CHAPTER 6
EFFECT OF INDUKANTHA GHRITHA IN TUMOR BEARING ANIMALS: ANTICANCER STUDIES
6.1. INTRODUCTION

Cancer is a serious health issue that affects each and every one of us in some way, whether it be directly or indirectly and the second largest killer disease of the humans. It is a disease with high morbidity and mortality. Cancer does not represent a single disease. Rather it is a group of diseases with as much different manifestation as there are tissues and cell types in the human body. It is now recognized that cancer, in its simplest form is a genetic disease, or more precisely a disease of abnormal gene expression (Woodhouse, E. C *et al*, 1997). Cancer refers to the hyperproliferation of cells that have lost the ability to be controlled by normal cell signals. Cancer cells have the ability to proliferate independent of their environment and are capable of metastasizing, or colonizing other tissues in the body. Recent research efforts have revealed that different forms of cancer share common molecular mechanism governing uncontrolled cellular proliferation, involving loss, mutation etc (Folkman J,1992).

![Diagram of cancer](image)

Figure 6.1. Acquired capabilities of cancer
The basic characteristics of cancer cells are uncontrolled growth (immortality), abnormal DNA (Mutations), inhibition of apoptosis, lack of differentiation, lack of anchorage dependence. Cancerous cells fail to coordinate to and may cause destruction to neighbouring normal cells. Compared to normal cell, cancer cell have a relatively larger nucleus and smaller cytoplasmic region. The smaller amount of cytoplasm is due to rapid cell division, whereas the daughter cells have insufficient time to synthesize new cytoplasm. Cancerous cells also fail to differentiate into specific cell types and fail to induce apoptosis (Bishop, J.M., and Weinberg, R.A., 1996)

6.1.1. Causes of cancer

The cause of cancer cell development is damage to DNA under normal circumstances, when DNA gets damaged; either the cell dies by apoptosis or the DNA is able to repair itself. In cancer cells, the damaged DNA is not repaired and the apoptotic pathways are disturbed (Bouck, N et al, 1996). It is more likely that some factor increases the susceptibility of cells to other inherent (endogenous) and external (exogenous), factor (environmental, cultural, and socioeconomic) and may push cells one or several steps towards malignancy. It has also been observed that cancer incidence varies greatly from one country to another. For example cancer in liver is more common in Africa than in Europe (Motola-Kuba D et al, 2006); Japan has a high incidence of stomach cancer as compared to the USA (Kurosawa M, 2006); cancer of the mouth is predominant in India (Patel MM and Pandya AN, 2006), bladder cancer is more common in Egypt (Khaled HM., 2005); whereas skin cancer is prevalent in countries with sunny climates. In some cases, specific environmental factors have been found to be associated with specific cancers; for example aflatoxin with cancer of the liver, the type of diet with cancers of the stomach, ultraviolet radiation in sunlight with skin cancer pan with cancer of the mouth etc. The incidence of these cancers is partly dependent on the sex, the genotype and the degree of the exposure to the environmental factors (Benigni R, 2006).
6.1.2. **Immune mechanisms in cancer**

The major function of the immune system is to destroy transformed cells before they can become tumors. This theory is known as immune surveillance. Cytotoxic T lymphocytes through TCR binds to HLA class 1 molecule carrying the tumour peptide and subsequent linking of CD8 receptor on the cytotoxic T cells to HLA molecule of the target T cell is necessary to lyse the target T cell. The stability of T cells to react against the tumour cell depends on the MHC molecule on the tumour cells (Park B, 2006). Tumour antigen specific immunoglobulin are secreted by activated B cells through the IL-2 released by the Helper T cells and they bind to the specific antigens. Since the tumour cells can lose the surface antigen, the antigen-antibody thus generated leaves the cancer cell. These complexes along with free tumour antigen specific antibodies block the antigens of tumour cells to be recognized by the receptors of cytotoxic T-cells and thus protect from cytolysis. (Uhlin M et al, 2006), (Offringa R, 2006).

![Diagram showing the interaction between T cells and tumour cells](image)

**Figure 6.2.** How the T cell destroys cancer

Macrophages play an important role in tumour mechanisms. Tumour cells secrete a macrophage migration inhibition factors (MIF) that brings the macrophage closer to the tumour cells (Yan X et al, 2006). The macrophage near tumour produces large quantities of plasminogen activators that helps the entry of tumour cells into the blood vessel and thus tumour spreads. Macrophages activated by T cells kill tumour cells by producing nitric oxide. NK cells take part in immunesurveillance by
recognizing fewer targets, tumour containing high densities of tumour specific antigens and cells that express low levels or exhibit absence of MHC class 1 expression on their membrane (Poggi A and Zocchi MR, 2006). NK cells release cytokines like IFNα and β, TNFα, IL-1, IL-4 and colony stimulating factors which perform many antimicrobial functions through their action on other cells. T-cells derived cytokines can activate the killer cells to perform their cytotoxic function and are called lymphokine activated killer cells (Valenti R et al, 2006).

