EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF *CURCULIGO ORCHIOIDES* AND IT'S USEFULNESS IN THE INHIBITION OF TUMOUR CELL PROGRESSION

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CERTIFICATE

This is to certify that the thesis entitled **"IMMUNOMODULATORY ACTIVITY OF *CURCULIGO ORCHIOIDES* AND IT'S USEFULNESS IN THE INHIBITION OF TUMOUR CELL PROGRESSION"** is a bona fide record of research work carried out by **Mrs. Vishnu Priya Murali**, under my guidance and supervision at Amala Cancer Research Centre, Amala Nagar, Thrissur, and no part thereof has been presented for the award of any other degree, diploma or other similar titles. The thesis has been checked for plagiarism and the similarity indices are within the allowed limits as recommended by the University of Calicut.

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DECLARATION

I hereby declare that the thesis entitled "**EVALUATION OF THE IMMUNOMODULATORY ACTIVITY OF *CURCULIGO ORCHIOIDES* AND IT'S USEFULNESS IN THE INHIBITION OF TUMOUR CELL PROGRESSION"** is based on the original research carried out by me at Amala Cancer Research Centre, Thrissur under the guidance of **Dr. Girija Kuttan, Ph.D,** Professor in Immunology, Amala Cancer Research Centre, Amala Nagar, Thrissur and no part thereof has been presented for the award of any other degree, diploma or other similar titles.

Amala Nagar
15 March 2016

Vishnu Priya Murali

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List of abbreviations

ACC	:	Antibody dependent complement mediated cytotoxicity
ADCC	:	Antibody dependent cell mediated cytotoxicity
Ag	:	Antigen
ALP	:	Alkaline phosphatase
APC	:	Antigen presenting cells
BM	:	Basement membrane
BUN	:	Blood urea nitrogen
b.wt	:	Body weight
CAMs	:	Cell adhesion molecules
CD	:	Cluster of Differentiation
CO	:	*C. orchioides*
Con A	:	Concanavalin A
COX	:	Cyclooxygenase
CPA	:	Cyclophosphamide
cpm	:	Counts per minute
CRP	:	C-reactive protein
CS	:	Curculigoside
CTL	:	Cytotoxic T Lymphocytes
D.D H_2O	:	Double distilled water
DC	:	Dendritic cells
DLA	:	Daltons lymphoma Ascites
DNA	:	Deoxyribonucleic acid
DTH	:	Delayed type hyper sensitivity
EAC	:	Ehrlich ascites carcinoma
ECM	:	Extracellular matrix
EGFR	:	Epidermal growth factor receptor
FAK	:	Focal adhesion complex
GM-CSF	:	Granulocyte Monocyte Colony Stimulating Factor
GPT	:	Glutamate pyruvate transaminase
GPx	:	Glutathione peroxidise
GSH	:	Reduced Glutatione
h	:	hours
HA	:	haemaglutination
IFN	:	Interferon

IL	:	Interleukin
iNOS	:	Inducible nitric oxide synthase
LOX	:	Lipoxygenase
LPO	:	Lipid peroxidation
LPS	:	Lipopolysaccharide
MHC	:	Major Histocompatibility Complex
min	:	Minutes
MMP	:	Matrix metalloproteinase
NF-κB	:	Nuclear factor- κB
NK cell	:	Natural Killer cells
NKT	:	Natural Killer T cells
nm23	:	Nonmetastatic gene 23
NO	:	Nitric oxide
NSAIDs	:	Non steroidal anti-inflammatory drugs
OD	:	Optical density
PFC	:	Plaque forming cells
PHA	:	Phytohemagglutinin
PWM	:	Pokeweed mitogen
ROS	:	Reactive oxygen species
RT	:	Room temparature
RT-PCR	:	Reverse transcription–PCR
SOD	:	Super oxide dismutase
SRBC	:	Sheep Red Blood Corpscles
STAT	:	Signal Transducer and Activator of Transcription
TAM	:	Tumour associated macrophages
TCR	:	T cell receptor
TGF-β	:	Transforming Growth Factor-β
TH cells	:	T-helper cells
TIMP	:	Tissue Inhibitors of Matrix metalloproteinase
TNF-α	:	Tumor Necrosis Factor –α
TRAIL	:	TNF-related apoptosis inducing ligand
UV	:	Ultra violet
VEGF	:	Vascular endothelial growth factor

Introduction

Immune system is a multifaceted surveillance system poised to protect our body from any non-self materials or antigens. This natural defence system plays an important role in maintaining homeostasis of our body and a proper functioning of immune system is required to protect against disease or other potentially damaging foreign bodies (Kuby, 2002). Integrity of immune system should be maintained to sustain good health (Ishizuka *et al.,* 1995) and a perfect balance of innate and adaptive arms is essential to avoid the occurrence and progression of inflammatory diseases like cancer (Rachmut *et al.,* 2013). Natural products that can provide a positive modulation of immune system are found be a good choice of study where these tuning effects can be employed to prepare the body itself to struggle against malignancies like cancer.

Cancer refers to a group of diseases arise from abnormal gene expression and remains as the major health problem to humanity. This genetic disease is mainly caused by environmental factors like food and water contaminated with chemical carcinogens or due to physical factors like UV exposure. Cells of our body are following a controlled growth pattern and functioning and when these cells lost their control over cell growth and division due to altered gene expression leads to the formation of tumours (Bishop, 1991). Tumours can be benign or malignant where the benign tumours are slow growing and less harmful which can be removed by surgical procedures. Malignant tumours have the capability of rapid growth and are able to colonize other vital organs by a process known as metastasis (Alison, 2001). Immune system has the ability to detect these abnormal cells and eliminate them and the key players behind these responses are Natural Killer (NK) cells and T cells.

NK cells are parts of innate immune system that can identify and kill any cells that lacks self MHC class I molecule. Thus NK cells do not require prior antigen stimulation to attack tumour cells which has an altered gene expression pattern. For these reasons NK cells are considered as promising natural agents to fight against cancer (Dahlberg *et al., 2015*). Since they represent only a minor population of blood cells, enhancing their activity is a good choice to counter tumour cells. We have tried to work with this hypothesis using a plant derived product as stimulator of NK cells. Meanwhile, T-cells are the chief players of

adaptive immune response against tumour development, especially cytotoxic T cells (CD8+T cells). CD8+T cells has the ability to identify tumour antigen but when these antigens are processed and presented by antigen presenting cells via MHC class I molecule, naive CD8+T cells get activated, differentiated and execute antitumour immune response (Castelli *et al.*, 2000).

During cancer progression cancer cells secretes immunosuppressive factors leads to decline in immunity of the host and also results in an impaired inflammatory and phagocytic response (Maneesh and Kuttan, 2003; Pratheeshkumar and Kuttan, 2011). Immune stimulatory agents can help the body to boost up the immune responses in this situation; at the same time inflammatory responses should be reduced since inflammation is a risk factor for most type of cancers. TNF-α, Interleukins (IL-1β and IL-6) and nitric oxides are the major proinflammatory factors that increase the risk of cancer (Aggarwal *et al.*, 2006).

Tumour cells invade through extra cellular matrix and metastasise into a new location, by degrading connective tissue and basement membrane. Matrix metalloproteanases are Zinc dependant proteolytic enzymes primarily responsible for extra cellular matrix (ECM) degradation (Hidalgo and Eckhart, 2001). Type IV collagen is a main component of basement membrane that can be degraded by MMP-2 and MMP-9 which are also known as gelatinases (Wei *et al.*, 2005). By inhibiting MMP production or activation, tumour metastasis can be blocked up to an extent; thus can be utilized as an excellent target for cancer therapies.

Even though researchers are working for years and years for the drug development, cancer treatment strategies are facing a number of challenges and consequently novel treatments lag behind (Peters, 2013). Most of the physicians remain unaware about the recent advances in the field and they suggest and go for the conventional treatment modalities including chemotherapy, radiotherapy or combination treatment and all directed at killing tumour cells or preventing their proliferation. Most of the anticancer drugs are targeting DNA synthesis and the major drawback is that, it also affects the proliferating normal cells of the body including bone marrow cells, epidermal cells etc. and most of the patients suffers from immune deprivation due to their anti-proliferative effects on the bone

marrow stem cells (Pratheeshkumar and Kuttan, 2010). Supplementation of an immune stimulatory agent can help in this situation.

In our studies we are focusing on the well known traditional Ayurvedic medicinal plant *Curculigo orchioides* Gaertn. coming under the Family Amaryllidaceae (Hypoxidaceae). The common name of this small herb is Golden eye grass or black Musali. It is a perennial plant grows in moist laetrile soil from sea level to 2300 m height and found in tropical regions of Asia and Australia (Daffodil *et al.,* 2012). It is one of the 'Dasapushpa' plants which is well known for its rejuvenating effects and also an important Rasayana drug for vigour and vitality (Irshad *et al.,* 2006). The leaves of the plants are used for its anti-inflammatory potential in folk medicines. The study was designed based on the previous reports on the immune stimulatory (Lakshmi *et al.,* 2003), anti-inflammatory (Asif and Kumar, 2010) and hepatoprotective (Venukumar and Latha, 2002) effects of the plant.

Aim of the study

- To evaluate the effects of *C. orchioides* on the cell mediated immune system especially on the NK cell and CTL activity and to study whether the immune-stimulatory and anti-inflammatory properties of the plant can be utilized to restrain tumour progression.
- To assess the efficacy of the plant as an adjuvant to minimise the toxic side effects of chemotherapeutic drug.

Objectives of the study

The following are the major objectives comprised,

- Collection, preliminary screening and toxicity analysis of the plant.
- To study the effect of *C. orchioides* on the nonspecific and specific immune responses in experimental animals.
- Effect of *C. orchioides* on the cell mediated immune system during tumour progression under experimental conditions.
- Evaluation of the anti-inflammatory potential of the plant in acute and chronic models along with the effect on the proinflammatory cytokine release by the activated macrophages.

- Screening of the plant for its anti-metastatic effects using C57BL/6 mice.
- Evaluation of the ameliorative effects of *C. orchioides* on multiple organ toxicities induced with Cyclophosphamide administration in experimental animals.

