 1985). Correspondingly, an increasing body of evidence indicates that antioxidants have anticancer activities. Antioxidants can inhibit tumor initiation, tumor promotion, and cell transformation (Steele et al., 1990; O'Brien, 1994). Scavengers and inhibitors of free radical processes have also been demonstrated to prevent or delay neoplastic process (Kensler et al., 1983; Salim, 1992; Perchellet and Perchellet, 1989). Furthermore, antioxidants have been shown to trigger apoptosis in smooth muscle cells independent of oxidative reactions (Tsai et al., 1996). In another study, sulfur-containing antioxidants are reported to induce p53-mediated apoptosis selectively in transformed cells (Liu et al., 1998). Apart from these, findings suggested that even nitric oxide (NO) has a cytostatic and/or cytotoxic effect on tumor cells. Another possible consequence of NO production is apoptosis, and this process has been implicated in the tumoricidal activity of NO (Nicotera et al., 1995). Cytotoxicity as a result of substantial NO-formation is established to initiate apoptosis, characterized by upregulation of tumor suppressor, changes in the expression of pro- and anti-apoptotic Bcl-2 family members,
cytochrome c relocation, activation of caspases, chromatin condensation and DNA fragmentation (Brune et al., 1999).

Thus, evidence-based studies suggested apoptosis with nontoxic antioxidants may have a direct clinical application. In the previous chapter, the Fa fraction from Tricholoma giganteum was demonstrated to possess potential in vitro antioxidant capacity and NOS activation property. Here in this present chapter, an attempt have been made to address the apoptogenic effect of Fa fraction from T. giganteum on Ehrlich's ascites carcinoma (EAC) cells grown in the peritoneal cavity of Swiss albino mice.

Materials and Methods

Extraction procedure:

Powdered T. giganteum (100 g) was extracted with 80% ethanol at room temperature overnight and was repeated 4 times, and then freeze-dried to obtain Fa fraction. The freeze-dried fraction Fa was reconstituted in distilled water at a concentration of 10 mg/ml. This stock solution was kept in the dark at 4°C for further use (Cui et al., 2005).

Animals:

Healthy male Swiss albino mice of approximately the same age weighing about 20 g were used for the study. They were fed with standard diet and water ad libitum. They were housed in polypropylene cages maintained under standard condition (12 h light/dark cycle; 25 ± 3°C temperature, 35-60% relative humidity). The animals were maintained according to the guidelines recommended by the Animal Welfare Board and approved by our institutional animal ethical committee. All procedures complied with the Declaration of Helsinki, as revised in 1996.

Acute toxicity studies:

The standard conditions of the mice were maintained during the experiment. They were housed in polypropylene cages maintained under standard condition (12 h light/dark cycle; 25 ± 3°C temperature, 35-60% relative humidity). The Fa fraction was fed orally with increasing dose up to 3,000 mg/kg body weight.
Tumor models:

Swiss albino mice (~ 20 g each; 10 mice in each group) were randomly divided into different groups (Das et al., 2002) including: (i) normal set (non-tumor bearing); (ii) tumor-bearing set which were intra-peritoneal injected with $1 \times 10^5$ exponentially grown EAC; (iii) Fa fraction-treated (150 mg/kg body weight, p.o.) non-tumor-bearing set, and (iv) Fa fraction-treated (150 mg/kg body weight, p.o.) tumor-bearing set. The treatment was started 30 days prior to EAC injection to evaluate the chemopreventive activity of the fraction. On 21st day after EAC cells administration, the animals were sacrificed.

Dosage optimization was done by primary antitumor effect with respect to tumor cell count of the EAC after treatment of the Fa fraction with ascending doses from 50 to 200 mg/kg body weight. EAC cells from peritoneal cavity were collected, freed from adherent cells and viable cells were counted by Trypan Blue exclusion test.

Isolation of EAC from mice peritoneal cavity:

The EAC cells were isolated from the peritoneal cavity of tumor-bearing mice (control or treated). 3 ml of sterile PBS was injected into the peritoneal cavity of the mice and the peritoneal fluid containing the tumor cells was withdrawn, collected in sterile Petri dish and incubated at 37°C for 2 h. The cells of macrophage lineage adhered to the bottom of the Petri dishes. The non-adherent population was aspirated out gently and repeatedly washed with PBS. The viability of EAC was assessed and counted in hemocytometer by trypan blue exclusion test. The viable EAC cells were processed for further experiments (Chattopadhyay et al., 2002).

