Chapter-6

Anticancer activity
Introduction

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell or die. Cancer cell develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is allowed to continue and spread, it can be fatal. In fact, almost 90% of cancer related deaths are due to tumor spreading process called metastasis (Kaku et al., 2011).

Cancer arises from the abnormal and uncontrolled division of cells, known as cancer cells, which then invade and destroy the surrounding tissues. The tissues most affected by cancer and which have a relative survival rate of five years are the esophagus, lung, pancreas, stomach, liver, ovaries and breasts (Chang and Kinghorn, 2001). Cancer cells refuse to stop multiplying and continue to increase in number, producing millions of cells, which constitute a tumor and is referred to as neoplasm.

Cancer is perhaps the most progressive and devastating disease posing a threat of mortality to the entire world despite significant advances in medical technology for its diagnosis and treatment. Global awareness of cancer and it being the greatest cause of death of people of various ages and racial backgrounds have led to much research and clinical studies in an effort to limit the spread of this disease. Recently, considerable attention has been focused on identifying natural occurring chemopreventive substance capable of inhibiting, retarding, or reversing the process of multistage carcinogenesis (Chatterjee et al., 2013).

The International Agency for Research on Cancer (IARC), the specialized cancer agency of the World Health Organization, released the latest data on cancer incidence, mortality, and prevalence worldwide. According to GLOBOCAN 2012, an estimated 14.1 million new cancer cases and 8.2 million cancer related deaths occurred in 2012, compared with 12.7 million and 7.6 million, respectively, in 2008. Prevalence estimates for 2012 show that there were 32.6 million people (over the age of 15 years) alive who had had a cancer diagnosed in the previous five years. The most commonly diagnosed cancers worldwide were those of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%) and colorectum (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%) and stomach (0.7 million, 8.8%).
Projections based on the GLOBOCAN 2012 estimates predict a substantive increase to 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population. More than half of all cancers (56.8%) and cancer deaths (64.9%) in 2012 occurred in less developed regions of the world and these proportions will increase further by 2025.

Type of tumors

Tumors are classified as either benign or malignant.

- **Benign tumors** are not cancerous and do not spread to other parts of the body.
- **Malignant tumor** can metastasize- a process during which cancer cells escape from the tumor, enter the bloodstream or lymphatic system, and spread to nearby parts of the body and eventually to sites far away from the original tumor. Most of the cancers are named for the organ or type of cell in which they being to grow, such as lung, stomach, breast, colon cancer etc.

- **Melanoma** is a cancer of cells in the skin, eye and some other tissues, known as melanocytes, which make pigment.

- **Carcinomas** are cancers that develop in the tissues such as lung, liver, skin or breast. This tissue is called epithelial tissue.

- **Sarcomas**: Malignant tumors derived from connective tissue, or mesenchymal cells.

- **Leukemia** and **Lymphoma**: Malignancies derived from hematopoietic cells.

- **Germ cell tumor**: Tumors derived from totipotent cells. In adults most often found in the testicle and ovary; in fetuses, babies, and young children most often found on the body midline, particularly at the tip of the tailbone; in horses most often found at the poll (base of the skull).

- **Blastic tumor or blastoma**: A tumor (usually malignant) which resembles an immature or embryonic tissue. Many of these tumors are most common in children.

Insights into cancer

Cancer arises from one single cell. Carcinogenesis is the transformation from a normal cell into a cancerous cell is a multistage process (Figure 6.1).

- **Initiation stage**, which involves the reaction between the carcinogens and the DNA of the cells. Inhibiting this early stage of cancer is an important strategy in cancer prevention or treatment.
➢ **Promotion stage**, it occurs slowly over an extended period of time, ranging from several months to years. Beneficial effects may arise from a change in lifestyle and diet, which may result in the individual not developing cancer during his or her lifetime.

➢ **Progressive stage**, involving the spread of the cancer. It is evident that, upon entering into this stage, preventative factors such as diet have less of an impact (Reddy et al., 2003).

![Figure 6.1: Transformation of normal cell to a cancerous cell](image-url)

Initiation and progression of cancer depends on both external factors in the environment (tobacco, chemicals, radiation and infectious organisms) and factors within the cell (inherited mutations, hormones, immune conditions and mutations that occur from metabolism) (Molina et al., 2008). These factors can act together or in sequence, causes abnormal cell behavior and excessive proliferation. As a result, cell masses grow and expand, affecting surrounding normal tissues (such as in the brain) and can also spread to other locations in the body (metastasis). However, it is important to remember that most common cancers take months/years for these DNA mutations to accumulate and result in a detectable cancer (Rosell et al., 2001).

**Predisposing factors to developing cancer**

Cancers are primarily an environmental disease with 90-95% of cases due to lifestyle and environmental factors and 5-10% due to genetics. Common environmental factors that lead to cancer death include: tobacco (25-30%), diet, obesity (30-35%), infections (15-20%), radiation, radon exposure, stress, lack of physical activity and environmental pollutants.

**Environmental risk factors**

**Ultraviolet (UV) radiation**: UV radiations from the sun are directly linked to melanoma and other forms of skin cancer. These harmful rays of the sun cause premature aging and damage the skin. Artificial sources of UV radiation, such as sun lamps also increase the risk of skin cancer. By wearing protective clothing and sunscreens and by avoiding prolonged exposure to...
the sun, one may reduce the risk of skin cancer. Many of the 1.3 million skin cancers diagnosed in the year 2000 could have been prevented by protection from the sun’s rays.