Scope of the Study

- Revealing the un-known valuable medicinal effects of *Curculigo orchioides.*
- A detailed study can be conducted, based on the reported immune stimulatory effects of *C. orchioides* to develop plant derived products with least toxicity but exhibits good anti-tumour efficacy via its immuno-modulatory effects.
- Initial screening for the development of adjuvant to chemotherapeutic drugs which can ameliorate the toxic side effects, without hindering their anti-proliferative property against abnormal cells.

Chapter 1

Review of literature

Nature blessed our planet with rich and diverse flora and fauna of which there are a number of imperative plants with healing power, known or unknown to the human population. During the early stages, medicinal science was built on the basis of these plants with curing effects. As a result of the continued research for the past years and centuries a huge number plants has been identified and studied for their use in disease management and for promoting health (Ballal *et al.*,2012). These medicinal plants and derived products are able to modify different aspects of human physiology and put forth an alleviating effect on several pathophysiological conditions, and the concepts of immunity and immunomodulation can be traced back several hundred years to the history of medicine (Gulati *et al.,* 2002).

1.1 Immune system and Cancer

Immune system is an orchestration of a number of cells and organs which consist of an innate (non-specific immune system) and adaptive (specific or acquired immune system) wings (Vesely *et al.,* 2011). The main function of the immune system is to protect against disease by identifying and killing pathogens and tumour cells. Innate immune system is the body's first line of defence against any antigenic encounter in a non-specific and generic manner by molecular interaction and expression of inducible cytokines and chemokines. Various cell types like macrophages, granulocytes, dendritic cells, natural killer (NK) cells etc are taking part in this defensive mechanism whereas the adaptive immune system consists of B lymphocyte mediated humoral and T lymphocyte mediated cell-mediated immune responses along with the antigen presenting cells (Rachmut *et al.,* 2013). Antigen presenting cells like macrophages and dendritic cells process and present antigens to these specialized effector cells (T and B cells), leading to the activation of cytotoxic T cells and generation of antibodies that are critically involved in the elimination or prevention of abnormal cells or pathogenic challenges (Gertsch *et al.,* 2011).

An immunomodulator can be any natural or synthetic substance that is able to stimulate, suppress or modulate any of the above said components of immune system including humoral or cellular components (Agarwal and Singh, 1999). Modulation of immune system is a complex process where, over activation of

immune system leads to deleterious pathological conditions including autoimmunity (Kamradt and Mitchison, 2001) and chronic inflammatory diseases like cancer (Aggarwal *et al.,* 2006; Galgani *et al.,* 2009). On the other hand, immune deficiencies will results in greater vulnerability to infection and tumour development (De Souza and Bonorino, 2009; Sethi *et al.,* 2009). Thus a perfect tuning of immune system is an essential aspect and one of the major streams in the field of immunomodualtion has been the search for agents that can be used to inhibit tumour development and progression (Yamamoto, 1996).

Cancer is a non-communicable disease of abnormal gene expression and constitutes a considerable public health problem of great concern. Efficacy of tumour cells to detach from the primary tumour and to metastasize at different locations forms the primary reason for the cancer associated mortality (Nathanson, 2003). Immune system plays the foremost role in prevention of tumours mainly by three routes, first it protects the host from viral infections, thus reduces the chance of virus induced tumours. Second mechanism is the timely elimination of pathogens and thus the avoidance of a chronic inflammatory condition which can lead to tumourigenesis. Finally the ability of immune system to target the abnormal cells on the basis of their altered surface markers or molecules induced by cellular stress and eliminates them from the body. This process is referred to as tumour immune surveillance (Swann and Smith, 2007). Even in the presence of a functioning immune system cancer development occurs, thus the theory of Immune-editing was emerged (Dunn *et al.,* 2002). According to Dunn *et al.,* (2004) the process of tumour immune-editing is a multifarious process that can be segregated into 3 phases, called elimination, equilibrium, and escape. Of this, the elimination phase is the same process of tumour immune surveillance, whereby abnormal cells that are developed due to abnormal expression of tumour suppressor genes are avoided by the immune system. The elimination phase can be complete, when all tumour cells are cleared, or incomplete, when some of the tumour cells are escaped from this elimination process. Incomplete elimination phase is followed by the equilibrium phase where tumour cells remains dormant or may be accumulating much more mutations results in altered surface antigen expression. Immune system again encounters the tumour cells with sufficient power to block tumour progression, but if the immune

system again fails to completely eliminate tumour cells, they will become capable to avoid anti-tumour immune response (Swann and Smith, 2007). The cells that drive the antitumour immune responses are lymphocytes, macrophages and other antigen presenting cells, mainly dendritic cells.

1.1.1 Lymphocytes

Lymphocytes form the major population of cells that plays a key role in integrating immune reactions. There are three major subsets of lymphocytes, B lymphocytes, T lymphocytes and null cells (natural killer cells, NK).

T lymphocytes

T-lymphocytes mature in thymus under the influence of thymic hormones and execute the cell mediated immune responses. T-cells are key players of anti-tumour immune response. T lymphocytes are the part of adaptive immune system which express CD-3 surface molecule and comprises mainly of two types, T-helper cells (TH cells, $CD4^+$) and Cytotoxic T cells (CTL, $CD8^+$) based on their surface molecular markers or functional variation (Coico and Sunshine, 2015).

T- Helper cells (TH cells/ CD4+ T cell)

T_H cells are get activated by small quantity of antigens that may be insufficient for the activation of other immune cells and secretes a number of cytokines thus help the activation of B cells, TC cells and macrophages. TH cells are more heterogeneous than TC cells and the differentiation is depending upon the stimulation provided by the antigen-presenting cell (APC). Based on the functions and cytokines produced, TH cells were divided into three sub types in the past, TH1, TH2 and TReg. But recent evidences suggests that besides TH1, TH2 and TReg there are TH3, TH17, TH9, TFH (for follicular helpers), NKT and γδ subsets (Purnama *et al.,* 2013).

Along with providing help for the functioning of B cells and cytotoxic T cells via cytokine secretion and cell-cell contact, TH cells also take part in the regulation and recruitment of innate cells such as macrophages, neutrophils, mast cells, and monocytes. Activation of naïve $CD4^+$T cells is brought about by the binding of T-cell receptor (TCR) on $CD4^+$T cells with MHC class II molecules on APC

along with the interaction of co-stimulatory molecule B7 on APC with CD28 receptor on T cells. The cytokine levels will influence the TH cell differentiation and each TH subset will have a signature cytokine profile. Earlier TH response was known to be of two types, TH-1 response towards viral infection (cellular immunity) and TH-2 response that promote humoral immunity. Later the T-regulatory cells were discovered which has the ability to suppress the immune response especially the cell mediated immune response. Recently other cell types like TH-3, TH-17, TH-9, TFH (for follicular helpers), NKT and γδ subsets were discovered along their functional validation (Purnama *et al.,* 2013).

Activation of TCR along with CD3 pursued intra-cellular signalling pathway leads to the proliferation and differentiation of naive cell into particular effector cell, and this lineage-specific differentiation depends on factors like cytokines levels type and concentration of antigen, type of APC and costimulatory molecules (Ashkar *et al*., 2000; Tao *et al*., 1997; Luckheeram *et al*., 2012). Dendritic cells are the most efficient APC in terms of naive T cell stimulation. IL-12 and IFN-γ induces TH-1 differentiation, of this IL-12 is mainly secreted by APCs and can induce NK cells to produce IFN-γ and the transcription factor involved is STAT3, whereas TH-2 differentiation is brought about by IL-4 along with GATA3 transcription factor (Zhu *et al.,* 2010; Huppa and Davis, 2003).
Elimination of the intracellular pathogens is the main function of TH-1 cells and the major cytokines secreted by them are IFN-γ, IL-2 and lymphotoxin α (Lf-α), and of these IFN-γ is crucial for the activation of phagocytic response and cell mediated immune response (Prete, 1992; Murray *et al.,* 1985). Whereas IL-2 enhances the $CD8^+$T cell proliferation and cytotoxic responses (Kim *et al.,* 2006). Along with this IL-2 also induces the expansion of $CD8^+$ memory cells after antigenic encounter, therefore facilitating the secondary immune response (Gattinoni *et al.,* 2005; Williams *et al.,* 2006).

Regulatory $CD4^+$T Cells are natural thymus derived subset which helps to maintain immune tolerance to self and non self antigens by negatively regulating the immune cells after the antigen clearance, providing important role in the maintenance of immunologic tolerance to self and foreign antigen thereby protecting against immunopathology (Lukheeram *et al*., 2012). Recent evidences

suggest that tumour cells can recruit Treg cells to inhibit antitumour immunity in the tumour microenvironment, thus limiting the efficiency of cancer immunotherapy. In recent times tumour-specific Treg cells are also recognized and characterized, providing convincing evidence that such antigen-specific Treg cells can induce tumour-specific local immune tolerance (Wang and Wang, 2012). Thus the suppression of these cells may provide better therapeutic output. Whereas the NK T-cells constitutes a heterogeneous population of T cells that shares the characteristics of both T cells and NK cells and has the capability of rapid release of various cytokines like IL-2, IFN-γ, TNF-α and IL-4. (Jerud *et al.,* 2006). Since $CD4^+$ T cells are also involved in the development of auto-immunity and inflammatory responses, fine regulation of these cells forms a vital obsession to attain.

Cytotoxic T-lymphocytes (CTL/$CD8^+$T cells)

The key players of adaptive cellular immune responses are T lymphocytes in general, and for the effector function, cytotoxic T lymphocytes (CTLs) in particular. CTLs, by virtue of their ability to detect quantitative and qualitative antigenic differences in the transformed cells, are capable to provide defence against spontaneous malignant tumours. $CD8^+$ T cells identify antigen which is processed by protein degradation inside the cell and presented by MHC class I-derived molecules on the cell surface to CTLs, thus enabling CTLs to scan for cellular alterations (Castelli *et al.,* 2000). CTL are often activated with the help of $CD4^+$T cells against cell-derived antigens for which $CD4^+$T and $CD8^+$T cells must recognize antigen on the same APC or the $CD4^+$T cells activate APCs through CD40/CD40-ligand interactions, so that APCs can activate CTLs (Schüler *et al.,* 2001). IL-2 is required for the proliferation and clonal expansion of CTLs and this expansion can be many thousand-fold at the peak of a primary immune response. This highly expansive property is mainly attributed by the easiness in activation by Ag-MHC class I complex and better survival in the circulation.

