Chapter-7

Apoptosis induction study
Introduction

The term “apoptosis” had been coined in order to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr, Wyllie and Currie (Kerr et al., 1972). Apoptosis is of Greek origin, having the meaning “falling off or dropping off”, in analogy to leaves falling off trees or petals dropping off flowers. Apoptosis is a highly organized process of programmed cell death for removing unwanted cells from the body during organ development, tissue remodeling and immune responses. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems (Leist and Jaattela, 2001). Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide).

Figure 7.1: Mechanisms contributing to evasion of apoptosis and carcinogenesis

Apoptotic processes are of widespread biological significance, being involved in e.g. development, differentiation, proliferation/homoeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. T-lymphocytes are cells of the immune system that are responsible for destroying infected or damaged cells in the body. They
mature in the thymus, but before enter to the bloodstream they are tested to ensure, that are effective against foreign antigens and are also not reactive against normal, healthy cells. Any ineffective or self-reactive T-cells are removed through the induction of apoptosis. Thus, dysfunction or dysregulation of the apoptotic program is implicated in a variety of pathological conditions (Figure 7.1). Defects in apoptosis can result in cancer, autoimmune diseases and spreading of viral infections, while neurodegenerative disorders, AIDS and ischemic diseases are caused or enhanced by excessive apoptosis (Qiao and Wong, 2009).

**Morphological features of apoptosis**

![Figure 7.2: Hallmarks of the apoptotic and necrotic cell death process.](image)

Apoptosis was originally defined by the morphological changes that transpire with notable trustworthiness in a wide variety of cells despite of cell lineage and the method of cell death induction. Apoptotic cells display distinctive morphology during the apoptotic process: cell begins to shrinks, shows deformation and looses contact to its neighboring cells, chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding and finally the cell is fragmented into compact membrane-enclosed structures, called
‘apoptotic bodies’ which contain cytosol. The apoptotic bodies are engulfed by macrophages and thus are removed from the tissue without causing an inflammatory response (Figure 7.2). Those morphological changes are a consequence of characteristic molecular and biochemical events occurring in an apoptotic cell, most notably the activation of proteolytic enzymes which eventually mediate the cleavage of DNA into oligonucleosomal fragments as well as the cleavage of a multitude of specific protein substrates which usually determine the integrity and shape of the cytoplasm or organelles (Saraste and Pulkki, 2000; Kurosaka et al., 2003). Apoptosis is in contrast to the necrosis involves loss of membrane integrity, cell swelling, formation of cytoplasmic vacuoles, swollen endoplasmic reticulum, distended or ruptured mitochondria, lysosomes, lysis and release of the cytoplasmic contents into the surrounding tissue (Trump et al., 1997) leading to inflammatory reaction.

**Molecular mechanisms of apoptosis pathways**

A hallmark of apoptosis is the activation of a caspase cascade, resulting in cleavage of numerous structural and signaling proteins. This can be achieved by two major cellular signaling pathways, known as the intrinsic (or mitochondrial) pathway and the extrinsic pathway also known as death receptor pathway (Figure 7.3).

**Intrinsic signaling pathway** that initiate apoptosis involve a diverse array of non-receptor mediated stimuli that produce intracellular signals that act directly on targets within the cell and are mitochondrial initiated events and is controlled by the pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family (Chipuk et al., 2010). The apoptotic signal, such as oxidative stress or DNA damage caused by many anticancer therapeutics, the pro-apoptotic Bcl-2 members Bax or Bak are activated at the mitochondria, thereby triggering a cascade of signaling events including cytochrome c release into the cytoplasm, which is released from the outer mitochondrial membrane through a channel called mitochondrial apoptosis-inducing channel (MAC). In the cytosol, cytochrome c binds with apoptotic protease activating factor 1 (Apaf1) and ATP, which then bind to pro-caspase-9 to create a protein complex known as apoptosome, where caspase-9 activates the executioners of apoptosis, effectors caspase-3, leading to cleavage of numerous cellular proteins and that ultimately ends with the death of the cell (Crompton, 2000). Anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1, prevent cell death by binding and sequestering pro-apoptotic proteins. The activity of caspases is also tightly regulated by cytosolic inhibitor of apoptosis (IAP) proteins.
(XIAP, cIAP1, cIAP2, survivin), which can directly bind to and block caspase activation (Srinivasula and Ashwell, 2008).