**Radiation:** Ionizing radiation releases enough energy to damage the DNA within each cell, which can result in malignant changes taking place in later life. The most common forms of radiation induced cancers are thyroid, basal and squamous cell carcinoma of the skin.

**Viruses and Bacteria:** Some viruses, including hepatitis B and C, human papilloma viruses (HPV) and the Epstein Barr virus, which causes infectious mononucleosis, have been associated with increased cancer risk. Immune system diseases, such as AIDS, can make one more susceptible to some cancers. *Helicobacter pylori* are linked to the development of gastric and duodenal ulcers.

**Chemicals:** Long term exposure to chemicals such as vinyl chloride, pesticides, uranium, nickel, asbestos, arsenic, radon, aniline dyes and benzene can increase the risk of cancer.

**Tobacco/Smoking:** There is overwhelming evidence to link tobacco with a variety of cancers. Cigarette smokers are more likely to develop several types of cancer like those of the mouth, larynx, esophagus, pancreas, bladder, kidney and cervix. Smoking may also increase the likelihood of developing cancers of the stomach, liver, prostate, colon and rectum.

**Alcohol:** Heavy drinkers face an increased risk of mouth, throat, liver, esophagus and larynx cancers. Some studies suggest that even moderate drinking may slightly increase the risk of breast cancer. The American Cancer Society (ACS) estimated that in the year 2000 about 171,000 cancer deaths were expected to be caused by tobacco use and about 19,000 cancer deaths were to be related to excessive alcohol use, frequently in combination with tobacco use.

**Diet:** High-fat, high cholesterol diets are proven risk factors for several types of cancer such as colon, uterus and prostate. Obesity may be linked to breast cancer among older women as well as to cancers of the prostate, pancreas, uterus, colon and ovary. Scientific evidence suggests that up to one-third of the 552,200 cancer deaths expected to occur in the US in the year 2000 were related to nutrition and other lifestyle factors.

**Hereditary risk factors**

The abnormal gene responsible for causing cancer is passed from parent to child, posing a greater risk for that type of cancer in all descendants of the family, 20% of cancers are hereditary.
Age: Although cancer can occur in persons of every age, it is common among the aging population. Sixty percent of new cancer cases and two thirds of cancer deaths occur in persons > 65 years. The incidence of common cancers (breast, colorectal, prostate and lung) increases with age (Vasto et al., 2009).

Genetics factors
Multiple genetic changes are basic for the development of cancer, most clearly exemplified by the stepwise genetic changes shown by many colon polyps progressing to cancer (Larsen and Minna, 2011). Lifetime exposure to estrogen may lead to breast or uterine cancer; exposure to testosterone leads to prostate cancer (Chen et al., 2011). The decline in cellular immunity may also lead to certain types of cancer that are highly immunogenic (lymphomas and melanomas).

The six hallmarks of cancer
DNA mutations result in defects in the regulatory path of a cell, which disrupt normal cell proliferation behavior (Flora et al., 1996). Individual cell behavior is not autonomous and it usually relies on external signals from surrounding cells in the tissue or microenvironment (Luca et al., 2003), which causes different types specific changes of normal cells. The common changes occur in cell physiology that results in cancer can be described as follows:

- **Immortality: Continuous cell division and limitless replication:** Normal cells have a finite lifespan. Cancer cells manipulate the cell to keep dividing indefinitely by producing proteins that enable them to do so (Cheng et al., 2004).
- **Produce ‘Go’ signals (growth factors from oncogenes):** Most cells wait for a ‘Go’ signal before dividing but cancer cells don’t bother waiting, they produce their own chemical message and continue dividing (Liou et al., 2011).
- **Override ‘Stop’ signal (Anti-growth signals from tumor suppressor genes):** Even if neighboring cells produce a ‘Stop’ signal, cancer cells override these signals and continue dividing (Sluis et al., 1994).
- **Resistance to cell death (Apoptosis):** Normal cells sometimes react to stress by triggering a ‘Self Destruct’ button and killing itself, but cancer cells sneak past these signals and continue divide.
- **Angiogenesis**: Cancer cells make sure they can keep growing by stimulating the developing of new blood vessels to keep their nutrient supply lines open.

![Figure 6.2: Angiogenesis in tumor growth](image)

- **Metastasis**: The final stage in tumor progression is the migration and spread of cancers to different sites from where they originated.

![Figure 6.3: Biological pathways of cancer metastasis](image)
Management of cancer

- **Surgery:** Non-hematological cancers can be cured if entirely removed by surgery. This has given rise to the popularity of local only treatments such as surgery for small cancers. Examples of surgical procedures for cancer include mastectomy for breast cancer, prostatectomy for prostate cancer and lung cancer surgery for non-small cell lung cancer. The goal of the surgery can be either the removal of only the tumor, or the entire organ.

- **Radiation therapy:** Radiation therapy (also called radiotherapy, X-ray therapy or irradiation) is the use of ionizing radiation (via external beam radiotherapy (EBRT) or internally via brachy therapy) to kill cancer cells and shrink tumors. Radiation therapy injures or destroys cells in the area being treated (the “target tissue”) by damaging their genetic material, making it impossible for these cells to continue to grow and divide. Radiation therapy may be used to treat almost every type of solid tumor, including cancers of the brain, breast, cervix, larynx, lung, pancreas, prostate, skin, stomach, uterus, or soft tissue sarcomas. Radiation is also used to treat leukemia and lymphoma. The goal of radiation therapy is to damage as many cancer cells as possible, while limiting harm to nearby healthy tissue.