Besides the rapid expansion, the capability of single $CD8^+$ CTL to obliterate more than one target cell, without effecting the normal cells make CTL very efficient Ag-specific effector cells. Direct cell contact with the target cell and Ag recognition leads to the release of cytolytic granules into the immunological

synapse which results in the target cell death. Costimulatory signals are not required for the killing effects of CTL upon antigen recognition, thus they can destroy a variety of target cells bearing “foreign” Ag. Activated $CD8^{+}$T cells contain cytolytic granules in its cytoplasm along with surface Fas ligand molecule (CD 178). It can kill the target cells by two methods either by executing Ca^{2+}-dependent perforin/granzyme- mediated apoptosis or Ca^{2+}-independent Fas ligand/Fas-mediated apoptosis where both the pathways has the priming step of TCR activation and signalling (Broere *et al.,* 2011). The predominant pathway of killing of target cell lysis involves the exocytosis of the granules that contains perforins and granzymes. Perforins makes trans-membrane channels on the target cell membrane, results in the increase in membrane permeability and eventually leads to target cell death. Whereas granzymes which are serine protease can enter in to the cells through the membrane channels made by perforins and induces apoptosis. The second mechanism involves the interaction of Fas (CD 95) molecule expressed on the surface of many cell types with the Fas ligand on $CD8^{+}$T cells which activates caspases in the target cell and causes apoptosis. After the antigen clearance activated T-cells induces apoptosis by Fas-Fas ligand interaction with each other, leaving only a minor population of long lived antigen specific T cells that are either memory $CD4^{+}$T cells or memory $CD8^{+}$T cells (Coico and Sunshine, 2015).

B lymphocytes

B cells mature in bone marrow or Bursa of fabricus, found to be concentrated in spleen and lymph nodes. B cells bring about the humoral immune responses and upon proper antigenic stimulation they rapidly divide to form antibody secreting plasma cells and memory cells. A particular clone of B lymphocyte proliferates and differentiates into many daughter cells and all produce a similar type of antibody, thus the antibodies produced by a specific group of B-lymphocyte is known as monoclonal antibody. Memory B cells are long living cells which are capable to mount immune response upon further encounter with the similar type of antigen in a faster way and helps to prevent disease recurrence (Fox, 2002; Solomon and Komanduri, 2001; Mautner and Huang, 2003). B cells also function as antigen presenting cells that presents processed tumour antigens to cytotoxic T lymphocytes and also produce antibodies against tumour specific antigens.

NK cells

Natural killer (NK) cells are cytokine producing lymphocytes of the innate immune system which have cytotoxic ability to kill both viral infected and tumour cells. Reports on Tumour-killing lymphocytes were published in 1968 by Hellström *et al*. and in 1975 Kiessling and colleagues, in parallel with Ronald Herberman's research laboratory, named this cytolytic population as NK cells that are able to target tumour cells. Eventhough the functions of NK cells are similar to CTL, they are distinct in many ways like NK cells do not express a TCR and their development is independent of the thymus. Despite of these differences experimental evidences suggests that T and NK cells are derived from a common progenitor (Ikawa *et al.,* 1999). Unlike T cells and B cells, target cell recognition and NK cell activation is not antigen specific but is mediated by the signals delivered through several activating and inhibitory receptors. Thus NK cell response is determined by the balance between these activating and inhibitory signals (Dahlberg *et al., 2015*).

NK cell can kill a target cell in a spontaneous fashion that did not require any priming and was not restricted by the target cell's expression of major histocompatibility complex (MHC) molecules. NK cells would kill any target that lacked self–major histocompatibility complex (MHC) class I molecules (the "missing self" hypothesis) (Caligiuri, 2008). Thus, NK cells indeed employs MHC molecules in identifying target cells and for this NK cells express a surface killer immunoglobulin-like receptors (KIRs) that interact with self MHC molecule and NK cells are inhibited. In the absence or low expression of self MHC molecules NK cells get activated due to lack of inhibitory signals leading to lysis of the host cell (Dahlberg *et al., 2015*). Once an NK cell has come in contact with a target cell, it releases effector molecules such as granzyme B and perforin to induce apoptosis or by the expression of death receptor ligands such as FasL and TNF-related apoptosis inducing ligand (TRAIL). At the same time they also can secrete effector cytokines such as IFN-γ that can activate and regulate other innate immune cells and lymphocytes (Fathman *et al.,* 2011). NK cells can also get activated by cross linking of Fc receptor CD16 to target cell leading to antibody-dependent cellular cytotoxicity (ADCC) and lysis of the target cell (Ashkar *et al*.,

2000; Drayton *et al*., 2006). Additionally, immunomodulatory drugs (IMiDs) may up regulate TRAIL expression on NK cells that potentially enhance the TRAIL-mediated elimination of tumour cells (*Dahlberg et al., 2015*).

1.1.2 Antigen presenting cells (APC)

APCs are specialised immune cells of heterogeneous types that can engulf, process and present antigens to effector immune cells especially T cells, thus can activate them. Macrophages, Dendritic cells, Langerhans cells and B cells are the major APCs and they express both the MHC class I and class II molecules on their surfaces. Most of the antigens including tumour antigens are not recognised by T cells, however APCs present processed antigens to both the TC and TH cells via MHC I and MHC II molecules respectively, and the naive T-cells get activated, proliferate and differentiate into sufficient number of effector cells. APCs can be categorized into two groups, professional APCs that express both MHC I and II molecules and non-professional APCs that include all the cell types express Class I MHC molecules (Kindt and Goldsby, 2007).

Dendritic cells (DC) are the most efficient professional antigen presenting cells. Even though tumour cells itself does not activate naive T cells, dendritic cells have the capability to enhance the T cell response against tumour cells. Along with these MHC molecules, they also express surface co-stimulatory molecule B7 that can interact with CD28 molecule on naive T-cell surface. Dendritic cells cross present the tumour antigens to the T-cells and activate them, but the fact is that DCs in cancer patients are often kept in an immature or dysfunctional state, thereby preventing the stimulation of tumour-specific T cells (Petersen *et al*., 2010). According to Palucka and Banchereau, dentritic cells are nature's adjuvant' that are at the centre of the immune system and can control both immune tolerance and immunity, hence can be employed in tumour immunotherapy (Palucka and Banchereau, 2012).

Macrophages are one of the first line responder cells reaches the infection site or tissue damage, where they perform several functions. Their effector mechanism is mainly by phagocytosis and cytokine and chemokine production. Recruitment of other immune cells and to release various growth factors, angiogenic factors

and proteases are also brought about by the macrophages. Since they are good APCs they activate T cells by cross priming and they are also capable to kill pathogens by the production of reactive oxygen and nitrogen radicals. Macrophages are generally not cytotoxic to tumour cells but when get activated especially by IFN-γ or bacterial products they can execute antitumour immune responses that is similar to delayed type hyper sensitivity response. Macrophages itself can exert proinflammatory and aniinflammatory responses according to the micro-environment (Siveen and Kuttan, 2009). Tumouricidal macrophages can distinguish and destroy neoplastic cells *in vitro* and *in vivo*, without affecting the normal cells (Knezevich and Silvetz, 2011). Although the exact mechanism(s) of discrimination is unknown, it is independent of tumour cell characteristics such as immunogenicity, metastatic potential, and sensitivity to cytotoxic drugs (Knezevich and Silvetz, 2011; Whitworth *et al*., 1990). At the same time, there are a number of evidences from the clinical and experimental trails to support the tumour promoting activity of macrophages. Macrophages are tuned by the tumour micro environment to adopt a trophic role that facilitates angiogenesis, matrix breakdown and tumour-cell motility — all of which are elements of the metastatic process (Pollard, 2004).

Macrophages are a major population of cells present in tumour mass that can play controversial role. Macrophages are derived from monocytes, but belong to an extremely heterogeneous population. Even though their origin is same, they can be differentiated into distinct types, known as M1 (or classically activated) and M2 (or alternatively activated). As per today's knowledge tumour associated macrophages belongs to M2 phenotype and show mostly pro-tumoral functions, promoting tumour cell survival, proliferation, and dissemination. TAMs are known to be associated with metastatic progression and angiogenesis and results in bad prognosis (Solinas *et al*., 2009; Pollard, 2004).

1.2 Inflammation and cancer

Inflammation is the process of innate immunity in response to physical, physiological and/or oxidative stress or it can be defined as the protective mechanism of the body to heal tissue injury (Hoesel and Schmid, 2013). The

Roman medic Aulus Cornelius Celsus defined inflammation as: “rubor” (redness), “tumour” (swelling), “calor” (heat) and “dolor” (pain). Over thousands of years ago Ayurveda described that continuous irritation over long periods of time can lead to cancer (Eiró and Vizoso, 2013) and it is now clear from the epidemiological studies that inflammatory diseases are frequently associated with increased risk of cancers. Cancer progression from inflammation may be driven by inflammatory cells as well as a variety of mediators, including cytokines, chemokines, and enzymes, which altogether establish an inflammatory microenvironment (Coussens and Werb, 2002). Sometimes this response may restrain tumours, but in most situations it facilitates cancer development via multiple signalling pathways (Yang *et al.*, 2005).

Early stages of inflammation is marked by the infiltration of neutrophils to the site, in response to the molecular messengers released by rapidly responding macrophages and mast cells pre-stationed in tissues (Lu *et al*., 2006). Various other types of WBCs including lymphocytes are attracted to the site by a signalling network involving a great number of growth factors, cytokines, and chemokines during the progression of inflammation. Tissue damage is resulted by these cell types for strengthening and maintaining the defence against infection. A rapid programmed clearance of inflammatory cells is required to prevent inflammation response from lasting too long. Neighbouring macrophages, dendritic cells, and backup phagocytes do this job by means of phagocytosis and apoptotic induction of target cell. When this inflammation resolution is dysregulated, cellular response changes to the pattern of chronic inflammation (Lu *et al.*, 2006).