**Figure 7.3:** Schematic representation of apoptotic signaling pathways.

**Extrinsic signaling pathways** that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor receptor (TNFR) gene super family, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (Stegehuis et al., 2010). These receptors are characterized by a cytoplasmic death domain that is activated upon ligand binding, leading to the formation of a death-inducing signaling complex (DISC) consisting of the adaptor protein Fas-associated death domain (FADD) and pro-caspase-8/10. In this process, pro-caspase-8/10 is self cleaved, activated and released into the cytoplasm and in turn activates the effectors caspases (caspase 3, 6, and 7) responsible for the proteolytic degradation of structural and signaling proteins that eventually results in cell death. The link between the caspase signaling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocate to the mitochondria where it acts in concert with the pro-apoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other
mitochondrial pro-apoptotic factors into the cytosol (Luo et al., 1998). Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with 7-fold symmetry that triggers the activation of the initiator procaspase-9. Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7 and caspase-6, ultimately resulting in cell death (Acehan et al., 2002).

**Caspases are central initiators and executioners of apoptosis**

The term caspases is derived from cysteine-dependent aspartate-specific proteases: are families of proteins that are one of the main executors of the apoptotic process (Table 7.1). They belong to a group of enzymes known as cysteine proteases and exist within the cell as inactive pro-forms or zymogens. These zymogens can be cleaved to form active enzymes following the induction of apoptosis (Richardson et al., 2002).

Induction of apoptosis via death receptors typically results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 and caspase 6. These caspases are responsible for the cleavage of the key cellular proteins, such as cytoskeletal proteins, that leads to the typical morphological changes observed in cells undergoing apoptosis (Leist and Jaattela, 2001; Kang et al., 2002).

**Table 7.1: Common known caspases involved in apoptosis (Rastogi et al., 2009)**

<table>
<thead>
<tr>
<th>Type</th>
<th>Names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiator (or apical)</td>
<td>Caspases-2, Caspases-8, Caspases-9, Caspases-10</td>
</tr>
<tr>
<td>Effectors (or executioner)</td>
<td>Caspases-3, Caspases-6, Caspases-7</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>Caspases-1, Caspases-4, Caspases-5, Caspases-11,</td>
</tr>
<tr>
<td></td>
<td>Caspases-12, Caspases-13, Caspases-14.</td>
</tr>
</tbody>
</table>

**Apoptosis and anticancer therapeutics**

Chemotherapy, surgery, radiation and drugs, singly or in combination are major existing modes of cancer treatment in modern medicine. Chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally cures. Unfortunately, cancer chemotherapeutic agents insidiously affect the host cells especially bone marrow,
epithelial tissues, reticule-endothelial system and gonads. An appropriate anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis. Thus, modulating apoptosis may be useful in the management and therapy or prevention of cancer. Synthesis or modification of known drugs continues as an important aspect of research. However, a vast amount of synthetic work has contributed relatively small improvements over the prototype drugs. There is a continued need for new prototypes - new templates to use in the design of potential chemotherapeutic agents. Significantly, natural products have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects (Gonzales and Valerio, 2006).

Recent studies on tumor inhibitory compounds of plant origin have yielded an impressive array of novel structures. Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells. It has become increasingly evident that apoptosis is an important mode of action for many antitumor agents, including ionizing radiation, alkylating agents such as cisplatin and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU), topoisomerase inhibitor etoposide, cytokine tumour necrosis factor (TNF) taxol and N-substituted benzamides such as metoclopramide and 3-chloroprocainamide (Jiang et al., 1996; Pero et al., 1997).