- **Targeted therapies:** Which first became available in the late 1990s, a significant impact in the treatment of some types of cancer, and is currently a very active research area. Prominent examples are the tyrosine kinase inhibitors imatinib (Gleevec/Glivec) and gefitinib (Iressa).

Photodynamic therapy (PDT) involving a photosensitizer, tissue oxygen and light. PDT can be used as treatment for basal cell carcinoma or lung cancer; PDT can also be useful in removing traces of malignant tissue after surgical removal of large tumors.

- **Immunotherapy:** Contemporary methods for generating an immune response against tumors include intravesical BCG immunotherapy for superficial bladder cancer and use of interferons and other cytokines to induce an immune response in renal cell carcinoma and melanoma patients. Vaccines to generate specific immune responses are the subject of intensive research for a number of tumors, notably malignant melanoma and renal cell carcinoma. Sipuleucel-T is a vaccine-like strategy in late clinical trials for prostate cancer in which dendritic cells from the patient are loaded with prostatic acid phosphatase peptides to induce a specific immune response against
prostate-derived cells. Allergenic hematopoietic stem cell transplantation (“bone marrow transplantation” from a genetically non-identical donor) can be considered a form of immunotherapy, since the donor’s immune cells will often attack the tumor in a phenomenon known as graft-versus-tumor effect.

- **Hormonal therapy:** The growth of some cancers can be inhibited by providing or blocking certain hormones like estrogen or testosterone is often an important additional treatment. In certain cancers, administration of hormone agonists, such as progestogens may be therapeutically beneficial.

- **Chemotherapy:** Chemotherapy is the treatment of cancer with anticancer drugs that can destroy cancer cells. The term “chemotherapy” usually refers to cytotoxic drugs which affect rapidly dividing cells in general. Chemotherapy drugs interfere with cell division in various possible ways, e.g. with the duplication of DNA or the separation of newly formed chromosomes.

Chemotherapy, radiotherapy and surgery are only three major existing modes of treatment in modern medicine. Unfortunately, currently available cancer chemotherapeutic agents insidiously affect the host cells especially bone marrow, epithelial tissues, reticule-endothelial system and gonads. Scientists are also turning towards developing clues for treatment from natural resources. India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as Ayurveda, Unani and Siddha (Gupta et al., 2004). The natural product drug development programmed of the United State National Cancer Institute has identified about 3000 species of plants and animals are useful in dealing with one or the other aspect of cancer management. Ethno medicines constitute a common substitute for cancer prevention and treatment in disparate countries around the globe. Due to the aforementioned concerns, such studies investigating medicinal plant have been steadily held with interests. The anticancer activity was done by methanol extract of *Anthocephalus cadamba* (MEAC) and β-sitosterol glucoside (BSSG) against Ehrlich ascites carcinoma (EAC) and Dalton’s lymphoma ascites (DLA) cells treated mice.
Materials 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 free access to 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 Jadavpur University Animal Ethics Committee.

Culture of tumor cells

Ehrlich ascites carcinoma (EAC) and Dalton’s lymphoma ascites (DLA) 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 (i.p.) transplantation of 2×10^6 cells per mouse after every 10 days and it is used for present study.

Treatment schedule for assessment of antitumor potential

- **Group I**: Normal saline control (5 ml/kg, i.p.)
- **Group II**: EAC control (2×10^6 cells/mouse, i.p.)
- **Group III**: DLA control (2×10^6 cells/mouse, i.p.)
- **Group IV**: EAC + MEAC (200 mg/kg body weight, i.p.)
- **Group V**: EAC + MEAC (400 mg/kg body weight, i.p.)
- **Group VI**: EAC + BSSG (50 mg/kg body weight, i.p.)
- **Group VII**: EAC + BSSG (100 mg/kg body weight, i.p.)
- **Group VIII**: DLA + MEAC (200 mg/kg body weight, i.p.)
- **Group IX**: DLA + MEAC (400 mg/kg body weight, i.p.)
- **Group X**: DLA + BSSG (50 mg/kg body weight, i.p.)
- **Group XI**: DLA + BSSG (100 mg/kg body weight, i.p.)
- **Group XII**: EAC + 5-FU (20 mg/kg body weight, i.p.)
- **Group XIII**: DLA + 5-FU (20 mg/kg body weight, i.p.)
Swiss albino mice (20-25 g) were divided into thirteen groups (n=12). Group-I served as normal saline control (5 ml/kg, i.p.). Group-II and group-III served as EAC (2×10^6 cells/mouse, i.p.) and DLA (2×10^6 cells/mouse, i.p.) control. This was marked as day ‘0’. After 24 h, EAC transplanted Group-IV-VII were being injected MEAC (200 and 400 mg/kg b.w, i.p.) and BSSG (50 and 100 mg/kg b.w, i.p.), other hand DLA transplanted Group-VIII-XI were being injected MEAC (200 and 400 mg/kg b.w, i.p.) and BSSG (50 and 100 mg/kg b.w, i.p.) once daily for nine consecutive days. Group-XII and group-XIII received standard drug 5-FU (20 mg/kg i.p) for nine consecutive days (Bala et al., 2010). After administration of last dose, 6 mice from each group were kept fasting for 18 h and blood was collected by direct cardiac puncture for the estimation of hematological and serum biochemical parameters and then sacrificed for collection of liver and kidney tissues to check the different antioxidant parameters. Rest of the animals in each groups were kept alive with food and water ad libitum to check percentage increase in life span of the tumor host to determine the mean survival time (MST).