Cancers and inflammation are inter-related to each other in two ways, extrinsic and intrinsic pathways. Extrinsic pathway involves the conditions that cause non-resolved chronic inflammatory responses whereas intrinsic pathway is determined by oncogenes or tumour suppressor genes involved in the expression of inflammatory gene products (Balkwill and Mantovani, 2012). Cancer development is a multistep process which is resulted from the accumulation of genetic alterations that provides specific types of growth advantage; thus the normal cells lost its control over cell cycle leads to the progressive transformation

from normal cells to malignant cancer cells. Self-sufficiency of growth signals, insensitivity to antigrowth signals, escaping from apoptosis, unregulated proliferation potential, enhanced angiogenesis, and metastasis are the hall marks of cancer cells (Hanahan and Weinberg, 2000; Lu *et al.,* 2006). Epidemiological studies explain that there are 10 fold greater risk of colo-rectal cancer for those who are suffering from inflammatory bowl diseases like ulcerative colitis. Chronic hepatitis also leads to hepatocellular carcinoma, whereas human papilloma virus infection is the leading cause of penile and anogenital cancers (Lu *et al.,* 2006).

Cytokine signalling could contribute to tumour progression in two ways: the stimulation of cell growth and differentiation and the inhibition of apoptosis of altered cells at the inflammatory site (Lu *et al.,* 2006). Uncontrolled release of inflammatory mediators like TNF-α and nitric oxide (NO) are believed to be the two major factors that drive inflammation mediated host tissue damages. These inflammatory molecules are also known to cause cell death because NO interacts with the free radicals such as superoxides to produce peroxynitrite to form irreversible damage to cell membranes (Ravipati *et al.,* 2012). Besides TNF-α, inter-leukines like IL-1, IL-6, growth factors, and differentiation factors (colony-stimulating factors) are also responsible for tumour progression.

1.2.1 TNF-alpha

Aggarwal *et al* in 2003 identified TNF- α as an anti-cancer cytokine and also reports that when expressed locally by the cells of the immune system, TNF-α has a therapeutic role. Dysregulated and systemic TNF levels leads to a wide variety of diseases, including cancer (Aggarwl *et al.,* 2003) where they can bring about inflammatory mediators and proteases that orchestrate inflammatory responses. Tumour cells also release TNF- α and can act as an endogenous tumour promoter (Balkwill, 2002). Researchers proposes that TNF- α induces cellular transformation, proliferation, and tumour promotion and according to Komori *et al* TNF- α is 1000 times more effective than the chemical tumour promoters okadaic acid and 12-O-tetradecanoylphorbol-13-acetate in inducing cancer (Komori *et al.,* 1993). Besides macrophages, most of the lymphoma and carcinoma cells can secrete TNF- α, thus act as an autocrine growth factor and in

many cell types TNF- α induces the expression of other growth factors like epidermal growth factor receptor (EGFR) and transforming growth factor (TGF-a), which mediate proliferation of tumours. TNF- α also promotes tumour metastasis and angiogenesis by increasing invasive properties of tumour cells along with an increased VEGF levels and has been even reported to mediate macrophage-induced angiogenesis (Aggarwal *et al.,* 2006).

1.2.2 Interleukins

Interleukins are one of the major mediators of tumourigenesis that include IL-1, IL-6, IL-8, and IL-18. Studies demonstrated that IL-1β released by pancreatic carcinoma cell lines promotes growth and confers chemo-resistance (Arlt *et al.,* 2002). IL-1 β secretion into the tumour micro-environment also enhances the tumour angiogenesis and promotes tumour growth through hyper neovascularization in lung carcinoma growth *in vivo* (Saijo *et al.,* 2002). IL-6 can be an autocrine or paracrine growth factor in some malignancies, especially those of hematologic origin where it helps to block apoptotic signals (Balkwill and Mantovani, 2012). At the same time IL-6 exerts paracrine effect that act as a tumour promoting growth factor in multiple myeloma, non-Hodgkin's lymphoma, bladder cancer, colorectal cancer, and renal cell carcinoma (RCC) (Aggarwal *et al.,* 2006).

1.2.3 Chemokines

Chemokines have an important role in cancer progression, especially in angiogenesis, inflammation, cell recruitment, and migration. Chemokines also regulates the recruitment and trafficking of leukocytes to sites of inflammation. There are four major categories of chemokines: C, CC, CXC, CX3C; and this classification are based on the positions of key cysteine residues (Aggarwal *et al.,* 2006). CCL2 (MCP-1) and inflammatory CC chemokines known to be linked to the recruitment and activation of Tumour associated macrophages (TAM) in tumours and these TAMs will favour the tumour progression directly or indirectly by encouraging angiogenesis and tumour growth. M2 polarization and survival of TAM are also promoted by CC chemokines, whereas CCL2 and its cognate receptor CCR2 are found to be crucial for the tumour development and progression in mouse especially, prostate cancer (Balkwill and Mantovani, 2012).

1.2.4 Cycloxygenase, Lipoxygenase and NO

Cyclooxygenase (COX)-2 is an important mediator of inflammation and tumourigenesis. It is an inducible enzyme of arachidonic acid metabolism which produces prostaglandins and its gene expression is regulated by NF-kB. COX-2 over expression is seen associated with the progression of various cancers like colon cancer and its expression is induced by various growth factors, cytokines, oncogenes, and other factors. Whereas 5-Lipoxygenase (5-LOX) is the key enzyme responsible for the conversion of arachidonic acid to leukotrienes and according a number of scientific studies, 5-LOX has an important role in carcinogenesis of humans and animals. Besides these, leukotrienes are one of the mediators in allergy and tumour inflammation; these compounds are also linked to pathophysiological events in the brain, including cerebral ischemia, brain oedema, and increased permeability of the blood-brain barrier in brain tumours (Aggarwal *et al.,* 2006). Evaluation of the colonic adenoma formation promoted by cigarette smoke was also shown elevated expression of COX-2 and 5- LOX. When colon cancer cells were pre-treated with cigarette smoke extract, it promoted colon cancer growth in the nude mouse xenograft model and inhibition of COX-2 or 5-LOX reduced the tumour size (Ye *et al.,* 2005; Aggarwal *et al.,* 2006).

Over expression of inducible Nitric Oxide Synthase (iNOS), an enzyme-catalyzing NO production is seen in association with chronic inflammatory diseases including cancer. NO has an important role in the regulation of tumour progression and inflammatory responses. Various proinflammatory cytokine like TNF-α and IL-1 can induce iNOS, where it acts as a downstream effector of cytokines and NF-κB in linking inflammation to cancer (Lu *et al.,* 2006). From experimental evidences it was found that when lipopolysaccharide induced NF-κB activation was hindered, it led to the inhibition of iNOS expression and NO generation, thus the inflammation was reduced (Kim *et al.,* 2005).

1.2.5 Antiinflammatory agents

Most of the anti-inflammatory drugs available in the market are cyclooxygenase pathway inhibitors where the nonsteroidal anti-inflammatory drugs (NSAIDs) are major category of them which are used to treat diseases like arthritis, asthma, and

cardiovascular disease. But prolonged administration of these medicines will cause a number of side effect including gastro-intestinal ulcers, bleeding, and renal disorders due to their nonselective inhibition of both constitutive (COX-1) and inducible (COX-2) cyclooxygenases enzymes. Thus alternatives to the NSAIDs and opiates are being searched worldwide and in this scenario medicinal plants are believed to be good candidates as a source of new chemical substances with potential therapeutic effects (Kaushik *et al.,* 2012).

From ancient time natural products with anti-inflammatory activity have been used as a folk lore for inflammatory conditions such as fevers, pain, migraine and arthritis. These plant derived products can be divided into terpenoids, flavonoids, allied phenolic and polyphenolic compounds and sulphur-containing compounds as per British Nutrition Foundation reports. Of these Curcumin a low molecular weight polyphenol found in the rhizomes of the plant turmeric (*Curcuma longa*), is the major one. Both preclinical and clinical studies provide scientific validation for the anti-inflammatory potential of curcumin (Satoskar *et al.,* 1986). Another compound is parthenolide which is the major sesquiterpene lactone found in Mexican India medicinal plants and in feverfew (*Tanacetum parthenium*). As it is used as a folk medicine, its mechanism of action was revealed from clinical studies and it was become clear that parthenolide inhibits the expression of genes involved in inflammation such as nitric oxide (NO) synthase, intracellular adhesion molecule-and various proinflammatory cytokine (Hwang *et al.,* 1996) along with its inhibitory effect on NF-kB (Hehner *et al.,* 1999). Triterpenes like Cucurbitacins, monoterpene oxide like 1,8-Cineole (cineole, eucalyptol) and diterpene glycosides like pseudopterosins etc. are also found to exhibit profound anti-inflammatory effects (Yuan *et al.,* 2006).

Lyprinol, the stabilized lipid extract of the New Zealand green-lipped mussel (NZGLM) is currently used to relieve symptoms of arthritis. Bromelain from pineapple, various flavanoids like quercitrin, rutin, anthocyanins, Ternatin etc. and different sapanins are also reported to have anti-inflammatory activities (Yuan *et al.,* 2006).

1.2 Metastasis

Metastasis is a complex process that represents the end products of a multistep cell-biological process where the cancer cells colonize anatomically to a distant organ sites and their subsequent adaptation to foreign tissue microenvironments (Valastyan and Weinberg, 2011). Primary tumours can be managed by surgical along with the adjuvant therapy whereas the metastatic disease is largely incurable because of its systemic nature and the resistance of disseminated tumour cells to existing therapeutic agents. More than 90% of cancer associated mortality is attributed to the metastasis, not the primary tumours from which these malignancy arise (Gupta and Massague , 2006; Steeg, 2006; Valastyan and Weinberg, 2011). For the successful colonization of tumour cells to a new location, they must complete a chronological chain of steps like separation from the primary tumour, invasion through surrounding tissues and basement membranes, entry and survival in the circulation, lymphatics or peritoneal space and arrest in a distant target organ followed by extravasation into the surrounding tissue, survival in the foreign microenvironment, proliferation, and induction of angiogenesis. These steps have to be attained before the primary tumour becomes a clinically detectable lesion (Hunter *et al.,* 2008). And these stages are marked by a variety of genetic and histopathological checkpoints, together with hyper activation or silencing of specific genes, expression of tumour markers, and stereotypic changes in cell and tissue architecture (Kumar and Weaver, 2009).