6.1.3. Programmed cell death (apoptosis)

The word 'apoptosis' is derived from Greek word meaning 'falling leaves' and was first used to describe new form of cell death distinct from necrosis in the early 1970s. Apoptosis is defined as programmed cell death, and involves the systematic disassembly of a cell. The main function of apoptosis is to dispose of a cell without causing damage or stress to neighbouring cells. There are several situations in which apoptosis are a desirable activity for cells in a multicellular organism: In every human, 10 billion cells must die every day to balance the 10 billion produced in mitosis. Apoptosis is a major cell regulatory mechanism, for which cancer has resistance. Cancer cells fail to undergo apoptosis, and therefore exhibit increased life spans compared to their normal counterparts (Kim R et al, 2006).

![Figure 6.3. Balance between cell replication and cell death.](image)

Harmonious survival of the entire organism depends on the rigorous preservation of the controls that regulate the balance between the cell’s replication and apoptosis. This balance depends largely, although not exclusively, on the integrity of the genome. Cancer is a flagrant illustration of the danger of imbalance between cell proliferation and cell death. As cells replicate unfettered by normal
controls, they invade the surrounding healthy population of cells, and they colonize distant organs. (Ashkenazi A. and Dixit V M, 1999).

Apoptosis is also induced by exposure of cells to various agents and aberrant physiological conditions. Differentiation inducing agents such as retinoid and sodium butyrate are known to induce apoptosis (Sadie and Hager GL, 1994). The DNA fragmentation associated with apoptosis is a Ca\(^{2+}\) dependent process (Nawaz M et al, 2006) have claimed that calcium ionophores induce apoptosis and calcium chelators suppress it.

Figure 6.4. Pathway involved in apoptosis

6.1.4. The nature of apoptotic change

Cells undergoing apoptosis display distinctive morphological changes involving the condensation of nuclear chromatin and shrinkage of the cytoplasm. The cells break up into apoptotic bodies comprising membrane bound cell fragments. The major biochemical feature of the apoptotic process is the fragmentation of the nuclear DNA by Ca\(^{2+}\)-Mg\(^{2+}\)dependent endonucleases, which cleaves the DNA at intranucleosomal sites. The fragmentation of the DNA is visualized in agarose gel electrophoresis by the ladder pattern of 180-200bp oligomers (Wyllie A H, 1993).
In the life of a multicellular organism, apoptosis is used for many purposes. During development, it is used to remodel tissues and remove cells that are no longer needed (Rosfjord EC and Dickson RB, 1999). During postnatal life, apoptosis is used to eliminate unnecessary or unwanted cells in the immune system (Foster J.R, 2000). Intracellular signals such as DNA damage or severe metabolic imbalance can trigger apoptosis by inducing the release of proapoptotic molecules from the intermembrane compartment of mitochondria (Hengartner M O, 2000), (krammer P H, 2000). These include cytochrome C, a flavoprotein known as apoptosis-inducing factor (AIF).

Failure of apoptosis can lead to disease hence apoptosis requires tight regulation. Lack of such control, leading to either too much or too little apoptosis can have pathological consequences: Cancer— it is now believed that some cancers are caused by cells not dying, rather than a greater rate of proliferation. The role of apoptosis implicates immense potential to treat various diseases (Meiler J and Schuler M, 2006), (Kim R et al, 2006). Research is already underway to harness apoptosis as a therapeutic tool in modern medicine to control of cancer.
6.1.5. Cancer- An Ayurvedic Perspective

An integrated approach is needed to manage cancer using the growing body of knowledge gained through scientific developments. Thousands of herbal and traditional compounds are being screened worldwide to validate their use as anticancer drugs. Review of literature on anticancer drugs of plant origin revealed identification of newer ayurvedic drugs that are not mentioned in the ancient texts. The details of experimental and clinical studies conducted on single and compound ayurvedic preparations for their anticancer efficacy strongly emphasize ayurvedic therapy as a scientifically driven one and not simply unconventional (Balachandran P and Govindarajan R, 2005).