Antitumor activity of MEAC and BSSG was assessed by observation of changes with respect to the following parameters (Haldar et al., 2010).

**Tumor volume and weight**

The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. Volume of the fluid was measured by taking it in a graduated centrifuge tube and expressed in milliliter (ml). Tumor weight was measured by taking the weight of the mice before and after the collection of the ascitic fluid from peritoneal cavity and expressed in gram (g).

**Tumor cell (Viable/nonviable) count**

The ascitic fluid was taken in a WBC pipette and diluted upto 20 times with PBS solution. Then a drop of the diluted cell suspension was placed on the Neubauer’s counting chamber and the numbers of cells in the 64 small squares were counted.

The viability and non-viability of the cell were determined by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the dye were nonviable. These viable and nonviable cells were counted by using the under-scribbled formula.

\[
\text{Cell count} = \frac{(\text{Number of cells} \times \text{dilution factor})}{(\text{Area} \times \text{thickness of liquid film})}
\]
**Percentage increase life span (% ILS)**

The effect of MEAC and BSSG on tumor growth was monitored by recording the mortality of the experimental mice. Percentage increase in life span (% ILS) was calculated by the following formula.

\[
\text{Mean survival time (MST) in days} = \frac{\text{Day of the first death} + \text{Day of the last death}}{2}
\]

\[
\text{ILS} \% = \left( \frac{\text{MST of the treated group}}{\text{MST of the control group}} \right) - 1 \times 100
\]

**Hematological parameters**

Collected blood was used for the estimation of hemoglobin (Hb), red blood cell (RBC) and white blood cell (WBC) count by standard procedures (D’Armour et al., 1965; Wintrobe et al., 1961).

**Hemoglobin (Hb) content**

0.1 ml of heparinized blood was taken in Sahlis Hemoglobinometer and diluted with 0.1N HCL until the color matched with standard. The reading was taken from the graduated cylinder and expressed as g/dL.

**Red blood cell (RBC) count**

**Procedure**

1) Clean and dry mixing pipette (dilution 1:200)
2) The blood was sucked with RBC pipette unto the 0.5 mark slowly and carefully
3) Immediately mixed with RBC diluting fluid and sucked in RBC pipette the diluting blood unto 101 marks. Diluting fluid prevents haemolysis, rouleaux formation, coagulation and bacterial growth.
4) Quickly run the diluting blood under the cover glass on to the central platform of Neubauer’s chamber.
5) Counted the cells in 16 squares in five different parts of field

**Calculation:**

\[
\text{Number of RBC per cubic mm} = \frac{\text{Number of cells counted} \times \text{dilution (200)}}{\text{area} \times 0.1 \times \text{number of small squares counted}}
\]

\[
= \frac{\text{Number of cells counted} \times 200}{0.0025 \times 0.1 \times 80}
\]

\[
= \text{Number of cells counted} \times 10000
\]
White blood cells (WBC) count

Procedure

1) The blood was sucked with WBC pipette upto the 0.5 mark slowly and carefully
2) Immediately mixed with WBC diluting fluid 20 times and sucked in WBC pipette the diluting blood upto 11 mark. Diluting fluid prevents haemolysis, rouleaux formation, coagulation and bacterial growth.
3) Rotated the pipette slowly so as to allow the blood to mix with diluting solution.
4) Quickly run the diluting blood under the cover glass on to the central platform of Neubauer’s chamber.
5) Counted the cells in the four corners of 1 sq. mm.

Calculation

\[
\text{Number of WBC per cubic mm} = \frac{\text{Number of cells counted} \times \text{dilution (20)}}{\text{area} \times 0.1 \times \text{number of 1 sq. mm counted}}
\]

\[
= \frac{\text{Number of cells counted} \times 20}{1 \times 0.1}
\]

\[
= \text{Number of cells counted} \times 20 \times 10
\]

Serum biochemical parameters

The blood samples were allowed to clot and the serum was separated by centrifugation at 5000 rpm for 10 min. Serum was utilized for the estimation of various biochemical parameters like total protein, serum glutamic oxaloacetate transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT) and alkaline phosphatase (ALP). All the analysis was performed by using commercially available kits manufactured by Span Diagnostics Ltd, Surat, India.

Tissue antioxidant assay parameters

The tissue antioxidant assay was performed with liver and kidney tissues and evaluation was carried out by measuring the level of lipid peroxidation (Ohkawa et al., 1979), the amount of enzymatic antioxidants Catalase (Aebi, 1974) and superoxide dismutase (Kakkar et al., 1984) and nonenzymatic antioxidant system such as reduced glutathione (Ellman, 1959).

About 200 mg of liver and 100 mg of kidney were homogenized separately in 10 ml and 5 ml of tris buffer (20mM, pH-7.0) and centrifuged at 12000 rpm for 30 min at 4 °C. The supernatant was collected and used for the estimation of said parameters.
Lipid peroxidation (LPO)

The level of thiobarbituric acid reactive substances (TBARS) in the liver and kidney tissue were measured as per reported method (Ohkawa et al., 1979). About 500 μl of tissue homogenate was mixed with PBS (0.02 M, pH-7.4) and TCA (10% w/v) and kept at room temperature for 30 minutes. Then the mixture was centrifuged at 3000 rpm for 10 minutes. 1 ml of supernatant was mixed with TBA (1% w/v) and heated at water bath (95 °C) for 1 hour until a stable pink color formed. Absorbance was measured at 532 nm.