Apoptosis induction has been a new target for innovative mechanism-based drug discovery. It is thus considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. Evidence has emerged from various studies that suggest, products derived from plants are useful in the treatment as well as in the prevention of cancer (Table 7.2). Chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but, their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention. Understanding the modes of action of these compounds should provide useful information.
Table 7.2: Some apoptosis inducing natural plant compounds used in cancer chemotherapy

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mechanism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamaldehyde (Cinnamomum osmophloeum)</td>
<td>Release of cytochrome-c into cytosol, by the imbalance of BCl-2 family of proteins</td>
<td>Huang et al., 2007</td>
</tr>
<tr>
<td>Aloe-emodin (Aloe vera)</td>
<td>Blocks casein kinase II activity and phosphorylation of Bid and induces Aif and cytochrome-c</td>
<td>Chen et al., 2007</td>
</tr>
<tr>
<td>Curcumin (Curcuma longa)</td>
<td>Induces the production of ROS and downstream activation of JNK as well as activation of caspases-9, 3 and 8</td>
<td>Johnson and Mukhtar, 2007</td>
</tr>
<tr>
<td>Hormonemate (Hormonema dematioides)</td>
<td>Induces caspase-3 activity</td>
<td>Filip et al., 2003</td>
</tr>
<tr>
<td>Cinnamaldehyde (Cinnamomum cassia)</td>
<td>Induces the ROS mediated mitochondrial permeability transition and resultant cytochrome-c release</td>
<td>Ka et al., 2003</td>
</tr>
<tr>
<td>Ellipticine (Ochrosia borbonica, Excavatia coccinea)</td>
<td>Accumulation of dephosphorylated mutant p53</td>
<td>Kuo et al., 2005</td>
</tr>
<tr>
<td>Dehydroeburicoic acid (Antrodia camphorate)</td>
<td>Induced apoptotic and DNA damaging markers, inhibited topo II isomerase</td>
<td>Du et al., 2012.</td>
</tr>
<tr>
<td>Shikonin (Lithospermum erythrorhizon)</td>
<td>loss of mitochondrial membrane potential, ROS generation, cytochrome c release, and subsequent induction of pro-caspase-9 and -3 processing</td>
<td>Hsu et al., 2004</td>
</tr>
<tr>
<td>Apicidin (Fusarium sp.)</td>
<td>Induces Fas and Fas-L, cytochrome c release into the cytosol, and activation of caspases-9 and 3</td>
<td>Kwon et al., 2002</td>
</tr>
</tbody>
</table>

The primary goal of chemotherapy is to kill cancer cells, one way or another, a secondary goal is to have those cells die by apoptosis so that they may be cleared quickly and “quietly” by neighboring phagocytic cells. In the previous chapter it was found that methanol extract of Anthocephalus cadamba (MEAC) and β-sitosterol glucoside (BSSG) from Castanopsis indica possesses significant inhibitory effect on the proliferation of cancer cells. This investigation was to evaluate the apoptogenic mechanistic studies of MEAC and BSSG on Ehrlich ascites carcinoma (EAC) cells treated mice.
Material and methods

Animals

Swiss albino mice of about 8 weeks of age with an average body weight of 20-25 g were used for the experiment. The mice were grouped and housed in poly acrylic cages (38 cm × 23 cm × 10 cm) with not more than six animals per cage. The animals were maintained under standard laboratory conditions (temperature 25-30 °C and 55-60% relative humidity with dark/light cycle 14/10 h) and were allowed to free access of standard dry pellet diet and water ad libitum. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All the described procedures were reviewed and approved by Institutional Animal Ethics Committee.

Cell culture

EAC cells were obtained from Chittaranjan National Cancer Institute, Kolkata, India. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (days 7-8 of tumor bearing) of the tumor cells. The EAC cells were maintained in vivo in Swiss albino mice by intraperitoneal transplantation of 2×10^6 cells per mouse after every 10 days and it is used for present experiment.

Tumor model

- **Group I** : EAC control (2×10^6 cells/mouse, i.p.)
- **Group II** : EAC + MEAC (200 mg/kg body weight, i.p.)
- **Group III** : EAC + MEAC (400 mg/kg body weight, i.p.)
- **Group IV** : EAC + BSSG (50 mg/kg body weight, i.p.)
- **Group V** : EAC + BSSG (100 mg/kg body weight, i.p.)