6.1.6. Clinical importance of herbal medicines

The use of herbs for medical benefit has played an important role in nearly every culture on earth. Certain plants have provided active principles, used to control advanced stages of several malignancies in clinical settings (Kinghorn A.D et al, 2004). Determining the pharmacological mechanisms of herbal medicines presents certain challenges distinct from the study of synthetic drugs. For eg, synthetic drugs are studied in isolation; whereas herbal medicines often contain multiple active substances that act in combination. Herbs contain a vast spread of pharmacologically-active ingredients and each herb has its own unique combination and properties. More than 80 percent of the people in South Asia rely on herbal remedies as a principle means of preventing and curing illnesses, and several traditional medical systems are based on the use of plants. There are several advantages to such systems: the plants involved are readily available, are easy to transport, and do not spoil quickly. Remedies based on these plants often have minimal side effects, and the relatively high cost of synthetic medicines in developing countries often makes traditional herbal medicines an affordable choice for the poor in these lands (Block KI and Mead MN, 2003).
6.1.7. Herbal aids for cancer

Cancer treatments are a paradox, on one hand delivering powerful toxicity to a tumor, but on the other spreading toxicity to the rest of the body. The side effects of such treatments may cause death even before the cancer does. Herbal drugs have unique effect on physiology and can reduce the side effects of cancer treatments, while at the same time increasing their effectiveness. These drugs repair damage to the body by stimulating the immune system to restore optimal function (Efferth T et al, 2001).

Many herbs have long been known to affect the immune system, but only recently have scientists considered them as possible adjunct cancer therapies. Such herbs often prompt the body’s cells to secrete cytokines, which then enhance the immune response. The most promising of these herbs include black cumin, mistletoe, ginseng, green tea, garlic; astragalus etc. (el-Aziz MA et al, 2005), (Rostock M et al, 2005)

The herbal supplements are generally taken for two reasons that are to alleviate symptoms of illness or to prevent illness. In the second case, herbal products are taken specifically in the hopes of preventing disease. In the area of cancer prevention herbs may act through several mechanisms to provide protection. Besides that they are also have been tested as possible cancer therapies (Melnick SJ, 2006), (Yance DR Jr and Sagar SM., 2006).

Figure 6.6. Possible mechanisms for chemoprevention by herbal medicines.
6.1.8. Potential benefits of Ayurveda during Cancer

Ayurvedic herbs used in cancer therapy results not only in total healing, but also reduce the side effects and cancer associated complications. Each herbal product contains multiple active principles that may operate synergistically, producing therapeutic benefits and lowering the risks on adverse effects (Singh RH, 2002). The anorexia or weight loss could be effectively managed by *Plumbago zeylanica*, *Zingiber officinale*, etc. These herbs have been shown to improve appetite, food intake, malnutrition, fatigue and sensation of well-being which could elicit bodyweight gain. These herbs might stimulate the flow of digestive juices, thereby improving digestion and increasing the appetite (Dai Y, 2004), (Sandur SK *et al*, 2006) (Yemitan OK and Izegbu MC, 2006), (Ihlaseh SM, 2006). *Aegle marmelos*, *Plumbago zeylanica* can be used as anti-diarrhoeals when diarrhoea becomes one of the complications of cancer. Use of specific herbs which are very helpful not only in preventing the progression of the disease but also makes the patients feel better and comfortable overcoming the symptoms (Tripathi YB *et al*, 2005). Thus, ayurvedic therapeutic regimen rejuvenates the body tissues, tones up the systems and act as a tonic to the body against cancer. This kind of orientation toward total healing and health promotion makes ayurvedic treatment approach to cancer therapy promising. In any case, studies on anticancer ayurvedic drugs will be popular from the economy point of view because cancer is becoming the major cause of death.

6.2. MATERIALS AND METHODS

Staining Solution

Acridine orange 6mg / ml
Citric acid 0.1M
Na₂HPO₄ 0.2 M pH 2.6
Prepare 90ml of citric acid solution; add acridine orange and 10ml Na₂HPO₄
Acridine Orange solution is stable for several weeks when stored at 4°C and in the dark.

6.2.1. Animals

Inbred strains of BALB/c mice weighing 20-25g were used for the present study.
6.2.2. Cell lines

EAC and DLA cells were maintained by transplanting the cells every two weeks as ascites in the peritoneal cavity of Balb/c mice.

6.2.3. Chemotherapy

Cyclophosphamide at a total dose of 250 mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg) was administered.

6.2.4. Tumor models

6.2.4.1. Ascites tumor model

Tumor cells were maintained and propagated by peritoneal inoculation of \(1 \times 10^6\) cells/mouse. The tumor cells were collected from the peritoneum in PBS in an aseptic condition and pelleted in cold centrifugation. The cells were counted in a hemocytometer. The EAC cells were allowed to grow in vivo for 14 days. At the end of the experimental period the animals were sacrificed. The mortality of the animals treated with and without drug was noted.