Detection of apoptosis by flow cytometry:

For the analysis of cell cycle phase distribution, EAC cells harvested from tumor-bearing mice were permeabilized followed by nuclear DNA labeling with propidium iodide (PI, 125 μg/ml). Cell cycle phase distribution of nuclear DNA was determined on BDFACS Calibur fluorescence activated cell sorter (FACS), fluorescent detector equipped with 488 nm argon-ion laser light source and 623 nm band pass filter (linear scale). Ten thousand events were acquired and analyzed. A histogram of DNA content (x-axis, PI fluorescence) versus counts (y-axis) has been
displayed (Pal et al., 2001). The percentage of apoptotic cells was determined by measuring the fraction of nuclei that contained a sub-diploid DNA content.

**Phenotypic analysis of EAC cells by confocal microscopy:**

For the assessment of chromatin condensation and nuclear blebbing, EAC cells were fixed and nuclear DNA was stained with DAPI, 4', 6'-diamidino-2-phenylindole (0.2 µg/ml for 15 min at room temperature). A laser scanning confocal microscope (Zeiss LSM 510 META) was used to visualize apoptotic cells. The experiments were performed three times (Choudhuri et al., 2002).

**Flow cytometric analysis of expression of pro- and anti-apoptotic proteins:**

EAC cells from mice were fixed and permeabilized and used further for the determination of the expression of proapoptotic proteins p53 and Bax or anti-apoptotic protein Bcl-2. Cells (1 × 10^5) from each group were incubated either with polyclonal anti-p53 or anti-Bcl-2 or anti-Bax (1 µg/ml) primary antibody for 1 h at room temperature and then with fluorescein isothiocyanate (FITC)-conjugated isotype specific secondary antibody for detecting the respective protein levels. Cells were washed thoroughly with PBS and analyzed on flow cytometer equipped with 488 nm argon laser light source and a 515 nm band pass filter for FITC-fluorescence. A total of 10,000 events were acquired and histogram plot of FITC-fluorescence (x-axis) versus counts (y-axis) has been shown in logarithmic fluorescence intensity.

**Analysis of protein expression by Western blotting:**

EAC lysate was loaded into a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, the gel was transferred to nitrocellulose membrane and blocked with non-fat milk in PBS containing Tween-20. The membrane was then incubated with specific primary antibody, mouse anti p53, anti Bax and anti Bcl-2 at appropriate dilution overnight at 4°C. The protein of interest was visualized by treating with alkaline phosphatase (AP) conjugated specific secondary antibody. The target protein band was then visualized using bromochloroindolyl phosphate (BCIP) and nitrobluetetrazolium (NBT) substrates. Equal loading of protein in each lane was established by α-actin antibody probing.
Analysis of gene expression by semi-quantitative RT-PCR:

Expression of the genes was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Total RNA of EAC was extracted with TRIZol Reagent (Invitrogen, USA). The cDNA was synthesized from the total RNA using RevertAid M-MuLV Reverse Transcriptase (Fermentas, USA) according to the manufacturer protocol. 20 µl reaction volume contained 1 µg of RNA, 0.5 µg of Oligo(dT), 20 units of RiboLock RNase Inhibitor (Fermentas, USA), 4 µl of 5x reaction buffer [250 mM Tris–HCl (pH 8.3), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT], 2 µl of 10 mM each deoxynucleoside triphosphates (dNTP Mix; Fermentas, USA) and 200 units RevertAid M-MuLV Reverse Transcriptase. The reaction was carried out at 45°C for 60 min followed by 70°C for 10 min. To analyze the expression of specific gene, 1 µl of the cDNA was taken in a 50 µl PCR mixture containing 1x DreamTaq PCR buffer, 0.2 mM of each dNTPs, 1 µM of each gene specific primer and 1.25 units DreamTaq DNA polymerase (Fermentas, USA). p53, Bax, Bcl-2 and p21 genes were co-amplified with the β-actin gene as control. Primers used in this experiment are listed in Table 4.1. The PCR cycle conditions were as follows: 95°C for 4 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 60 s with a final extension step of 7 min at 72°C in a thermal cycler (Applied BioSystem, USA). The PCR products were electrophoresed in 2% agarose gel, stained in ethidium bromide, visualized in UV transilluminator and then photographed.