Reduced glutathione (GSH)

Reduced glutathione level of liver and kidney tissue was determined as per reported method (Ellman et al., 1959). Briefly, 100 μl of tissue homogenate and 1.3 ml of EDTA (0.02 M) were mixed and kept on ice bath for 10 minutes. Then 1 ml distilled water and TCA (50%) were added and again kept on ice bath for 10 minutes. After that reaction mixture was centrifuged at 3000 g for 15 minutes. 1 ml of supernatant was mixed with 0.4 M tris buffer (pH-8.9) followed by DTNB (0.01 M) and absorbance was measured at 412 nm. Standard graph was drawn from the different concentration of GSH solution and GSH contents in liver and kidney homogenates were calculated from this graph.

Catalase (CAT)

Catalase activity was assayed according to the reported method (Aebi et al., 1974). Reaction mixture contents 100 μl tissue homogenates, 20 mM H₂O₂ and phosphate buffer (500 mM, pH-7.0). The estimation was done spectrophotometrically following the decrease in absorbance at 240 nm. The specific activity of catalase (CAT) was expressed in terms of μmoles of H₂O₂ consumed/min/mg of tissue.

Superoxide dismutase (SOD)

Superoxide dismutase level in liver and kidney tissue was assayed as per reported method (Kakkar et al., 1984). 1 ml of nitroblue tetrazolium solution (156 μg NBT in 100 mM Phosphate buffer, pH 7.4), 1 ml NADH solution (468 μg in 100 mM PBS, pH 7.4) and 0.1 ml of tissue homogenates were mixed well. The reaction started by adding 100 μg of phenazine methosulphate (PMS) solution (60 μg NBT in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min and the absorbance was measured at 560 nm. SOD activity was expressed as units/mg of tissue.
Statistical analysis

All the experimental data are expressed as the mean ± standard error of mean (SEM). Data was statistically analyzed by using one way analysis of variance (ANOVA) followed by Dunnett’s post hoc test by Instat software (version 4). \( p < 0.05 \) and \( p < 0.01 \) were considered as statistically significant and highly significant, respectively.

Results

Tumor growth and survival parameters

Antitumor activity of MEAC (200 and 400 mg/kg) and BSSG (50 and 100 mg/kg) against EAC and DLA tumor bearing mice was assessed by the parameters such as tumor volume, tumor weight, cell count (viable and non-viable), mean survival time and % increase in life span. The tumor volume, tumor weight and viable cell count were found to be increased and non-viable cell count was declined in EAC control animals (Table 6.1). Administration of MEAC and BSSG significantly \( (p < 0.01) \) decreased the tumor volume, tumor weight and viable cell count. Non-viable cell count was significantly \( (p < 0.01) \) higher in MEAC and BSSG treated animals when examined with respect to EAC control animals. The median survival time of EAC was 20.5 days which was increased to 29.5 \( (% \text{ ILS} = 43.90) \) and 36.0 \( (% \text{ ILS} = 75.61) \) days on administration of MEAC in a dose dependant manner. Furthermore, the median survival time was increased to 31.0 \( (% \text{ ILS} = 51.20) \) and 37.5 \( (% \text{ ILS} = 82.92) \) days on administration of BSSG in a dose dependant manner.

The tumor volume, tumor weight and viable cell count were found to be increased and non-viable cell count was declined in DLA control animals (Table 6.2). Treatment with MEAC and BSSG significantly \( (p < 0.05, p < 0.01) \) decreased the tumor volume, tumor weight and viable cell count. Non-viable cell count was significantly \( (p < 0.01) \) higher in MEAC and BSSG treated animals when examined with respect to DLA control animals. The median survival time of DLA was 20 days which was increased to 28.0 \( (% \text{ ILS} = 40.00) \) and 33.5 \( (% \text{ ILS} = 67.50) \) days on administration of MEAC in a dose dependant manner. Furthermore, the median survival time was increased to 29.5 \( (% \text{ ILS} = 47.50) \) and 36.0 \( (% \text{ ILS} = 80.00) \) days on administration of BSSG in a dose dependant manner.
Table 6.1: Effect of MEAC and BSSG on tumor volume, tumor weight, viable and nonviable cell count, median survival time (MST) and percentage increase in life span (% ILS) in EAC cells bearing mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor volume (ml)</th>
<th>Tumor weight (g)</th>
<th>Viable cell (\times 10^7) cell/ml</th>
<th>Nonviable cell (\times 10^7) cell/ml</th>
<th>MST (days)</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAC control (2\times10^6) cells/mouse</td>
<td>2.92 ± 0.20</td>
<td>2.75 ± 0.25</td>
<td>8.35 ± 0.31</td>
<td>0.33 ± 0.05</td>
<td>20.5</td>
<td>00</td>
</tr>
<tr>
<td>EAC + MEAC (200) mg/kg</td>
<td>1.76 ± 0.18*</td>
<td>1.30 ± 0.14*</td>
<td>3.61 ± 0.19*</td>
<td>1.23 ± 0.12*</td>
<td>29.5</td>
<td>43.90</td>
</tr>
<tr>
<td>EAC + MEAC (400) mg/kg</td>
<td>1.18 ± 0.17*</td>
<td>0.95 ± 0.09*</td>
<td>1.36 ± 0.18*</td>
<td>2.53 ± 0.22*</td>
<td>36.0</td>
<td>75.61</td>
</tr>
<tr>
<td>EAC + BSSG (50) mg/kg</td>
<td>1.53 ± 0.67*</td>
<td>1.13 ± 0.32*</td>
<td>3.08 ± 1.17*</td>
<td>2.03 ± 0.59*</td>
<td>31.0</td>
<td>51.20</td>
</tr>
<tr>
<td>EAC + BSSG (100) mg/kg</td>
<td>0.98 ± 0.27*</td>
<td>0.88 ± 0.17*</td>
<td>1.08 ± 0.19*</td>
<td>2.84 ± 0.52*</td>
<td>37.5</td>
<td>82.92</td>
</tr>
<tr>
<td>EAC + 5-FU (20) mg/kg</td>
<td>0.63 ± 0.07*</td>
<td>0.61 ± 0.10*</td>
<td>0.80 ± 0.06*</td>
<td>3.03 ± 0.15*</td>
<td>40.5</td>
<td>97.56</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM, where n = 6 mice in each group. \* treated groups vs EAC control group, \# p <0.05, \*p <0.01.