6.2.4.2. Solid tumor induction

For establishment of solid tumor, Dalton's lymphoma ascites cells (DLA) \((1 \times 10^6)\) were injected subcutaneously on the hind limb of mice. The perpendicular tumor diameters were measured at regular intervals and tumor volume was estimated. The average tumor volume was determined by measuring the 3 perpendicular diameters with calculation of the volume by the formula,

\[
\text{Tumor volume (V)} = (d_1 \times d_2 \times d_3) \pi/6.
\]

The difference of the tumor volume between the control and treatment group was examined by student's t-test.

6.2.5. Treatment protocol

6.2.5.1. Determination of effect of IG on solid tumor

All animals received DLA cells \((0.2\text{ml of } 1 \times 10^6 \text{ cells/mouse})\) injected subcutaneously on the hind limb of mice to develop solid tumor. Twenty four hours after tumor implantation, mice were randomized into four groups: Group I animals received tumor cell alone (DLA alone, \(n=8\)), Group II animals were treated with 250 mg/kg of IG for 28 days (DLA + IG, \(n=8\)), Group III animals were treated cyclophosphamide at a total dose of 250 mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg) was administered (DLA + CTX, \(n=8\)),
Group 1V animals were treated with cyclophosphamide at a total dose of 250 mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg) and also received IG with 250mg/kg for 28 days (DLA + CTX + IG).

6.2.5.2. Determination of effect of IG on ascites tumor

All animals received EAC cells (0.2ml of 1 x 10^6 cells/mouse) intraperitoneally. Twenty four hours after tumor implantation, mice were randomized into four groups: Group I animals received tumor cell alone (EAC alone, n =12), Group 11 animals were treated with 250mg/kg of IG for 14 days (EAC + IG, n =12), Group 111 animals were treated cyclophosphamide at a total dose of 250 mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg) was administered (EAC + CTX, n= 12), Group 1V animals were treated with cyclophosphamide at a total dose of 250mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg) and also received IG with 250mg/kg for 14 days (EAC + CTX + IG).

All animals were weighed at the start of the experiment. The dosing schedule for IG (250mg/kg body weight) was chosen after preliminary experiments demonstrated maximal antitumor activity without significant toxicity at this dose. At the end of the experimental period (15th day) animals from each group were sacrificed by cervical decapitation for anticancer studies. Four animals from each group were maintained for survival analysis.

6.2.5.3. Determination of effect of IG on immunological parameters in tumor bearing mice.

Six groups of animals were used in this study consisting of 8 animals in each group for both DLA induced solid and EAC induced ascites tumor. All the groups were administered with 250mg/kg of IG orally except Group I and Group 111. Group I served as Control animals received vehicle-plain ghee (control), Group 11 were administered with 250mg/kg of IG for 14 days (IG). Group 111 animals were injected with either DLA or EAC cells (DLA alone or EAC alone), Group 1V animals were administered with 250mg/kg of IG for 14 days and injected with either DLA or EAC cells (IG + DLA or IG +EAC). Group V animals were treated with cyclophosphamide at a total dose of 250mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and 100mg/kg on day 4 and injected with either DLA or EAC cells (CTX +DLA or
CTX +EAC). Group V1 animals were also treated with cyclophosphamide at the same time intervals as described earlier in tumor bearing animals (DLA and EAC) along with 250mg/kg of IG orally for 14 days (DLA+ CTX + IG or (EAC + CTX + IG). At the end of the experimental period (15th day) animals from each group were sacrificed by cervical decapitation for immunological studies in tumor bearing mice. Parameters such as total WBC count, Bone marrow cellularity and lymphocyte proliferative response, Immunophenotypic analysis of T cell subsets etc were evaluated on the 15th day as done in chapter 3.

6.2.6. Determination of WBC count in tumor bearing animals
As described previously in the chapter 3.

6.2.7. Determination of Bone marrow cellularity in tumor bearing animals
As described previously in the chapter 3.

6.2.8. Determination of lymphocyte proliferative response in tumor bearing animals
As described previously in the chapter 3.

6.2.9. Flow cytometric analysis of lymphocyte subsets in tumor bearing animals
As described previously in the chapter 3.

6.2.10. Cytokine analysis in tumor bearing mice
As described previously in the chapter 4.

6.2.11. Effect of IG on Tumor growth response
The antitumor effect of IG was assessed by change in the body weight, mean survival time (MST), percentage increase in life span (% ILS) and body weights were measured at regular intervals. Mice were maintained on regular diet and examined daily for evidence of tumor growth.

MST and ILS was calculated using following formula
MST= (Day of first death + Day of last death)/ 2
ILS (%) = [Mean survival time of treated group/ mean survival time of control group) – 1 x 100]

6.2.12. Detection of apoptosis in EAC tumor cells by Indukantha Ghritha
The detection of apoptosis in EAC cells by IG was determined using DNA fragmentation and acridine orange staining.
6.2.12.1. DNA fragmentation

Principle

Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of apoptosis. Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation and nuclear DNA fragmentation. Detection of DNA fragmentation is a widely accepted method to assay for apoptosis: DNA fragmentation can be visualized by agarose gel electrophoresis following DNA extraction.