Table 4.1: Primer sequences used in RT-PCR analysis (Manna et al., 2006)

<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Sequences of primer</th>
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<tbody>
<tr>
<td>p53</td>
<td>FP 5' ATG ACT GCC ATG GAG GAG TCA CAG T 3'</td>
</tr>
<tr>
<td></td>
<td>RP 5' GTG GGG GCA GCG TCT CAC GAC CTC C 3'</td>
</tr>
<tr>
<td>Bax</td>
<td>FP 5' AAG CTG AGC GAG TGT CTC CCG CG 3'</td>
</tr>
<tr>
<td></td>
<td>RP 5' GCC ACA AAG ATG GTC ACT GTC TGG C 3'</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>FP 5' CTC GTC GCT ACC GTC GTG ACT TGG C 3'</td>
</tr>
<tr>
<td></td>
<td>RP 5' CAG ATG CCG GTG CAG GTA CTC AGT C 3'</td>
</tr>
<tr>
<td>p21</td>
<td>FP 5' AAT CCT GGT GAT GTC CGA CC 3'</td>
</tr>
<tr>
<td></td>
<td>RP 5' AAA GTT CCA CCG TTC TCG G 3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>FP 5' GTG GGC CGC TCT AGG CAC CAA 3'</td>
</tr>
<tr>
<td></td>
<td>RP 5' CTC TTT GAT GTC ACG CAC GAT TTC 3'</td>
</tr>
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Results

Acute toxicity studies:

The Fa fraction of *T. giganteum* when administered up to a dose of 3,000 mg/kg body weight did not exhibit any signs or symptoms of either toxicity or mortality.

Effect of Fa fraction on tumor cell number:

In an attempt to determine the effect of Fa fraction of *T. giganteum* in tumor regression in murine model system, it was indicated that administration of the fraction decreased the mortality rate in tumor-bearing mice. The treatment of tumor-bearing mice with fraction Fa of *T. giganteum* resulted in considerable reduction of EAC cell count in comparison with untreated tumor-bearing mice. The Fa fraction of *T. giganteum*, when given in a dose-dependent manner lowered tumor burden showing the optimal activity at a dose 150 mg/kg body weight (Table 4.2). On day 21, a total of $463.33 \times 10^6$ EAC cells were measured in the peritoneal fluid of untreated tumor-bearing mice, whereas in treated set mice showed decreased EAC cell count at a concentration of 150 and 200 mg/kg body weight $\sim 24 \times 10^6$. Therefore, further studies were performed with a concentration of 150 mg/kg body weight.

**Table 4.2: Effect of *T. giganteum* Fa fraction on EAC cell number in the peritoneal cavity of tumor-bearing mice**

<table>
<thead>
<tr>
<th>Doses of Fa fraction (mg/kg body weight)</th>
<th>EAC number ($\times 10^6$) after 21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>$463.33 \pm 12.47$</td>
</tr>
<tr>
<td>50</td>
<td>$171.30 \pm 6.7$</td>
</tr>
<tr>
<td>100</td>
<td>$97.36 \pm 4.9$</td>
</tr>
<tr>
<td>150</td>
<td>$24.41 \pm 8.11$</td>
</tr>
<tr>
<td>200</td>
<td>$24.09 \pm 5.98$</td>
</tr>
</tbody>
</table>

Flow cytometric analysis of EAC cell cycle phase distribution:

To determine the effect of Fa fraction of *T. giganteum* on the distribution of cells in different cell cycle phase, treated and untreated cells were analyzed by flow cytometry and results are shown in Figure 4.1. On 21st day after EAC inoculation,
the content of hypoploid DNA (<2n DNA) was 5.08% [Figure 4.1(a)] in case of only tumor-bearing set, whereas, it was increased to 15.45% (Fig. 1b) as a result of Fa fraction treatment. Simultaneously, the cell population of G0/G1 phase was decreased proportionately [41.8%, Figure 4.1(a) versus 23.37%, Figure 4.1(b)] as well as the DNA content in S and G2/M phase are also decreased in the treated set [33.70%, Figure 4.1(a) versus 18.15%, Figure 4.1(b)]. These results suggested Fa fraction of *T. giganteum*-induced EAC DNA breakdown. This obviously led to the growth arrest of EAC.