Swiss albino mice (20-25 g) were divided into five groups (n=12). Group-I served as EAC control and group II-V served as treated group. All the animals in each group were being injected EAC cells (2×10^6 cells/mouse, i.p.). This was marked as day ‘0’. After 24 h, EAC transplanted group-II and III received MEAC (200 and 400 mg/kg b.w, i.p.) and group-IV and V received BSSG (50 and 100 mg/kg b.w, i.p.) respectively, once daily for 14 consecutive days (Chatterjee et al., 2013). After administration of last dose, the mice were kept fasting for 18 h and then sacrificed for collection of EAC cells to check the apoptogenic properties of MEAC and BSSG.
Detection of apoptosis

Isolation of tumor cells from mice peritoneal cavity

The EAC cells were isolated from the peritoneal cavity of tumor-bearing mice (control or treated). 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 cells was assessed by trypan blue exclusion test. The viable tumor cells were processed for further experiments (Bhattacharyya et al., 2003).

Acridine orange (AO) and ethidium bromide (EB) double staining

Principle

Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Acridine orange stains live cells will appear uniformly green. Early apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Late apoptotic cells will also incorporate ethidium bromide and therefore stain orange, but, in contrast to necrotic cells, the late apoptotic cells will show condensed and often fragmented nuclei. Necrotic cells stain orange, but have a nuclear morphology resembling that of viable cells, with no condensed chromatin (Zhang et al., 1998; Ribble et al., 2005). Morphology of exposed cells was determined under fluorescent microscope after labeling with AO and EB double staining method.

Procedure

DNA-binding dyes AO and EB (Sigma Aldrich, USA) were used for the morphological detection of apoptotic and necrotic cells (Attari et al., 2009). EAC cells (1×10⁶) were collected from sacrificed mice. The cells were detached, washed by cold PBS and then stained with a mixture of AO (100 μg/ml) and EB (100 μg/ml) at room temperature for 5 min. The stained cells were observed by a fluorescence microscope (Leica DM 3000, Germany) at 40X magnifications. The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).
DNA damage by comet assay

Principle

Inter-nucleosomal DNA fragmentation is considered a hallmark of apoptosis. During apoptosis, activated nucleases degrade the higher order chromatin structure of DNA into fragments of 50 to 300 kilo bases and subsequently into small DNA pieces of about 200 base pairs in length. These DNA fragments can be extracted from cells and visualized by horizontal gel electrophoresis followed by ethidium bromide staining. The detection of DNA fragments by single cell gel electrophoresis (SCGE) is one method to identify cells undergoing apoptosis (Das and Chaudhuri, 2014).

The comet assay is a rapid, simple and sensitive quantitative technique by which visual evidence of DNA damage in eukaryotic cells may be measured. It is based on quantification of the denatured DNA fragments migrating out of the cell nucleus during electrophoresis. This assay has been use in various areas including human biomonitoring, genotoxicology, ecological monitoring and as a tool for research into DNA damage or repair in different cell types in response to a range of DNA damaging agents (Liao et al., 2009).

Procedure

The extent of DNA was quantified by alkaline single cell gel electrophoresis (SCGE), also known as comet assay. Briefly, cells suspended in 0.5% (w/v) low melting agarose were layered over a frosted microscopic slide coated with a layer of 1% normal melting agarose. The slides were left in a lysing solution overnight at 4 °C. Electrophoresis was carried out for 30 min (280 mA, 20 V) at 4 °C. The slides were washed thrice with neutralizing buffer (Tris 0.4 M, pH 7.5), stained with EB, examined under a fluorescence microscope (Leica DM3000, Germany) and subjected to image analysis using CometScore software (Das et al., 2014).

Flow cytometric analysis (FACS)

Principle

Flow cytometry measures optical and fluorescence characteristics of single cells. Physical properties, such as size (represented by forward angle light scatter) and internal complexity (represented by right-angle scatter) can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside
cells. Apoptosis is distinguished from necrosis or accidental cell death, by characteristic morphological and biochemical changes, including compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry (Schiller et al., 2008).