Procedure

Briefly EAC (3 x 10⁶) cells were harvested from tumor bearing mice of different groups and centrifuged at 1000x for 10 minutes. The cell pellet were suspended in 1 ml of lysis buffer (5ml/L Triton X-100,10mmol/L EDTA and 10 mmol/L Tris-HCl, pH 8.0) and proteinase K 40ug/L at 37°C for 2 hours. The lysate was extracted with 0.5% NaCl 5 mol/L and 50% 2-propanol and incubated overnight at -20°C, and centrifuged at 7000x for 20 min. The supernatant was washed with 70% ethanol and centrifuged. The pellets were dried and suspended in Tris-EDTA buffer (10mmol/L Tris-HCl, pH 8.0 and 1 mmol/L EDTA). DNA was analysed after separation by gel electrophoresis (20g/L agarose).

Agarose Gel Electrophoresis

1. 1.2% agarose gel was prepared in 1X TBE buffer
2. The molten agarose was cooled to a temperature of 60°C. Ethidium bromide was added to the gel (from a stock solution of 10mg/ml), to obtain a final concentration of 0.5% ug/ml.
3. The gel was poured into the mold and the comb was placed immediately.
4. When the gel was completely set, it was mounted in the electrophoresis tank.
5. The comb was removed and enough electrophoresis buffer (1X TBE) was added to cover the gel.
6. 10ul of DNA sample was mixed with 3 ul of 1X gel loading dye and loaded to the wells.
7. 100 base pair molecular weight marker was also run along with the samples.
8. The gel was run at 80 V till the bromophenol blue migrated to an appropriate distance.

9. Gels were photographed using the Gel-Doc 1000 photo documentation system (Bio Rad, Philadelphia) and the resulting images were analyzed using quantity one software.

6.2.12.2. Acridine Orange Staining

Principle

Acridine orange (AO) is a commonly used dye to detect apoptosis. AO differentially stains double-stranded DNA to a green fluorescence (around 520 nm) and single-stranded DNA and RNA to an orange fluorescence (around 610 nm). This metachromatic fluorescence is sensitive to DNA conformation, making it a useful probe for detecting apoptotic cells. In mammalian cell culture, apoptotic cells stained by AO show reduced green fluorescence and enhanced orange fluorescence in comparison to normal cells. This is believed to result from the breakdown of DNA to fragments that can more easily denature and bind AO as single-stranded nucleic acid. The technique used to identify the type of cell death involved staining of chromosomal material in cells that had been fixed in 0.4% formaldehyde. Cells were cytospun on to glass slides and stained with acridine orange to detect apoptosis, followed by propidium iodide to detect necrosis.

Procedure

1. Wash cells \( (1 \times 10^6) \) in PBS and centrifuge at 200g for 5 min
2. Resuspend the cell pellet in 1 ml PBS
3. Fixed cells by transferring the cell suspension in 9 ml 1% Para formaldehyde in PBS, on ice. Incubate for 15 min on ice
4. Centrifuge at 200g for 5 min and resuspend the cell pellet in 5 ml PBS, centrifuge
5. Suspend the cell pellet in 1 ml PBS and transfer the suspension in 9 ml 70% ethanol, on ice
6. Incubate for 4 hours (The cells can be stored in ethanol for weeks)
7. Centrifuge at 200g for 5 min and resuspend the cell pellet in 1 ml PBS
8. After this add 2 ml AO staining solution
9. Observe the cells under fluorescence microscope with an appropriate filter set

6.3. RESULTS

The data obtained from the present study indicates that the IG showed significant anticancer activities against both solid and ascites tumor in Balb/c mice.

6.3.1. Effect of Indukantha Ghritha on body weight, mean survival time and percentage increase in life span of EAC (Ehrlich ascites carcinoma) induced ascites bearing Balb/c mice.

The average body weight of mice receiving IG treatment was significantly reduced suggesting that the amount of ascitic fluid produced in the mice of experimental groups were less than those of EAC alone. The body weight of tumor induced mice with IG was significantly decreased compared to control mice, suggesting IG influenced antitumor activity against ascites bearing mice. As depicted in figure 6.7 when IG was administered along with CTX a significant reduction in tumor growth and thereby reduction in body weight was observed.

Treatment with either IG or CTX alone led to a statistically significant, increase in survival in these animals relative to the EAC control group. However, treatment of the tumor-bearing animals with a combination of IG and CTX significantly improved the overall survival rate and increased the mean survival time from 17 days to 31 days (Table 6.1).