1.3.1 Local invasion

Tumour cells detach from the primary tumour mass, invade into surrounding stromal layer and then into adjacent, healthy tissue. Invasive cells often display a reduced cell to cell and/or cell to matrix adhesion (Cavallaro and Christofori, 2001). For this the neoplastic cells has to cleave the basement membrane first. Basement membrane (BM) is the fundamental part of extra cellular matrix (ECM) that helps to organise the epithelial tissue in their position and also separates the cells from stromal compartment. Besides proving structural integrity BM has an imperative role in signal transduction as it contains a number of growth factors that are liberated upon the proteolysis by the invasive cells. It also influence the pathways initiated by integrin-mediated cell-matrix adhesions, leading to alterations in cell polarity, proliferation, invasiveness, and survival. Besides

proteolytic cleavage, modulation of ECM stiffness, physical displacement of normal cells by myosin dependant fraction force and integrin-dependent mesenchymal invasion are also employed by the aberrant cells for their "collective invasion" (Valastyan and Weinberg, 2011; Leber and Efferth, 2009). On another hand according to Friedl individual tumour cells may invade either by mesenchymal or amoeboid invasion can be observed (Friedl and Wolf, 2003). Proteolytic degradation of BM barrier is driven by the following groups of proteases, Serine proteases, Cathepsins, and Matrix metalloproteinases (MMPs) and of these key players are MMPs (Valastyan and Weinberg, 2011).

As per Laubli and Desgrosellier selectins and integrins are important mediators of cancer progression (L¨aubli and Borsig, 2010; Desgrosellier and Cheresh, 2010), since selectins contribute to tumour cell arrest and adhesion whereas integrin-mediated interaction from both tumour cells and the surrounding environment further contribute to cancer progression (Bendas G and Borsig, 2012).

1.3.1.1 Matrix metalloproteinases (MMP)

Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases reported as the chief EM degrading enzymes. Regulated and minimal expression of MMPs is usually occurring under normal conditions in order to maintain homeostasis. MMP expression is controlled by hormones, growth factors, and cytokines while the tissue inhibitors of MMPs (TIMPs) are the endogenous MMP inhibitors (MMPIs) that strictly control these enzymes (Verma, Hansch, 2007). Soluble MMPs are always secreted as inactive proenzymatic forms and are classified into 5 groups based on the preferential extracellular matrix substrate (fibronectin, gelatine, collagen, laminin and proteoglycans) (Kessenbrock *et al.*, 2010). The following are the group of MMPs, (1) interstitial collagenases, (2) stromelysins, (3) gelatinases (type IV collagenases), (4) matrilysins and (5) Membrane-type MMPs. MMPs are the only reported enzymes with the ability to cleave fibrillar collagen (Curran and Murray, 2000).

MMP 2 and 9 are gelatinases that degrade a variety of ECM macromolecules especially type IV collagen of basement membrane. Gelatinase A (MMP-2) is a 72 kDa protein and gelatinase B (MMP-9) is a 92 kDA protein. MMP-2 is

expressed by almost all cells including endothelial or epithelial cells where as MMP-9 is released by inflammatory cells, including blood neutrophils and tissue macrophages, as well as by stimulated connective tissue cells.

In order to penetrate ECM, the cells get elongated and forms invadopodia by actin polymerization which get attached to the ECM substrate to form focal adhesion complex, FAK (Gupta and Massagué, 2006; Pratheeshkumar and Kuttan, 2011). Localization of MMPs to these specialized cell surface structures (invadopodia) is required for their ability to promote invasion. These structures represent the site where active ECM degradation takes place. Invadopodia utilize transmembrane invadopodia- related proteinases, including MMP-14 [membrane type (MT)1-MMP], several members of the ADAM (zinc-dependent family of proteinases related to the MMPs) family, as well as secreted and activated MMPs at the site, such as MMP-2 and -9, to degrade a variety of ECM macromolecules and facilitate cell invasion (Weaver, 2006; Gialeli *et al.,* 2011). MMP-2 and 9 are also reported as the key MMPs involved in tumour angiogenesis (Rundhaug, 2003) and their over expression leads to the epithelial-mesenchymal transitions by the proteolytic activation of TGF-β (Illman *et al.,* 2006; Egeblad and Werb, 2002).

Transcription and translation of MMP genes are strictly controlled by a number of factors and when these systems go out of control it will facilitate the tumour cell proliferation and spreading. Since MMPs are produced as inactive zymogen form, the activation step forms another control point, along with these production of endogenous inhibitors like tissue inhibitors of metalloproteinases (TIMPs) also inhibits over activation of MMPs (Cathcart *et al.*, 2015).

1.3.1.2 TIMP

TIMPs are a family of proteins known as the natural inhibitor of MMPs and were identified and characterised as the specific inhibitor MMP but now it is clear that TIMPs perform a vast series of functions including the regulation other proteases like ADAMs and ADAMTSs. Activation of almost all types of metallo-proteases is controlled by TIMP through multiple site interaction with the target enzyme which triggers the transcription of TIMP variants that selectively inhibit different types of MMPs. Other biological activities performed by TIMP include its effect

on cell growth and differentiation, cell migration, antiangiogenesis, anti- and pro-apoptosis, and synaptic plasticity. TIMPS are involved in the signal transduction pathways as well as the antiangiogenic activity is partially attributed to the MMP inhibition especially gelatinase inhibition (Brew and Nagase, 2010).

1.3.2 Intravasation: For this the tumour cells attaches to the endothelial cells via adhesion molecules and secretes various proteolytic enzymes to infiltrate into the blood and lymphatic vessels (Leber and Efferth 2009). Intravasation can be facilitated by molecular changes that promote the ability of carcinoma cells to cross the pericyte and endothelial cell barriers that form the walls of microvessels.

1.3.3 Circulation: Within the blood vessel, the neoplastic cells have to withstand the unfavourable conditions present in the blood. High oxygen concentration and cytotoxic lymphocytes are the two major obstacles for the cell survival. A selection for particularly resistant and aggressive tumour cells takes place here.

1.3.4 Extravasation: When the cells reaches minute capillaries of an organ, the cells often get stuck in the capillaries and depart the blood stream by penetrating the endothelium through proliferation and/or proteolytic enzymes.

1.3.5 Colonization, proliferation and angiogenesis: the neoplastic cell settles at a distant tissue site and builds a secondary tumour. The latter proliferates and induces neoangiogenesis in order to ensure sufficient vascularization (Leber and Efferth, 2009).

Cancer therapy

Cancer management and treatment strategies include four main areas which are surgical removal, radiation therapy (including photodynamic therapy), chemotherapy (together with hormonal therapy and targeted therapy), immunotherapy and gene therapy.

1.4.1 Chemotherapy

Chemotherapy is one of the major conventional treatment modality which has been changed enormously after time allowing treatments for many previously fatal cancers. Hormonal therapy and targeted therapy are the new area that helped to improve the treatment quality and survival of the patients. Alkylating agents are the first category of chemotherapeutic drug that interacts with genomic DNA and effects its replication thus results in cell cycle arrest and finally ends up in the apoptosis of the target cell.

1.4.1.1 Alkylating agents

Nitrogen mustard alkylating agents

Cyclophosphamide (CPA) is the most successful nitrogen mustard anticancer agent with a modification in its chemical structure (Emadi *et al.,* 2009). Food and Drug Administration (FDA) approved CPA as an anticancer agent for than 50 years ago and till now it is one of the most successful anticancer drugs used to treat leukaemia (Hillmen, 2011), lymphoma (Mey *et al.,* 2012), breast cancer (López-Tarruella and Martin., 2009) and ovarian cancer (Clowse *et al.,* 2009).

Others

Nitrosoureas synthesized by National Cancer Institute (NCI)is another type of alkylating agent which is widely used to treat brain tumours as it can cross blood brain barrier (Wasserman *et al.,* 1975). Triazenes, are other group of anticancer agents with limited toxicity and good pharmaco-kinetic properties (Marchesi *et al.,* 2007).

1.4.1.2 Platinum based compounds

Thousands of platinum based anti-cancer agents were synthesised and tested out of which cisplatin was the most successful candidate but with a number of toxic side effects like renal and neutotoxicity. Platinum resistance was also a challenge for these agents. Carboplatin was developed as derivative with similar efficacy but showed hematopoietic toxicity (Hong *et al.,* 2010).

1.4.1.3 Antimetabolites

Antimetabolites like folic acid analogues (Aminopterin, methotrexate), purine analogs (6-mercaptopurine, *Fludarabine*), pyrimidine analogs (*Fluouracil, Capecitabine*) and cytidine analogs, all these are found to be very effective to treat various types of cancers like childhood cancer but are associated with a number of toxic side effects including hepato, hemato and intestinal toxicities (Guimarães *et al.,* 2013).

1.4.1.4 Microtubule targeting agents

They are mitosis inhibiting agents that are broadly classified into microtubule stabilizing (e.g. taxanes and epothilones) and microtubule-destabilizing (e.g. vinca alkaloids) drugs. Vincas are the first natural anticancer agents used in clinical practice. There are three major vinca alkaloids in clinical use: vinblastine, vincristine and vinorelbine. Vincas cause microtubule depolymerisation, thereby inhibits mitotic progression and leads to apoptosis of the cell and is used to treat lymphoma and breast cancer whereas myelosupression is the limitation. Taxanes including Paclitaxel are the microtubule-stabilizing anticancer agents used to treat metastatic breast cancer but a number of undesirable side effects, as well as resistance in cancer cells are caused by them (Guimarães *et al.,* 2013).