Annexins are a family of calcium-dependent phospholipid-binding proteins that preferentially bind phosphatidylserine (PS). Under normal physiologic conditions, PS is predominantly located in the inner leaflet of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution across the phospholipid bilayer and is translocated to the extracellular membrane leaflet marking cells as targets of phagocytosis. Once on the outer surface of the membrane, PS can be detected by fluorescently labeled Annexin V in a calcium-dependent manner. In early stage of apoptosis, the plasma membrane excludes viability dyes such as propidium iodide (PI). These cells will stain with Annexin V but not a viability dye, thus distinguishing cells in early apoptosis. However, in late stage of apoptosis, the cell membrane loses its integrity thereby allowing Annexin V to access PS in the interior of the cell. PI can be used to resolve this late-stage apoptotic and necrotic cells (Brown and Wittwer, 2000).

**Procedure**

Assay was performed using procedure described in the reagent-kit purchased from BD Biosciences and protocol was followed by manufacturer’s instruction. To distinguish between apoptosis and necrosis, in a double labeling system, EAC cells from untreated or MEAC and BSSG treated tumor-bearing mice were washed twice with cold PBS and then re-suspended in 1X binding buffer at a concentration of $1\times10^6$ cells/ml. Then 100 µl of the cell suspension was transferred to the 5 ml culture tube. After that, added 25 µl of Annexin V-FITC and/or propidium iodide (PI) solution to the cell suspension. The cell were gently mixed by vortexing and incubated for 15 min at 37 °C. Then 400 µl of 1X binding buffer was added to each tube and analyzed by FACS within one hour using flowcytometer (BD LSRFortessa™ Cell analyzer, USA).

**Apoptotic protein expression by Western blotting**

**Principle**

Western blot (immunoblot) is an analytical technique used in molecular biology to identify specific protein in the protein mixture. Western blot was performed using the alkaline phosphatase method. The technique comprises of gel electrophoresis (SDS-PAGE) to separate
native or denatured proteins by the size and molecular weight of the polypeptide of the protein. Then the gel were allowed to transferred on a polyvinyl difluoride (PVDF) membrane producing a band for each protein, this process is known as blotting. The membrane is then incubated through primary antibody conjugated with alkaline phosphatase specific to the protein of interest. The unbound antibody was washed off leaving only the bound antibody to the protein of interest. Then membrane incubated with secondary antibody fallowed by washing and subsequently membrane was developed with bromochloroindolyl phosphate (BCIP)/ nitrobluetetrazolium (NBT) substrate. The thickness of the band corresponds to the amount of protein present and normalization of the protein expression was performed by immune blotting for β-actin with the same amount of lysate (Findley et al., 1997).

**Procedure**

EAC lysate was loaded into a 10% SDS-polyacrylamide gel. After electrophoresis, the gel was transferred to nitrocellulose membrane and blocked with 5% Bovin serum albumin (BSA) in 1X tris buffer saline (TBS). The membrane was then incubated with specific primary antibody of p53, p21, Bax, Bcl-2 and β-actin (1:1000) for overnight at 4 °C. The protein of interest was visualized by treating with alkaline phosphatase (ALP) 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 β-actin antibody probing (Chatterjee et al., 2013).

**Statistical Analysis**

Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using ANOVA, followed by Dunnett’s post hoc test by GraphPad Prism software. *p <0.05, **p <0.01 and ***p <0.001 were considered as statistically significant.

**Results**

**Acridine orange (AO) and ethidium bromide (EB) double staining**

Alteration in morphological features of apoptosis such as chromatin condensation, nuclear fragmentation, alterations in the size and shape of cells, as revealed by fluorescence microscopic analysis, were observed predominantly after MEAC and BSSG treatment. AO penetrates into living cells, emitting green fluorescence after intercalation into DNA. The second dye, EB emits red fluorescence in the cells with an altered cell membrane. Treatment with
MEAC at doses 200 and 400 mg/kg has shown maximum increases in the number of apoptotic cells $39.3 \pm 2.52$ and $43.6 \pm 1.82\%$ (Figure 7.4B and 7.4C). Dose dependent treatment with BSSG (50 and 100 mg/kg) has shown the number of apoptotic cells $35.3 \pm 1.2$ and $38.6 \pm 1.82\%$ respectively (Figure 7.4b and 7.4c).