6.3.2. Effect of IG on DLA induced solid tumor in mice.

Inoculation of DLA cells subcutaneously on the hind limb resulted in solid tumor growth in all animals. Animals receiving IG or CTX treatment were compared with DLA control groups. The mean tumor size of the DLA group animals were compared with IG or CTX administered groups. The result of treatment on tumor growth is shown in Fig. 6.8. Tumors were measured with calipers from day 7, 14 and 21. Although mean tumor growth was initially equal in all groups, by day 14 a significant reduction in mean tumor size was noted in mice that received combined treatment of IG plus CTX. This reduction was greater than that seen with either treatment alone suggesting that IG and CTX act synergistically to reduce tumor growth.
Figure 6.7. Effect of Indukantha Ghritha on body weight in Ehrlich ascites carcinoma bearing Balb/c mice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean survival time (days)</th>
<th>Percentage increase in life span (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAC</td>
<td>17 ± 1.33</td>
<td>-</td>
</tr>
<tr>
<td>EAC + IG</td>
<td>25 ± 1.56</td>
<td>47</td>
</tr>
<tr>
<td>EAC + CTX</td>
<td>26 ± 2.10</td>
<td>52</td>
</tr>
<tr>
<td>EAC + IG + CTX</td>
<td>31 ± 1.18</td>
<td>82</td>
</tr>
</tbody>
</table>

Table 6.1. Effect of Indukantha Ghritha on mean survival time and percentage increase in life span of Ehrlich ascites carcinoma bearing Balb/c mice.
6.3.3. IG induces apoptosis in EAC cells.

DNA fragmentation assay and acridine orange staining was done to understand the nature cell death in EAC during IG treatment. Result shows that IG induces tumor killing by apoptosis characterized by cell shrinkage, chromatin compaction, plasma membrane blebbing and collapse of cell into small DNA fragments (apoptotic bodies) (figure 6.15A). We also found that IG induces DNA fragmentation (figure 6.15B).

The results from the present investigation have shown that IG effectively induces apoptosis in EAC cells and also stimulates immune response. Some changes of cellular shape were microscopically observed in EAC cells when cells were treated
with IG. IG showed growth inhibition and changed EAC cells into shrinking and multiblebbing shapes at various doses of IG. These morphological changes are typical of apoptosis. IG induced DNA laddering in EAC cells in concentration dependant manner. These DNA fragmentation and characteristic morphological changes indicated that growth inhibition of IG is mediated through apoptosis. Apoptotic and necrotic cells were identified morphologically using fluorescence microscopy. Percentage apoptosis and necrosis was calculated by counting 100 cells from non-overlapping views.

6.3.4. IG enhances the level of WBC count in both solid and ascites tumor.

Total number of leukocytes (cells/mm3) in peripheral blood of mice from each group were determined and compared with control group. As depicted in Figure 6.9, the total WBC count was found to be significantly decreased in the EAC and DLA control group in comparison to the normal mice (P<0.01). The results (Figure 6.9) showed complete inhibition of WBC count in the CTX treated tumor bearing group, but upregulation with IG and partial inhibition with CTX + IG. This would suggest that IG might be required for the complete recovery of total leukocyte count in both solid and ascites tumor after CTX treatment.

6.3.5. IG induces Bone marrow cellularity in solid and ascites tumor.

The dosing schedule for IG (250mg/kg body weight) was chosen after preliminary experiments demonstrated maximal antitumor and immunomodulatory activity without significant toxicity at this dose. Oral administration of IG significantly increased bone marrow cellularity in control mice. However CTX administration significantly reduced bone marrow cellularity in tumor bearing mice. As shown in figure 6.10 CTX treatment along with IG administration significantly restored the bone marrow cellularity to the normal level in tumor bearing mice.
6.3.6. IG induces lymphocyte proliferative response in solid and ascites tumor.

Lymphocyte proliferative response to PHA was significantly increased in control mice after IG treatment as showed in figure 6.11. However CTX treatment decreased stimulation index of lymphocytes. In addition, IG treatment was found be very effective in repairing the proliferative response of lymphocytes after CTX treatment in both solid and ascites tumor bearing mice.