**Figure 4.1:** Flow-cytometric analysis of cell cycle phase distribution of EAC nuclear DNA labeled with propidium iodide (PI). (a) Control (untreated) and (b) treated (Fa fraction treated). Histogram display of DNA content (x-axis; PI-fluorescence) versus counts (y-axis) has been shown.

**Effect of the fraction on phenotypic alterations of EAC:**

The morphological examination of EAC cells were assessed using confocal microscope after staining with DAPI. EAC from Fa fraction treated set evidently showed morphological changes like blebbing formation, nuclear condensation, cell shrinkage and collapse of the cell into small apoptotic bodies [Figure 4.2(b)], which are regarded as the typical hallmark of apoptosis (Aravind *et al.*, 2012). These observations lend further support to the hypothesis that the fraction induces apoptosis in EAC cells.
Effect of the fraction on the expression of pro- and anti-apoptotic proteins:

To determine whether the reduction in cell proliferation or an increase in cell death in EAC cell seen with Fa fraction of *T. giganteum* was due to apoptosis, an attempt was made to examine the EAC cells after treatment for evidence of apoptosis. It is now well recognized that pro- and anti-apoptotic proteins are hugely responsible for induction of apoptosis. Other studies have indicated that the p53 protein regulates its effects on apoptosis and cell cycle arrest, through up-regulation of Bax. p53 is also capable of down-regulating death suppressor Bcl-2. The expression of pro-apoptotic proteins p53 and Bax and as well as anti-apoptotic protein Bcl-2 in our mice model were examined to study the effect of the fraction. Flow-cytometric data revealed the expression of p53 and Bax increased markedly [Figure 4.3(a), (b)]. Interestingly, Bcl-2 level showed no significant change [Figure 4.3(c)], resulting a decrease in Bcl-2/Bax ratio, it is the ratio of Bcl-2 to Bax that determines sensitivity to apoptosis, rather than the absolute levels of either protein. These results suggest that Fa fraction induces a change in the pattern of expression of the pro- and anti-apoptotic proteins favoring apoptosis in the EAC cells, which
supports other reports on the interplay between these factors in the onset of apoptosis. Furthermore, western blot analysis was done to determine whether treatment with Fa fraction altered the expression of the apoptotic mediators, p53, Bax, and Bcl-2. The flow cytometric data was further attested by the western blot [Figure 4.3(d)]. It supported the notion that as a result of the treatment, the balance between positive and negative regulators of apoptosis shifted towards cell death.

**Figure 4.3:** EACs from control or treated tumor-bearing mice were labeled with (a) anti-p53; (b) anti-Bax and (c) anti-Bcl-2 primary antibody followed by FITC-conjugated secondary antibody. Cells were then analyzed on a flow cytometer and histogram display of FITC-fluorescence (x-axis) versus counts (y-axis) has been shown in logarithmic fluorescence intensity. (d) EAC lysates [Lane 1: control (untreated); Lane 2: treated (Fa treated)] were subjected to western blot analysis with anti-p53, anti-Bax and anti-Bcl-2 primary antibodies and visualized by AP-conjugated secondary antibody. The α-actin band confirmed equal protein loading.

**Effect of the fraction on some apoptosis-associated genes:**

To gain insight into the molecular effector pathway of apoptosis and elucidate whether the level of the critical determinants of apoptosis and cell cycle arrest were changed in Fa fraction-induced apoptosis, expression in the transcript level was determined by semi-quantitative RT-PCR. Figure 4.4 reveals that
differential alteration of apoptosis associated genes occurred on treatment. An increase in expression of p53 level treatment set is suggested to cause cell cycle arrest mediated by p21, moderate elevation of which is observed as well. p53-mediated apoptosis is suggested as pro-apoptotic Bax gene was shown to be upregulated when compared to the untreated tumor-bearing set. However, the expression status of Bcl-2 remains unchanged on treatment.