Table 6.2: Effect of MEAC and BSSG on tumor volume, tumor weight, viable and nonviable cell count, median survival time (MST) and percentage increase in life span (% ILS) in DLA cells bearing mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor volume (ml)</th>
<th>Tumor weight (g)</th>
<th>Viable cell (\times 10^7) cell/ml</th>
<th>Nonviable cell (\times 10^7) cell/ml</th>
<th>MST (days)</th>
<th>% ILS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLA control (2\times10^6) cells/mouse</td>
<td>2.99 ± 0.40</td>
<td>2.85 ± 0.21</td>
<td>9.12 ± 0.45</td>
<td>0.38 ± 0.07</td>
<td>20.0</td>
<td>00</td>
</tr>
<tr>
<td>DLA + MEAC (200) mg/kg</td>
<td>1.96 ± 0.28#</td>
<td>1.93 ± 0.18#</td>
<td>3.99 ± 0.39*</td>
<td>1.24 ± 0.15*</td>
<td>28.0</td>
<td>40.00</td>
</tr>
<tr>
<td>DLA + MEAC (400) mg/kg</td>
<td>1.26 ± 0.15*</td>
<td>0.91 ± 0.07*</td>
<td>1.76 ± 0.28*</td>
<td>3.03 ± 0.72*</td>
<td>33.5</td>
<td>67.50</td>
</tr>
<tr>
<td>DLA + BSSG (50) mg/kg</td>
<td>1.83 ± 0.24#</td>
<td>1.22 ± 0.18*</td>
<td>3.44 ± 0.91*</td>
<td>2.07 ± 0.29*</td>
<td>29.5</td>
<td>47.50</td>
</tr>
<tr>
<td>DLA + BSSG (100) mg/kg</td>
<td>1.18 ± 0.44*</td>
<td>0.99 ± 0.11*</td>
<td>1.52 ± 0.51*</td>
<td>2.69 ± 1.02*</td>
<td>36.0</td>
<td>80.00</td>
</tr>
<tr>
<td>DLA + 5-FU (20) mg/kg</td>
<td>0.78 ± 0.17*</td>
<td>0.81 ± 0.13*</td>
<td>0.84 ± 0.09*</td>
<td>3.53 ± 0.35*</td>
<td>41.0</td>
<td>105.0</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM, where n = 6 mice in each group. \* treated groups vs DLA control group, \# p <0.05, \*p <0.01.
Hematological parameters

Table 6.3: Effect of MEAC and BSSG on haematological parameters like RBC, WBC and Hb content in EAC cells bearing mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>RBC (cells × 10⁶/µl)</th>
<th>WBC (cells × 10³/µl)</th>
<th>Hemoglobin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (5ml/kg)</td>
<td>6.03 ± 0.08</td>
<td>5.16 ± 0.98</td>
<td>12.33 ± 1.21</td>
</tr>
<tr>
<td>EAC control (2×10⁶ cell/mouse)</td>
<td>2.26 ± 0.40 a,*</td>
<td>11.16 ± 0.75 a,*</td>
<td>5.25 ± 0.61 a,*</td>
</tr>
<tr>
<td>EAC + MEAC (200 mg/kg)</td>
<td>4.11 ± 0.28 b,*</td>
<td>8.33 ± 0.51 b,*</td>
<td>8.16 ± 0.98 b,*</td>
</tr>
<tr>
<td>EAC + MEAC (400 mg/kg)</td>
<td>5.06 ± 0.22 h,*</td>
<td>6.36 ± 1.16 h,*</td>
<td>10.91 ± 0.80 h,*</td>
</tr>
<tr>
<td>EAC + BSSG (50 mg/kg)</td>
<td>4.31 ± 0.9 h,*</td>
<td>7.19 ± 1.01 h,*</td>
<td>9.83 ± 1.04 h,*</td>
</tr>
<tr>
<td>EAC + BSSG (100 mg/kg)</td>
<td>5.16 ± 0.42 h,*</td>
<td>6.13 ± 0.91 h,*</td>
<td>11.13 ± 1.11 h,*</td>
</tr>
<tr>
<td>EAC + 5-FU (20 mg/kg)</td>
<td>5.65 ± 0.44 h,*</td>
<td>5.81 ± 0.32 b,*</td>
<td>11.83 ± 0.98 b,*</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM, where n = 6 mice in each group. a EAC control group vs normal group, b treated groups vs EAC control group, * p < 0.01.