6.3.7. Effect of IG on lymphocyte subsets in solid and ascites tumor model.

In the present study it was observed that oral administration of IG resulted an increase in CD3, CD4, CD8, CD19 CD56 cell populations in both solid (figure 6.13) and ascites (figure 6.12) tumor model. The result indicates that IG (250mg/kg for 14 days) stimulates the percentage of lymphocyte subsets were significantly increased in both the tumor model treated with CTX when compared with DLA control or EAC control. The results in the current study clearly indicate that oral administration of IG enhances immunomodulatory function in tumor bearing mice. The present study was done in order to evaluate the efficiency of IG as an adjuvant therapy with the conventional chemotherapeutic agents.
Figure 6.9. Effect of IG on total WBC count (cells/mm$^3$) in chemotherapy treated tumor bearing mice. Mice were treated with cyclophosphamide at a total dose of 250mg/kg intraperitoneal injections scheduled at day 1 (150 mg/kg) and day 4 (100 mg/kg). IG was administered orally at 250mg/kg for 14 days.
Figure 6.10. Effect of IG on bone marrow cellularity of control and tumor bearing mice.

Figure 6.11. Effect of IG on lymphocyte proliferative response in solid and ascites tumor models.
Figure 6.12. Effect of IG on lymphocyte subset analysis in EAC induced ascites tumor model. The percentage of lymphocyte subsets were determined by flowcytometric analysis. Results presented are the mean of three independent experiments.

Figure 6.13. Effect of IG on lymphocyte subset analysis in DLA induced solid tumor model. The percentage of lymphocyte subsets were determined by flowcytometric analysis. Results presented are the mean of three independent experiments.
6.3.8. Reverse of type 2 cytokine predominant status of solid and ascites tumor bearing mice after treatment Indukantha Ghritha.

The cytokine levels in solid and ascites tumor bearing mice was performed by ELISA method. As showed in Figure 6.14A (solid) and Figure 6.14B (ascites), the levels of type 1 cytokines, IFN-γ and IL-12 and GM-CSF in tumor bearing mice were significantly lowered when compared with those in control group. In contrast, the levels of type 2 cytokines, IL-4 in tumor bearing mice were significantly higher than those in control. These results further demonstrated that type 2 cytokine predominance was present in both solid and ascites tumor bearing mice. The results demonstrated that IG induces a Th1 cell-dominated immune response by decreasing IL-4 level and increasing the levels of IFN-γ in serum. This observation implies that the IG is effective in various diseases associated with Th2 cell development like cancer, allergic diseases etc.
Figure 6.14. Effect of IG on cytokine levels in DLA induced (A) solid tumor and (B) EAC induced ascites tumor model by ELISA method. * denotes significant difference from control group with $p \leq 0.01$. 
Figure 6.15(A) Detection of IG induced EAC apoptosis. For the determination of membrane blebbing, fragmentation, apoptotic bodies, EAC cells of (1) EAC control, (2) 30, (3) 200, (4) 250 mg/kg of IG treated mice were stained with acridine orange and visualized under a fluorescence microscope. (B) DNA fragmentation induced by IG in EAC cells. The DNA was isolated, electrophoresed on 2% agarose gel and then visualized by ethidium bromide staining. Lane 1: 100 base pair, Lane 2: EAC control, Lanes 3-5: EAC tumor bearing mice treated 30mg/kg, 100mg/kg and 250mg/kg of IG, respectively.
6.4. DISCUSSION

The elimination of transformed cells before they develop into tumors and the elimination of tumor cells is one of the major functions of the immune system. However, even though T cell dependent immunity against tumors exists, in many cases tumors are not eradicated by the immune system. The aim of cancer immunotherapy is therefore to exploit the capacity of the immune system to kill and eradicate tumor cells. There are several reasons, as to why immune responses frequently fail to prevent the growth of tumors. One major obstacle is the fact that tumor cells are derived from the host and resemble normal cells in many aspects. Thus, they express only few antigens that could possibly be recognized as non-self. Therefore, most tumors prove to be of very low immunogenicity. Only tumors that are induced by viral infections, and therefore bear foreign antigens, are able to elicit a potent immune response (Passalacqua R et al, 2006). Although the immune surveillance hypothesis, which states that the immune system can recognize and destroy transformed cells, has been generally confirmed, a majority of tumors induces very weak or even undetectable immunity. Thus, there is a need for the development of strategies to activate the immune system to effectively lyse tumor cells and eradicate tumors. In order to overcome these different types of immune evasion, strategies have been developed to actively stimulate the host immune response to the tumor (Betts GJ et al, 2006). This treatment would be of great advantage compared to the still unsatisfactory form of treatment with surgery, irradiation or chemical intervention. The classical pharmaceutical reagents, like for example alkylating agents and antimetabolites that unspecific target dividing cells, are necessary tools for tumor chemotherapy, but unfortunately cause severe side effects and are in many cases not able to heal the patients might provide an alternative way of cancer treatment without causing severe side effects (Elson LA, 1958), (Fried W, 1977), (Brookes P and Law ley PD, 1963). The present study was performed to evaluate the efficacy of IG when combined with chemotherapy in treating DLA induced solid tumor and EAC ascites in order to substantiate its therapeutic link with regard to their clinical utility.
Despite advances in cancer chemotherapy, the treatment of peritoneal carcinomas remains a significant failure. Unlike solid tumors, which can be removed by surgery, the peritoneal tumors result in inoperable accumulation of malignant ascites, and hence to date their treatment remains a challenge. Peritoneal concentrations of intravenously administered drugs remain low owing to poor drug penetration into the peritoneal cavity and therefore would be insufficient to eliminate such tumors. Intraperitoneal chemotherapy of peritoneal tumors would likely be a better method owing to high local concentration in tumor regions and low systemic toxicity (Sadzuka Y et al, 2000). However, effective prolonged treatment of tumors by conventional intraperitoneal chemotherapy with anticancer agents is doubtful because of their rapid elimination from the peritoneal cavity and distribution to other organs, as well as their lower half-life and stability. Techniques such as chemotherapeutic peritoneal perfusion of anticancer agents have been used with little success, and such techniques complicate the therapy by decreasing patient compliance (Koga S et al, 1988), (Fujimura T et al, 1994).