![Figure 4.4: Semi-quantitative RT-PCR of p53, Bax, Bcl-2 and p21 gene expressions as represented in Lane 1: control; Lane 2: treated. β-actin band represented equal loading.](image)

**Discussion:**

New discoveries in molecular oncology along with rapid expansion of our knowledge concerning the processes that govern differentiation, apoptosis, immune surveillance, angiogenesis, metastasis, cell cycle, and signal transduction control have unveiled an abundance of specific molecular targets for cancer therapy, including a variety of small-molecule compounds that inhibit or stimulate these molecular targets (Zaidman et al., 2005). Natural products have proved to be an infinite source for remedies over the ages. The concept that certain diet-derived substances can be used to prevent cancer or postpone its onset is currently eliciting considerable interest. In vitro and clinical studies have indicated that mushrooms exhibit cancer-chemoprevention and anticancer activity, which may be ascribed to
antioxidative and radical-scavenging effects of several edible macrofungi. This study was initiated to investigate intricately the mechanism(s) for reduction of EAC cells viability in Swiss albino mice by treatment with Fa fraction of T. giganteum in vivo. The fraction was found to produce significant induction of apoptosis in EAC cells by increasing content of hypoploid DNA. Simultaneously, the number of cells in G0/G1 and G2/M phases was reduced. It seems that fraction Fa does not block the cells in G0/G1 and G2/M phases, rather it induces apoptosis in these phases and thus the number of hypoploid cells increases. Thus it can be inferred that the fraction Fa was effective in imparting growth inhibition, cell cycle deregulation and apoptosis in EAC cells. The proliferation inhibition was as a result of apoptotic induction, as evidenced by the cell morphology analysis showing nuclear shrinkage, chromatin condensation and apoptotic bodies in fraction treated cells in contrast to control cells (Kerr et al., 1972). To elucidate the mechanism of apoptosis in EAC cells, the expression of different apoptosis-associated genes and their proteins were analyzed and results illuminated that the expression of both p53 and Bax in the modulatory circuit were up-regulated with treatment. p53-dependent induction of p21 restricts entry of cell in S phase (Helt et al., 2001), which is indicated by increase in p21 level in Fa fraction-treated set. Further data revealed, Bcl-2 status remained unchanged on treatment and thus could even signify that the Fa fraction is bypassing the Bcl-2 checkpoint and overrides its protective role on apoptosis. The apoptogenic action of the fraction might be expressed as a result of its antioxidant potential. The same fraction was an activator of the NOS enzyme. It had been reported beforehand that the nitric oxide synthase (NOS) activation had an inhibitory effect on diverse types of cancer including EAC (Sinha et al., 2002; Chattopadhyay et al., 2002). EAC has a resemblance with human tumors which are most sensitive to chemotherapy due to the fact that it is undifferentiated and it has a rapid growth rate. Due to this resemblance, EAC cell line can be used as a model for human cancer (Ozaslan et al., 2007). Various earlier reports of apoptosis induction in Ehrlich’s ascites carcinoma (EAC) cells by dietary natural products or compounds such as curcumin, black tea etc were already been demonstrated in a mice model (Pal et al., 2001; Bhattacharyya et al., 2003). Recently, even medicinal mushrooms Auricularia auricula and Astraeus hygrometricus has been shown to exert apoptosis inducing ability against EAC cell lines (Gurusam and Arthe, 2012; Biswas et al., 2012).
It can be thus concluded from this study that Fa fraction of *T. giganteum* could induce apoptosis in EAC cells grown *in vivo* through cell cycle arrest and as well as by modulation of balance between pro- and anti-apoptotic proteins. This might also be a benefit on the inhibition of EAC. The most notable implication of the work was that oral infusion of the Fa fraction could result in significant inhibition in progression of cancer in animal model that emulates human disease, suggesting development of a chemopreventive agent in cancer therapy.
Chemopreventive activity of Tricholoma giganteum Musset on EAC cells, forestomach and lung carcinogenesis in mice

References