Table 6.4: Effect of MEAC and BSSG on haematological parameters like RBC, WBC and Hb content in DLA cells bearing mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>RBC (cells × 10⁶/µl)</th>
<th>WBC (cells × 10³/µl)</th>
<th>Hemoglobin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control (5ml/kg)</td>
<td>6.03 ± 0.08</td>
<td>5.16 ± 0.98</td>
<td>12.33 ± 1.21</td>
</tr>
<tr>
<td>DLA control (2×10⁶ cell/mouse)</td>
<td>2.16 ± 0.60 a,*</td>
<td>10.16 ± 1.05 a,*</td>
<td>5.12 ± 0.91 a,*</td>
</tr>
<tr>
<td>DLA + MEAC (200 mg/kg)</td>
<td>3.81 ± 0.18 b,*</td>
<td>7.43 ± 0.31 b,*</td>
<td>9.05 ± 0.90 b,*</td>
</tr>
<tr>
<td>DLA + MEAC (400 mg/kg)</td>
<td>4.66 ± 0.42 h,*</td>
<td>6.13 ± 0.86 h,*</td>
<td>9.91 ± 0.80 h,*</td>
</tr>
<tr>
<td>DLA + BSSG (50 mg/kg)</td>
<td>4.22 ± 1.09 h,*</td>
<td>7.29 ± 1.51 h,*</td>
<td>9.55 ± 1.84 h,*</td>
</tr>
<tr>
<td>DLA + BSSG (100 mg/kg)</td>
<td>4.92 ± 0.42 h,*</td>
<td>6.03 ± 0.77 h,*</td>
<td>10.83 ± 1.11 h,*</td>
</tr>
<tr>
<td>DLA + 5-FU (20 mg/kg)</td>
<td>5.43 ± 0.44 h,*</td>
<td>5.71 ± 0.32 b,*</td>
<td>11.66 ± 1.49 h,*</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SEM, where n = 6 mice in each group. a DLA control group vs normal group, b treated groups vs DLA control group, * p < 0.01.

There was significantly (p <0.01) elevated WBC count and reduced level of RBC and hemoglobin (Hb) in EAC and DLA control group as compared to normal control group (Table
6.3 and Table 6.4). But, treatment with MEAC (200 and 400 mg/kg) and BSSG (50 and 100 mg/kg) in EAC and DLA cells bearing mice significantly \( (p < 0.01) \) increased both the RBC count and Hb content while WBC count was reduced significantly \( (p < 0.01) \) as compared with the EAC and DLA control group.

**Serum biochemical parameters**

![Bar charts showing serum biochemical parameters](image)

**Figure 6.4:** Effect of MEAC and BSSG on serum biochemical parameters such as Total protein (A), SGOT (B), SGPT (C) and ALP (D) in EAC bearing mice. Values are represented as mean ± SEM, where \( n = 6 \). \( ^{a} \) EAC control group vs normal control group, \( ^{b} p < 0.01; \) \( ^{b} \) All treated groups vs EAC control group, \( ^{*} p < 0.01. \)

Biochemical parameters like SGOT, SGPT and ALP level were significantly \( (p < 0.01) \) increased and total protein content was found to be significantly \( (p < 0.01) \) declined in EAC and DLA control when compared with normal group. Administration of MEAC and BSSG
significantly \((p < 0.01)\) increased total protein content as compared with both EAC (Figure 6.4A) and DLA control (Figure 6.5A). Treatment with MEAC and BSSG in EAC and DLA bearing mice significantly \((p < 0.01)\) decreased the SGOT, SGPT and ALP level in a dose dependant manner as compared to EAC (Figure 6.4B, C and D) and DLA control (Figure 6.5B, C and D).

**Figure 6.5:** Effect of MEAC and BSSG on serum biochemical parameters such as Total protein (A), SGOT (B), SGPT (C) and ALP (D) in DLA bearing mice. Values are represented as mean ± SEM, where \(n = 6\). \(^a\) DLA control group vs normal control group, \(^\# p < 0.01\); \(^b\) All treated groups vs DLA control group, \(^* p < 0.05\), \(^*\* p < 0.01\).

**Tissue antioxidant assay parameters**

Cancer leads to lipid peroxidation in liver and kidney tissues due formation of ROS species and subsequently elevate the level of malondialdehyde (MDA). In present study the MDA level were significantly \((p < 0.01)\) increased in EAC and DLA control animals when compared with normal control animals. Interestingly, treatment with MEAC and BSSG
significantly ($p < 0.05$, $p < 0.01$) reduced the MDA levels as compared with EAC control (Figure 6.6A) and DLA control group (Figure 6.7A).

The levels of Catalase, reduced GSH and SOD were significantly ($p < 0.01$) decreased in EAC and DLA control group when compared with normal control group. Administration of MEAC and BSSG in a dose dependent manner significantly ($p < 0.05$, $p < 0.01$) raised the catalase, reduced glutathione and SOD levels as compared with EAC (Figure 6.6B, C and D) and DLA control group (Figure 6.7B, C and D).

**Figure 6.6:** Effect of MEAC and BSSG on tissue antioxidant defense parameters like Lipid peroxidation (A), Reduce glutathione (B), Catalase (C) and Superoxide dismutase (D) in EAC bearing mice. Values are represented as mean ± SEM, where n = 6. $^a$ EAC control group vs normal control group, $^# p < 0.01$; $^b$ All treated groups vs EAC control group, $^{*}p < 0.01$. 