**Figure 7.4**: MEAC and BSSG induce apoptotic morphological changes on EAC cells. [(A) EAC control, (B) MEAC 200 mg/kg and (C) MEAC 400 mg/kg] and [(a) EAC control, (b) BSSG 50 mg/kg and (c) BSSG 100 mg/kg]. The cells were stained with AO/EB (100 μg/ml). Blue arrows next to "L" point to live cells; Yellow arrows next to "A" indicate apoptotic cells; and Red arrows next to "N" indicate necrotic cells (magnification at 40X).

**DNA damage by comet assay**

Treatment dependent DNA damage in EAC cells was observed after MEAC and BSSG treatment compared with the untreated EAC control (Figure 7.5A and 7.5a). MEAC and BSSG induced DNA damage even at a low concentration, as indicated by the presence of the DNA tail (Figure 7.5B and 7.5b). A greater percentage tail length (distance from DNA head to the end of DNA tail), more extensive were the DNA damage (Figure 7.5C and 7.5c). Experimental results
revealed that MEAC induced substantial DNA damage which was evident from the appearance of comet length 9.0 ± 1.50 and 12.1 ± 2.93 µm in MEAC at the doses 200 and 400 mg/kg with respect to the untreated control (2.05 ± 0.45 µm) group (Figure 7.5D). BSSG induced DNA damage, appearance of comet length 7.32 ± 1.70 and 10.61 ± 1.97 µm in BSSG at the doses 50 and 100 mg/kg with respect to the EAC control (2.31 ± 0.50 µm) group (Figure 7.5d).

**Figure 7.5:** The DNA damage was measured by comet assay after MEAC and BSSG treatment on EAC cells. [(A) EAC control, (B) MEAC 200 mg/kg, (C) MEAC 400 mg/kg and (D) The extent of DNA damage was expressed in terms of comet % tail length] and [(a) EAC control, (b) BSSG 50 mg/kg, (c) BSSG 100 mg/kg and (d) The extent of DNA damage was expressed in terms of comet % tail length]. Data are the mean ± SD from three replicate measurements. Treated groups vs. EAC control group, **p <0.01 and ***p <0.001.

**Flow cytometric analysis (FACS)**

Cells stained with Annexin V-FITC and PI were classified as necrotic cells (the upper left quadrant; Annexin−/PI+), late apoptotic cells (the upper right quadrant; Annexin+/PI+), intact cells (the lower left quadrant; Annexin−/PI−) or early apoptotic cells (the lower right quadrant; Annexin+/PI−). The result showed that MEAC quantitatively induce apoptosis on EAC cells at the doses of 200 and 400 mg/kg were found to be 41.9% and 47.7% respectively (Figure 7.6B and 7.6C). Apoptosis ratios for BSSG at the doses of 50 and 100 mg/kg were found to be 24.3 and 36.2% respectively (Figure 7.6b and 7.6c).
Figure 7.6: Flowcytometric analysis of EAC cells apoptosis after MEAC and BSSG treatment to detect increases the apoptotic rate. [(A) EAC control, (B) MEAC 200 mg/kg and (C) MEAC 400 mg/kg] and [(a) EAC control, (b) BSSG 50 mg/kg and (c) BSSG 100 mg/kg]. Quadrants: lower left- live cells; lower right- apoptotic cells; upper right- necrotic cells.

**Apoptotic protein expression by Western blotting**

The expression of pro-apoptotic proteins Bax and as well as anti-apoptotic protein Bcl-2 in our mice model were examined to study the effect of the MEAC and BSSG on EAC cells. The levels of p53, p21 and Bax protein expression up-regulated significantly and down-regulation of Bcl-2 was observed after MEAC and BSSG treated group (Figure 7.7A and Figure 7.7a). Interestingly, the Bcl-2/Bax ratio was significantly decreased by MEAC and BSSG (Figure 7.7B and Figure 7.7b) in a dose dependent manner.
Figure 7.7: Western blots analysis of pro-apoptotic proteins p53, p21, Bax and anti-apoptotic protein Bcl-2 and visualized by ALP-conjugated secondary antibody [MEAC- (A) and BSSG- (a)]. The β-actin band confirmed equal protein loading. Quantitative expression and ratio of western blots [MEAC- (B) and BSSG- (b)]. Data are the mean ± SD from three replicate measurements. Treated groups vs. EAC control group, *p <0.05 and **p <0.01.