1.4.1.5 Camptothecin analogs

Camptothecin is a pentacyclic quinoline alkaloid present in wood, bark, and fruit of the Asian tree *Camptotheca acuminate*, that inhibit topoisomerase I enzyme thus blocks DNA replication (Pommier, 2006). Irinotecan and topotecan are the main analogues in clinical use and are found to be less toxic and currently used in a wide spectrum of cancers. Nausea, vomiting and diarrhoea are the major side effects (Grochow *et al.,* 1992; Ormrod and Spencer, 1999).

Besides these, various Podophyllotoxin (etoposide and teniposide), antibiotics like doxorubicin, daunorubicin (anthrocyclines), Bleomycins etc, enzymes (L-Asparaginase) and Thalidomide are also used in cancer treatment. Eventhough a number of agents are available for cancer treatment the therapy remains a challenge for physicians and researchers, especially with regards to the tumour resistance and side effects (Guimarães *et al.,* 2013).

1.4.1.6 Targeted therapy

Targeted therapy is a kind of chemotherapy that excludes main side effect of conventional chemotherapy that is the toxic side effects to the normal cells. Drugs used in targeted therapies can be enzyme inhibitors like tyrosine kinase inhibitor, growth factor inhibitor or receptor inhibitor. Apoptosis inducing agents or anti-angiogenic substances are also used as targeted therapy agents. Targeted therapy drugs may be antibody to the specific target molecule or small molecule inhibitors of specific proteins. Examples are imatinib mesylate against gastric cancer coming under the category tyrosine kinase inhibitor, Gifitinib against non small lung cancer which is a VEGF receptor inhibitor. Drug is administered either by intra venously or by catheterisation. Side effects are there for targeted therapy and the severity is depends upon the target selected, but it is not as much severe as that of standard chemotherapy (Agha and Kinahan, 2007; Braiteh *et al.,* 2008; American Cancer Society, 2013).

1.4.2 Radiation therapy

Radiation therapy is an important treatment strategy for cancer patients that primarily affect the actively dividing cells. It may takes days or weeks to kill the cancer cells at the same time it also affects other actively dividing cells including skin, bone marrow, and the lining of the intestines etc. whereas nerve, breast, brain, and bone tissue are less affected. The term ‘radiosensitivity’ explains the probability of a cell to get damaged by radiation and it is depended upon the cell cycle stage in which the cell is present. Radiation used in cancer treatments are ionising radiation and and it is mainly of two types, Photon radiations (X-rays and gamma rays) and particle radiation (electrons, protons, neutrons, carbon ions, alpha particles, and beta particles) (American Cancer Society, 2014). Ionising radiation induces the production of reactive oxygen species (ROS), which include superoxide, hydroxyl radicals, singlet oxygen and hydrogen peroxide. These free radicals react with critical cellular components, such as DNA, RNA, proteins and membranes, resulting in cell dysfunction and death (Aljanabi *et al*., 2013). Major side effects of radiotherapy comprise tissue injury in target and non-target cells, especially cells of immune system (Pratheeshkumar and Kuttan, 2010).

1.4.3 Immunotherapy

Immunotherapy works on the principle of tuning patients own immune system to fight against cancer and is also kwon as biotherapy. Monoclonal antibodies, immune checkpoint inhibitors, cancer vaccines, cytokine therapy etc are coming under this treatment strategy.

1.4.3.1 Monoclonal antibodies (mAb)

Nacked mAbs like alemtuzumab (for chronic lymphocytic leukemia), trastuzumab (binds to HER2 protein) or conjugated antibodies can be used against specific target. Conjugated mAb can be radio labelled (Ibritumomab tiuxetan against CD 20) or chemo labelled (Brentuximab vedotin to treat Hodkins lymphoma) (American cancer society, 2015).

1.4.3.2 Immune check point inhibitors

PD1 molecule on T-lymphocyte targets cells lacks PD-L1, as an immune escape mechanism cancer cells over express PD-L1. Antibodies against PD-1or PD-L1 are used to treat melanomas (Pembrolizumab and Nivolumab) (American cancer society, 2015).

Various cancer vaccines to prevent (HPV and Hepatitis B vaccines) and treat cancer (Sipuleucel-T for prostate cancer) have been developed. Cytokine therapy is another method where IL-2 and Interferons are mainly used to boost antitumour immune response (American cancer society, 2015).

1.4.3.3 Complementary and alternative medicines

Conventional cancer treatment modalities are always bound to undesirable side effects sometimes that may be deleterious. Radiation treatment and chemotherapy drugs are toxic to the adjacent normal cells. These side effects can be acute or intermediate or late along with the chance of secondary tumour development. Complementary and alternative medicines are used to improve the health and survival of the patient along with the quality of life (Metri *et al.*, 2013).

1.5 *Curculigo orchioides* Gaertn.

Curculigo orchioides Gaertn (Family: Amaryllidaceae) is a tiny herbal plant widely distributed in China, India, Malaya, Japan, Australia and is popularly known as black musali in India (Wu *et al.,* 2005; Nagesh and Shanthamma, 2009). It is a geophilous perennial plant of about 30 cm height with short elongated root stock bearing lateral fleshy root. The genus consists of 10 species, out of which 3 species are present in India. The plant is believed to be originated from Asian thick forest and found to be distributed all over the India, especially in Western Ghats, Eastern Ghats and sub-tropical regions of Himalaya. Majority of the botanist classifies this plant under the Family Amaryllidaceae but some botanists include this plant under the family Hypoxidaceae (Asif, 2012) (Figure 1.1).

1.5.1 Phyto-constituents of the plant

Phytochemical screening of the plant was done by Agrahari *et al.,* and revealed the presence of carbohydrate & glycosides, saponins in high concentration followed by alkaloids, protein & amino acid, phytosteroids and gums & mucilage in extracts where as the phenolic compounds are present in lower concentration. Powder analysis parameters like extractive value, total ash, acid insoluble ash, water soluble ash and loss on drying were also determined by them (Agrahari *et al.,* 2010). Asif reported the presence of various mono saccharides and glucuronic acid in *C. orchioides* tubers along with other polysaccharides and glycosides (Asif, 2012). Evaluation of the hexane extract also showed the presence of alkaloid-lycorine, sterols including β-sitosterols and sapogenin called yuccagenin. A flavone glycoside from the rootstock has been isolated and identified as 5,7-dimethoxy glucopyranoside along with a number of fatty acids from *C. orchioides* root oil by Misra and team (Misra *et al*., 1984; Asif, 2012). GC-MS analysis of the methanolic extract of the plant was done by Daffodil *et al.,* 2012.

In 1992, four new cycloartane-type triterpene glycosides were isolated from rhizomes of *C. orchioides* and named as curculigosaponins G, H, I, and J by Xu *et al*. Their structures were elucidated by FAB-MS, 1H-, 13C-NMR, and 2D-NMR analysis and chemical evidence. From pharmacological study they found that

curculigosaponin G increased the weight of the thymus *in vivo* in mice (Xu *et al.,* 1992).

Two phenolic glucosides were isolated from the plant, named orchiosides A and B and their structures were elucidated by the combination of 2D-NMR analysis, mass spectrometry and chemical evidences. Along with this, four known compounds were also isolated by Gupta *et al.,* in 2005. A new hepatotoxic cycloartane-type triterpenoid ketone was identified and isolated by Jiao in 2013 (Jiao *et al.,* 2013). Wu in 2005 isolated a new orcinol glucoside, from the rhizomes of *C. orchioides* together with seven known compounds including Curculigoside and syringic acid. The structures of these compounds were elucidated using spectroscopic methods. The antioxidant activities of these isolated compounds were evaluated and all the compounds showed potent antioxidative activities (Wu *et al.,* 2013).

Six new cycloartane glycosides were isolated from the rhizomes of *C. orchioides* and their structures were determined by spectroscopic analyses by Yokosuka and colleagues. Cytotoxic activity was determined against HL-60 human leukemia cells and two compounds 1 and 1a showed cytotoxic activity against HL-60 cells with IC_{50} values of 9.0 and 1.8 μM, respectively (Yokosuka *et al.,* 2010).

1.5.2 Pharmacological properties

Curculigo orchioides Gaertn. is an important Rasayana drug according to the Ayurveda system of medicine. Rasayana is a unique concept of Ayurveda which means vital nourishment (Rasa + Ayana) representing a holistic approach, responsible for preventive aspects against ageing as well as curative aspect against diseases (Chauhan and Dixit, 2008; Mehrotra and Ojha 2006). In other words Rasayana is based on the concept of modulation of immune responses to alleviate the diseases (Patwardhan *et al.,* 1990; Bafna and Misra, 2006).The rhizome, as well as the tuberous roots of the plant has been extensively used in indigenous systems of medicine in India, Pakistan and China for the treatment of various diseases, including cancer, jaundice, asthma and diarthrosis wound healing (Dhar *et al.,* 1968).

Acute toxicity studies of the plant extract was conducted and it was found to be safe up to 2 g/kg body weight dose (Asif and Kumar, 2010). Immune stimulatory effects of the plant were reported by Lakshmi *et al*. (2003). They evaluated the plant extract as well as two isolated glycosides to study their effect on macrophage migration index (MMI), haemagglutination (HA) titre, PHA-induced blast transformation of lymphocytes (BTL) and delayed type hypersensitivity (DTH). Significant immunostimulant activity was found in purified glycoside-rich fraction isolated from the ethyl acetate extract of the plant. On another hand Bafna and Mishra evaluated the methanolic extract of the plant for their immune-stimulatory effects in animals treated with cyclophosphamide to suppress their immune system. Total WBC count, HA titre and DTH response were evaluated and the study was concluded with the statement that "the immunostimulatory effect produced by methanol extract of *Curculigo orchioides* in cyclophosphamide-induced immunosuppression may be due to cell mediated and humoral antibody mediated activation of T and B cells" (Bafna and Mishra, 2006).