The result from the present investigation has shown that IG effectively induces apoptosis and stimulates immune response in EAC induced ascites tumor model. Some changes of cellular shape were microscopically observed in EAC cells when cells were treated with IG. IG showed growth inhibition and changed EAC cells into shrinking and multiblebbing shapes at various concentrations. These morphological changes are typical of apoptosis. IG induced DNA laddering in EAC cells in concentration dependant manner. These DNA fragmentation and characteristic morphological changes indicated that growth inhibition of IG was mediated through apoptosis. IG and CTX act synergistically to improve survival of both solid and ascites tumor bearing mice. Hence the present study was carried out to elucidate the mechanism of anticancer and immunomodulatory effect of IG in mice. IG has aroused more interests in the treatment of tumors due to the changes of cell immune status, efficacy and low toxicity in tumor bearing animals.

While the chemotherapeutic drug cyclophosphamide (CTX) was associated with reduced lymphocyte proliferative response and lymphocyte subsets (CD3,CD4,CD8,CD19 and CD56) in tumor bearing mice, administration of IG and
CTX together restored the lymphocyte proliferative response and lymphocyte subsets in mice to normal levels. Thus IG can restore the immune potential to the normal level after the host was depressed by tumour burden or anticancer chemotherapeutic agent. IG influenced macrophage response as it significantly increased both the percentage of phagocytosis and phagocytic index of mice. This may explain in part, why IG has been shown to improve the immune condition of patients receiving chemotherapy. Oral administration of IG can improve the impaired antitumour CD4+ T-cell response in both DLA induced solid and EAC induced ascites tumor models.

The Th1 and Th2 cytokine patterns have now been implicated in several immune responses concerning infections, allergy and autoimmunity (Mosmanm TR and Sad S, 1996). Recent clinical information also suggests that Th1 response is suppressed and Th2 response is elevated systemically in tumor patients, suggesting that Th2 cytokines may mediate immunosuppression (Yamamura M et al, 1993), (Kharkevitch DD et al, 1994). It is generally considered that the immune response to malignantly transformed cells requires the collaboration of cytokines produced by T cells. Th1 cytokines appear to have a protective function, whereas Th2 cytokines seem to favor tumor growth (Romagnoni S, 1996). A shift from Th1 to Th2 cell function has been observed in several animal tumor models (Yu YH et al, 2005) and also in human tumors (Nakamori M et al, 2003). Th2-mediated immunosuppression reduced the protective cellular immunity. Type 2 cytokines are usually predominant in tumor patients and associated with tumor progression. To explore whether reversing of type 2 predominance could be a promising strategy in tumor immunotherapy, In the present study, results showed that lung cancer patients represented an obvious type 2 cytokine predominance, Indukantha Ghritha might reverse the type 2 predominant status in vivo. So the present study demonstrated that IG directly influences CD4 T cells and ameliorates Th2 cell lineage development by decreasing IL-4 level.

Moreover tumor bearing mice administered with IG showed a significant increase in WBC count with characteristic change in T cell subpopulations. Therefore we support the view that the antitumor effect of these samples on tumor bearing mice is mostly dependent upon the enhancement or restoration of immuno-competent cells.
IG may be useful a chemotherapeutic agent. Further exploration of IG as an antitumor drug is worthwhile.

IG was administered along with CTX in both EAC ascites and DLA solid bearing mice showed significant restoration of values near to basal levels in almost all groups indicating the ability of IG to counteract immunosuppression and thus could be used as an adjuvant to cancer chemotherapy. Thus, we conclude that oral administration of IG could be of benefit for the tumor-bearing organism resulting in immunomodulation combined with tumor growth inhibition.