Discussion

Apoptosis was originally defined by the morphological changes that transpire with notable trustworthiness in a wide variety of cells despite of cell lineage and the method of cell death induction. Consideration of morphology is the most important method for detection of apoptosis (Yang et al., 2013). In the present experiments, apoptosis associated morphological changes were observed on EAC cells after MEAC and BSSG treatment by AO/EB double staining method. It should be considered that healthy cells have green nuclei and uniformly
chromatin with intact cell membrane, the cells undergoing apoptosis have orange or green nuclei with condensed chromatin and the necrotic cells have red nuclei with damaged cell membrane (Attari et al., 2009). Most of the cells treated with MEAC and BSSG were apoptotic with green or orange fragmented nuclei which were also in accordance with relatively low cell viability. From the results of AO/EB double staining confirmed that MEAC and BSSG could induce cell death through apoptosis in a dose dependent manner.

DNA damage-induced apoptosis mainly proceeds through the mitochondrial pathway of caspase activation (Liao et al., 2009). Hence in order to understand the level of DNA damage followed by apoptosis had been observed by comet assay. DNA damage in proliferating cells activates a pathway that arrests cell division to allow either DNA repair or induction of cell death by apoptosis. Under alkaline conditions, necrotic or apoptotic cells can result in comets with small or nonexistent head and large diffuse tails (called a “hedgehog” comet) as observed on EAC cells after treatment with MEAC and BSSG (Liao et al., 2009; Das et al., 2014). MEAC and BSSG induced substantial DNA damage which was evident from the appearance of comet like features with considerable comet tail length.

To investigate the percentage of cell undergoes apoptosis by MEAC and BSSG, performed double labeling techniques using Annexin-V/PI staining followed by FACS to distinguish between apoptotic and necrotic cells. Annexin V binds to phosphatidylserine (PS) in a calcium-dependent manner. PS was normally found only on the intracellular leaflet of the plasma membrane in healthy cells (Yang et al., 2013). In early apoptosis, membrane asymmetry is lost and PS translocate to the external leaflet. Fluorochrome-labeled Annexin-V then specifically target and identify apoptotic and excludes necrotic cells as PI unable to bind. At late stage, apoptotic cells and necrotic cells will stain positively, due to the passage of these dyes into the nucleus where they bind to DNA (Mondal and Saha, 2011). Flowcytometric data revealed that, MEAC and BSSG quantitatively induce apoptosis of EAC cells in a dose dependent manner that suggesting the mode of cell death is due to apoptosis.

Programmed cell death or apoptosis of a cell becomes committed to apoptosis partly depends upon the balance between proteins that mediate growth arrest and cell death, e.g. p53, p21, Bax and proteins that promote cell viability, e.g. Bcl-2. Treatment with MEAC and BSSG to EAC cells increased the level of Bax with a concomitant decreased in Bax/Bcl-2 ratio. This suggested that a critical determinant of the overall propensity of cells were undergoing apoptosis.
at aforementioned treatment (Chatterjee et al., 2013). An important function of wild-type p53, the tumor suppressor gene, act as a transcription factor by binding with p53-specific DNA consensus sequence in responsive genes, which would be expected to increase the synthesis of p21 (Bhattacharyya et al., 2003; Chen et al., 1995). It was evident from various studies, that up-regulation of cell growth-regulating genes, upon p53 induction, may block the cell cycle but increased expression of pro-apoptotic factors can override the growth-arresting message and thereby ultimately leads to apoptosis (Helt et al., 2001).

**Conclusion**

The present study brings into focus on methanol extract of *Anthocephalus cadamba* (MEAC) and β-sitosterol glucoside (BSSG) from *Castanopsis indica* as an unique antitumor agents. The tumor suppressing mechanism involved the induction of apoptosis and/or cell death followed due to DNA damage property of MEAC and BSSG. Further we proved that MEAC and BSSG could induce apoptosis in EAC cells through modulation of balance between pro-apoptotic and anti-apoptotic proteins. The apoptosis inducing property of MEAC and BSSG on EAC cells in animal model suggests the development of an active and safe chemopreventive agent in cancer therapy.
References


Chapter 7

Induction of apoptosis