C. orchioides was reported to exhibit antibacterial activity against pathogenic strains of Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhimurium*) bacteria (Nagesh and Shanthamma, 2009; Asif, 2012). Marasini and colleagues evaluated the the *in vitro* antibacterial activity of ethanolic extracts of 16 different traditionally used medicinal plants. And they found that *C. orchioides* along with three other medicinal plants exhibit the maximum growth inhibitory effects on different bacterial strains with the minimum inhibitory concentration less than 100 µg/ml (Marasini *et al.,* 2015). Antifungal, antibacterial and antitumour activity of the plant was measured by Singh and Gupta. Various extracts of the plant was prepared and antifungal activity was screened using agar plate method, antitumour activity was screened against a human breast cancer cell line (MCF-7) and reported that methanolic extract showed maximum activity due to the saponins present in it (Singh and Gupta, 2008). Balamurugan and co-workers analysed the cytotoxic effect of the methanolic extract of the plant and reported that the extract doesnot exhibit cytotoxic effects against normal cells like Vero and cancer cells that is Hep2 (Balamurugan *et al.,* 2009).

The plant is reported to have anti-asthmatic effect in various *in vivo* and *in vitro* models by Patil *et al* (2010). Mast cell stabilization and antihistaminic potentials of the plant was evaluated and found that the plant inhibits the mast cell-derived immediate-type allergic reactions and mast cell degranulation under experimental condition (Venkatesh *et al.,* 2009). Asif and Kumar (2010) evaluated the effects of hydroalcoholic extract (HE) of *C. orchioides* rhizome and its alkaloidal and non-alkaloidal fractions were evaluated in carrageenan-induced paw edema experimental models of inflammation. Scientific investigation on the effects of *C. orchioides* on acute reflux esophigitis (RE) in rats induced by pylorus and forestomach ligation operation was performed by Ku and co-workers. The results suggested that the extract down regulated the proinflammatory cytokine levels in the rat and thus attenuate the severity of reflux esophagitis and prevent esophageal mucosal damage (Ku *et al.,* 2012).

From ancient time the plant is well known for its aphrodisiac properties. There are a number of scientific evidences for these effects of which some are listed below. ethanolic extract of rhizomes was evaluated for its effect on sexual behavior in rats by Chauhan and co-workers. Administration of the extract changed significantly the sexual behavior as assessed by determining parameters such as penile erection, mating performance, mount frequency and mount latency. Moreover a pronounced anabolic and spermatogenic effect was evidenced by weight gains of reproductive organs (Chauhan *et al.,* 2007). Thakur *et al*. found that the extract treatment was helpful in ameliorating the damage happened to the sexual functions caused by sustained hyperglycemia which was evidenced by the principle parameters like, male sexual behavior, sperm count, penile erection index and seminal fructose content and concluded that antioxidant and anabolic activities of the extract could be a major attribute in preserving the sexual functions in hyperglycemic male (Thakur *et al.,* 2012). The plant was also found to exhibit oestrogenic potential in experimental animals (Vijayanarayana *et al.,* 2007).

The plant has an immense capability to resist osteoporosis which is supported by a number of literatures. Cao and team reported the protective effect of *C.*

orchioides on bone loss in ovariectomized rats by inhibiting bone re-sorption and increasing serum phosphorus and calcium levels, without affecting bone formation. They suggested that the plant can be considered a potential antiosteoporosis herbal plant (Cao *et al.,* 2008). Jiao and colleagues isolated the constituent phenolic compound from the plant that is responsible for the anti-osteoporotic effects (Jiao *et al.,* 2009). Wound healing effect of the plant was studied by Agrahari under experimental conditions (Agrahari *et al.,* 2010) and reported its effectiveness.

Anti-oxidant and hepato protective effects of the plant in carbon tetra chloride induced toxicity was analysed by Venukumar and Latha (2002) and found that methanol extract of rhizomes of *C. orchioides* exhibit a liver protective effect against CCl4- induced hepatotoxicity and possess anti-lipid peroxidative and antioxidant activities. Ramchandani and co-workes revealed the neuro-protective effects of the plant against cyclophosphamide induced toxicity and proposed that the flavonoids and polyphenols present in the plant may be the responsible agents (Ramchandani *et al.,* 2014).

1.6 Curculigoside

In 1983 Kubo *et al.,* isolated a phenolic glucoside from the plant and named as Curculigoside. They determined both the IR and NMR spectra and its structure was elucidated as 5-hydroxy-2-O-β-D-glucopyranosylbenzyl 2,6-dimetoxybenzoate (Figure 1.1).The isolated compound was found to enhance the phagocytic activity of mice peritoneal macrophages (Kubo *et al.,* 1983). Curculigoside is reported as the major bioactive component of the plant *C. orchioides* (Ma *et al.,* 2011; Wang *et al.,* 2010; Liu *et al.,* 2014). Lu *et al.,* in 2002 standardised the HPLC protocols for the determination of the presence and percentage of Curculigoside in *C. orchioides* extracts and reports it as a quality determination method of the crude medicine prepared from the plant. Application of preparative high-speed counter-current chromatography (HSCCC) for the isolation of Curculigoside was described by Peng (Peng *et al.,* 2006). They standardised all the chromatographical conditions for the separation of highly pure Curculigoside from the plant.

Protective effect of Curculigoside against adjuvant induced arthritis in rat was evaluated by the team of Ding and found that the inhibition of NF-κB as the mechanism of action. They also found that the compound has inhibitory effect on the proinflammatory cytokine levels (Ding *et al.,* 2016). Osteoblast-protective mechanisms of Curculigoside through inducing proliferation, differentiation and reducing the inflammatory responses were revealed by Zu *et al.,* 2015.

Alzheimer's disease (AD) and osteoporosis represents two closely related multifactorial progressively degenerative diseases that predominantly affect aged people. Curculigoside was found to be an effective remedy for these two degenerative diseases. Curculigoside enhances calcium deposition and elevate the levels of ALP and Runx2 in osteoblasts under oxidative stress through its antioxidant potential (Zhao *et al.,* 2015). Another effect of Curculigoside is the ability to inhibit MMP-1 production in skin fibroblast cells and thereby it effects the skin ageing (Tundis *et al.,* 2015; Lee *et al.,* 2009).The compound also showed protective effects against H_2O_2 induced damages in endothelial cells which emphasize its anti oxidant potential (Wang *et al.,* 2010). A study was designed by Wu *et al.,* in 2012 to evaluate the ameliorative effect of the plant on learning and memory in aged rat and found that Curculigoside can improve cognitive function in aged animals, possibly by decreasing the activity of AchE (Acetylcholine esterase) in the cerebra and inhibiting the expression of BACE1 (Beta site Amyloid Precursor Protein Cleaving Enzyme) in the hippocampus.

Bone formation and fracture healing ability of Curculigoside was evaluated by Ma and coworkers and found that treatment with Curculigoside enhanced the proliferative ability of MC3T3-E1 cells with an enhanced release of vascular endothelial growth factor (VEGF), Fms-like tyrosine kinase-1 (Flt-1) and bone morphogenetic protein-2 (BMP-2) by the cells (Ma *et al.,* 2011). Kang *et al.,* 2014 evaluated the mechanism of angiogenesis brought about by Curculigoside and found that it is mediated through the VCAM-1/Egr-3/CREB/VEGF signaling pathway.

Based on these literatures, the present study was designed to analyse the efficacy of *C. orchiodes* and its derived product to prevent the tumour progression, at the same time the ameliorative effects of the plant on chemotherapy induced side effects were also investigated.

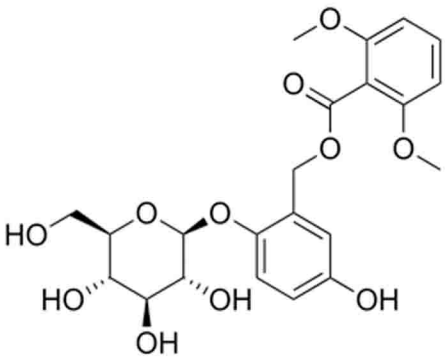
FIGURE 1.1. *CURCULIGO ORCHIOIDES GAERTN.* AND CURCULIGOSIDE

C. ORCHIOIDES PLANT

WITH TUBER

WITH INFLORESCENCE

CURCULIGOSIDE-STRUCTURE



Chapter 2

Methodology

2.1. Test Materials

2.1.1 *Curculigo orchioides* Gaertn.

Curculigo orchioides whole plant was collected locally and a voucher specimen was deposited at Amala Ayurveda Pharmacy after authentification.

2.1.2 Curculigoside

Curculigoside ($C_{22}H_{26}O_{11}$) is a phenolic glucoside of molecular weight 466.44 was obtained from Shanghai Standard Biotech, China, at a purity of 97.227%.

2.2. Cell lines

Dalton lymphoma ascites cells (DLA) and Ehrlischs Ascites carcinoma cells (EAC) were obtained from Adayar Cancer institute and maintained in Swiss albino mice as ascites tumour. L929 (lung fibroblast cells), B16F10 (mouse metastatic melanoma cells), EL-4 (mouse lymphoma) and K562 (Human myelogenous leukemic cells) were obtained from National Centre for Cell Sciences (NCCS), Pune, India.

2.3.Experimental Animals

Balb/c and Swiss albino mice were obtained from small Animal Breeding station, Veterinary University, Mannuthy, Kerala, India. C57Bl/6 mice were obtained from Sri Venkateshwara Enterprices, Subramanya Nagar, Bangalore (CPCSEA No. 237/2000).

The animals were kept in a controlled environment (Temperature: 23°C±1°C, 12-h light/12-h dark), fed with normal mouse chow (Krish Scientific Stores, Bangalore, India) and water *ad libitum*. Prior approval of the Institutional Animal Ethics Committee (IAEC) was procured and animal experiments were conducted strictly adhering to the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) constituted by the Government of India.