![Graphs showing effects of MEAC and BSSG on antioxidant parameters](image-url)
Figure 6.7: Effect of MEAC and BSSG on tissue antioxidant defense parameters like Lipid peroxidation (A), Reduce glutathione (B), Catalase (C) and Superoxide dismutase (D) in EAC bearing mice. Values are represented as mean ± SEM, where n = 6. \( ^a \) DLA control group vs normal control group, \( ^# \) \( p < 0.01; ^b \) All treated groups vs DLA control group, \( ^# \) \( p < 0.05, ^* \) \( p < 0.01 \).

Discussion

The present study was carried out to evaluate the antitumor activity of MEAC and BSSG in EAC and DLA cells bearing mice. The EAC and DLA cells were initially described as a spontaneous murine mammary rapidly growing adenocarcinoma with very aggressive behavior and can proliferate in almost all strains of mice (Karmakar et al., 2013). The EAC and DLA tumor implantation induces per se a local inflammatory reaction, with increasing vascular permeability, which results in an intense edema formation, cellular migration, progressive ascitic
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fluid formation which is essential for tumor growth since it constitutes a direct nutritional source for tumor cells. From the present experiment, it is limpid that treatment with MEAC (200 and 400 mg/kg) and BSSG (50 and 100 mg/kg) significantly reduced the tumor volume, tumor weight, tumor cell count (viable and non-viable) when compared to the tumor control group. These results could connote either a direct cytotoxic effect of MEAC and BSSG on tumor cells or an indirect local effect, which may involve macrophage activation and vascular permeability inhibition ((Haldar et al., 2010).

Prolongation of the animal life span was being considered a reliable criterion for the depiction of efficacy of an anticancer agent (Bala et al., 2010). The increase of life span of tumor bearing mice by reduction of nutritional fluid volume and seization of the tumor growth is a positive result and further corroborates the antitumor effect of MEAC and BSSG.

Major problems encountered in cancer chemotherapy are myelosuppression and anemia. The anemia exhibited in tumor bearing mice is mainly due to reduction of RBC or hemoglobin percentage and etiology is either iron deficiency or hemolytic/myelopathic conditions (Haldar et al., 2010). Pharmacotherapy with MEAC and BSSG replenishes the hemoglobin (Hb) content, RBC and WBC count towards the normal levels. It is evident from the result that MEAC and BSSG possess protective action on hemopoietic system.

From many years, serum enzymes have been studied as both early possible indicators of neoplasia and as an aid in following the progression and regression of disease. In certain circumstances they can be carcinogenic and may engender hepatotoxicity (Karmakar et al., 2013, Kathiriya et al., 2010). Furthermore, results of the experiments concluded that EAC and DLA control group exhibited raised levels of liver enzymes such as SGOT, SGPT, ALP and the levels of total protein were declined due to hepatocellular damages. Treatment with MEAC and BSSG restored the elevated biochemical parameters almost within the normal range, indicating the protection against tumor cell induced hepatotoxicity.

The oxidative stress may leads to damage of the macromolecules such as lipids and can induce lipid peroxidation in vivo (Haldar et al., 2010). In EAC and DLA cells bearing mice, the level of lipid peroxide in liver were significantly raised, which was however reduced near to normal level in MEAC and BSSG treated groups. This reflects the decline in free radical production and subsequent reduction in oxidative stress, which are the main risk factors of the ailment.
Glutathione (GSH), a potent inhibitor of neoplastic proliferation process, plays a crucial role as an endogenous antioxidant system. It was found particularly in high concentration in liver and is known to have key function in the protective process (Karmakar et al., 2013). The level of GSH was depleted in EAC and DLA induced mice which probably due to its utilization by the excessive amount of free radicals. Treatment with MEAC and BSSG was found to raise the GSH content in the liver and kidney as compared to EAC and DLA control animals.

Cells are also equipped with enzymatic antioxidant mechanisms that play an imperative role in the elimination of free radicals. It has been reported that a decrease in SOD activity in EAC and DLA cells bearing mice may be due to loss of Mn$^{2+}$ containing SOD activity and the loss of mitochondria which finally leads to a decrease in total SOD activity in liver (Haldar et al., 2010, Rushmore and Picket, 1993). The inhibition of both SOD and CAT activities as a result of tumor growth was also reported (Karmakar et al., 2013). The administration of MEAC and BSSG not only significantly elevated the SOD and CAT levels in a dose dependent manner but also restores the lipid peroxide and GSH content near to normal level; which indicates the antioxidant and free radical scavenging property of MEAC and BSSG.

Conclusion

Present investigation is quite encouraging as it explores that methanol extract of *Anthocephalus cadamba* and β-sitosterol glucoside possesses significant inhibitory effect on the proliferation of cancer cells. MEAC and BSSG have potent antitumor activity on cancer cells. Phytochemical study confirmed the presence of compounds namely triterpenoid, glycosides, saponins, alkaloids and flavonoid which may possess this property. Cytotoxicity is one of the main targets to produce antitumor activity and a number of anticancer drugs possess significant cytotoxic activity. It was found that both the compound possess direct cytotoxicity effect towards EAC and DLA cells. The mechanistic studies needs to be further clarified and established scientific evidence for the antitumor activity of MEAC and BSSG.

The methanol extract of *Anthocephalus cadamba* and β-sitosterol glucoside showed better antitumor effect on Ehrlich ascites carcinoma than Dalton’s lymphoma ascites cells. Therefore, further investigations are being carried out to trace the exact antitumor mechanistic pathways with MEAC and BSSG on EAC cells.
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References