2.4.Chemicals, Reagents and Culture medium

2.4.1. Tissue culture Medium and Medium components

Dulbecco's Modified Eagles Medium (DMEM)	:	Sigma Chemicals, St. Louis, USA
Minimum Essential Medium (MEM)	:	-do-
Rosewell Park Memorial Institute medium (RPMI-1640)	:	-do-
Fetal bovine Serum	:	Biological Industries, Israel.
Trypsin	:	HiMedia Laboratories, India.
L-Glutamine	:	-do-
Fluid Thioglycollate Medium	:	-do-
HEPES Buffer	:	-do-

Culturewares

Canted neck culture flasks	:	BD Falcon, India
24 well plate	:	Tarsons, India
96 well plate	:	-do-

2.4.2. Chemicals

2-mercaptoethanol	:	Sigma, USA
Acrylamide	:	-do-
Benzamidine	:	-do-
Bromophenol blue	:	-do-
Collagen solution Type I (from calf skin)	:	-do-
Chloramine T hydrate	:	-do-
DEPC (Diethylpyrocarbonate)	:	-do-
ECM-Gel (from Engelbreth Holrn-Swarm mouse sarcoma)	:	-do-
Ethidium bromide	:	-do-
Gelatin Type A (from porcine skin)	:	-do-
Glycyl-glycine	:	-do-
Hydroxyproline	:	-do-

γ-GT substrate	:	-do-
(L-Glutamic acid γ-p-nitroanilide/		
L-γ-Glutamyl-p-nitroanilide)	:	-do-
MTT (Methyl thiazol tetrazolium Bromide)	:	-do-
Phytohaemagglutinin	:	-do-
Sodium acetate	:	-do-
Molecular grade water	:	Genei, Bangalore
dNTPs	:	-do-
Oligo (dT)	:	-do-
Taq Polymerase enzyme and buffer	:	-do-
AMV RT buffer and enzyme	:	-do-
$MgCl_2$	:	-do-

2.4.3. Reagents

2.4.3.1 Phosphate Buffered Saline (PBS)

NaCl	: 8.00 g
KCl	: 0.20 g
$Na_2H_2PO_4$	: 1.44 g
KH_2PO_4	: 0.20 g

Dissolved and made up to 1L with distilled water (pH - 7.2) (Manu, 2009)

2.4.3.2 PBS-EDTA

EDTA	: 0.02 g
PBS	: 100ml

2.4.3.3 Trypsin (0.2%)

Trypsin	: 0.2 g
Glucose	:0.02 g
PBS-EDTA	:100 ml

Prepared in cold and sterilized by filtration.

2.4.3.4 Turks Fluid (Diluting Fluid)

Glacial acetic acid	: 1.5 ml

0.1 % Crystal violet : 1 ml

(in 20% methanol)

Made up to 100 ml using distilled water, kept for overnight stirring and filtered.

2.4.3.5 Griess Reagent

Solution A.

N- (1-Naphthylethylene diamino dihydrochloride) (NNED) : 1%

Solution B.

Sulfanilic acid in 5% H_3PO_4 : 1%

Mixed A and B in 1:1 ratio. (Manu, 2009)

2.4.3.6 Alsevier's solution

Dextrose	: 2.05g
Sodium citrate	: 0.80g
NaCl	: 0.42g

Dissolved in distilled water, adjusted the pH to 6.1 with 10% citric acid and made up to 100 ml with distilled water.

2.4.3.7 Scintillation fluid (Manu, 2009)

PPO	: 2.5g
POPOP	: 0.25g
Naphthalein	: 100g
Dioxan	: 1000ml

2.4.4. Stains

2.4.4.1 Trypan blue

Trypan blue : 1g in 100 ml normal saline, stirred over night and filtered.

2.4.4. 2 Crystal violet solution

Crystal violet	: 50mg
Methanol	: 20ml
Distilled water	: 80ml

2.4.4.3 Eosin

Eosin	: 500mg
Ethanol	: 100ml (Final volume)

2.4.4.4 Harris haematoxylin

Haematoxylin	: 5g
Ethyl alcohol	: 50ml
Potassium alum	: 50mg
Potassium iodide	: 50mg

Haematoxylin was dissolved in alcohol by gentle heating. Alum was dissolved in distilled water by heating with frequent stirring and kept overnight at 40^{o} C. Alcoholic haematoxylin was added to the alum solution. The mixture was cooled and potassium was added and filtered (Manu, 2009).

2.4.4.5 Leishman stain : 0.15% in methanol

2.5. Instruments

Autoclave	:	Kemi, India
Automatic Gamma Counter	:	PerkinElmer, USA
Rack Beta Fluid scintillation counter	:	Wallac, Finland
CO_2 Incubator	:	Napco, Canada
Colony counter	:	Equitron, India
Counting chamber	:	Rohem instruments Pvt Ltd. India
Deep Freezer	:	Remi, Chennai, India
Disc electrophoresis unit	:	Balaji Scientific Service, India
Distillation unit	:	Rotek, India
Electronic Balance	:	Schimadzu, Japan
ELISA- Reader	:	Thermolab systems, USA
Gel Documentation system	:	Vilber Lourmat, France
High speed cooling centrifuge	:	Remi, Chennai, India
Hot air oven	:	Amur Instrumentation, India
Inverted Microscope	:	Leica, Germany
Lyophilizer	:	Labconco Inc, USA
Minicycler – Thermocycler	:	MJ Research, USA

Modified Boyden Chamber	:	Nucleopore, USA
Modulator incubator chamber	:	Billups Rothenberg, Del Mar, CA
pH meter	:	Elico, India
Rotory Evaporator	:	Superfit Continental Pvt. Ltd, India
Shaking waterbath	:	Pooja Lab Equipments, Mumbai
Spectrophotometer	:	Elico, India
Spin win Microcentrifuge	:	Tarson, India
Submerged electrophoresis unit	:	Genei, Bangalore, India
Tissue homogenizer	:	York Scientific Industries, Delhi
Transilluminator	:	Vilber Lourmat, USA

2.6. METHODS

2.6.1 Preparation of *C. orchioides* extract

C. orchioides whole plant with maximum tuber size was collected locally, washed, shade dried and powdered. Plant powder was extracted with 70% methanol using soxhlet apparatus for 24 hr, alcohol was evaporated under reduced pressure at 42^{o}C using rotary evaporator. The yield of extract was 10.2% (w/w). For *in vivo* studies the dose was determined by detailed toxicity analysis, the extract was resuspended in 1% gum acacia and administered intra peritoneally (i.p.) to the animals. For *in vitro* studies, the extract was dissolved in DMSO and diluted with medium to get required concentrations so that the final DMSO concentration is 0.1% or below.

2.6.2 Curculigoside

Curculigoside obtained from Shanghai Standard biotech is dissolved in Phosphate buffered saline for *in vivo* analysis and administered i.p. to the animals. The dosage was determined after a detailed toxicity analysis. For *in vitro* analysis, the compound was dissolved in medium and diluted to get required concentration.

2.6.3 Tissue Culture

2.6.3.1 Processing and sterilization of culture wares

All autoclavable culture wares, glass bottles and filtration apparatus were soaked in 1% extran and washed thoroughly, rinsed with distilled water and dried in a hot air oven. Culture wares were sterilized by autoclaving at 15 pounds/square inch pressure for 15 min and dried in a hot air oven before using (Manu, 2009).

2.6.3.2 Preparation of culture media

All media were prepared in autoclaved double distilled water. Medium powder (DMEM – 9.98 g/L; RPMI - 10.3 g/L) was dissolved in water, 2mM L-glutamine was added and pH was adjusted using sodium bicarbonate to 7.2. Medium was filtered with cellulose acetate membrane (pore size – 0.22μm) using negative pressure. One ml medium was inoculated to 5 ml fluid thioglycolate medium (29.75 g/L) and incubated at 37^{o}C to check the sterility by observing for any visible contamination. Antibiotics like Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the medium along with 10% foetal bovine serum prior to use (Manu, 2009).

2.6.3.3 Maintenance of K562 and EL-4 cell lines in tissue culture

These cell lines were maintained as suspension culture, and incubated at 37^{o}C providing proper agitation. RPMI medium was used to culture the cells and were subcultured every third day by adding aliquots to fresh bottle with RPMI medium (with 10% FBS and antibiotics) after scattering the cell clumps (Manu, 2009).

2.6.3.4 Maintenance of adherent cell lines in culture

B16F10, L929, *etc*. were maintained in *in vitro* condition as monolayer culture. Culture bottles containing confluent healthy cells were taken; spent medium was removed and washed thrice with PBS. Cells were trypsinised with 0.1 ml trypsin (0.2%) and incubated at 37^{o}C for 3 min. Bottles were then tapped to detach the cells, DMEM with 10% FBS was added and pipetting was done repeatedly to make single cell suspension. Fresh bottle containing medium was taken and a small volume from the above suspension was added to the new bottle and incubated at 37^{o} C. Subculturing was performed once in a week. *In vitro* culture

of 70-80% confluency was used to perform different experiments. Cells were collected using a cell scraper by mechanical dislodgment, cell number was determined followed by the viability checking by trypan blue exclusion method (Talwar, 1974). Cell suspension with more than 95% viability was used for experiments (Manu, 2009).

2.6.3.5 Cell viability determination by trypan blue exclusion method (Kuttan *et al*., 1985**)**

Cell suspension (0.1 ml) was mixed with 1% trypan blue (0.1 ml) and kept at 37^{o}C for three min. Cell was then observed under microscope by loading on a haemocytometer and the cell count of stained and unstained cell was taken separately. Dead cells take up the dye and appeared as blue in colour whereas viable cells exclude the dye, thus appeared unstained **(**Kuttan *et al*., 1985**)**.

% cell death = [No. of dead cell / (No. of dead cells + No. of viable cells)] x 100

2.6.3.6 Long term *in vitro* cell viability studies in tissue culture (Cole, 1986; Campling *et al*., 1991)

Healthy cells were seeded at a cell density of 5000 cells/well in a 96-well flat bottom tissue culture plate. Total medium on each well is adjusted to 0.2 ml and incubated for 24h at 37°C with 5% CO_2 atmosphere. Different concentrations of the test materials were added to the wells after 24 h and again incubated under standard conditions for 48h. On 44th h 20µl of MTT (5mg/ml) was added to each well. After 48 h, the plates were centrifuged to remove supernatant and the crystals were dissolved by adding 0.1 ml DMSO to each well. The plates were then incubated at room temperature for 15 min and the optical density was measured at 570 nm (Cole, 1986; Campling *et al*., 1991; Manu, 2009).

The percentage of viable cells was determined using the formula,

% Viable cells = 1 – (OD of the drug treated/OD of the control) x 100

2.6.4 Haematological parameters

2.6.4.1 Determination of Haemoglobin (Cheesbrough and McArthur, 1976)

Principle

Potassium ferricyanide reacts with haemoglobin (oxyhaemoglobin /carboxyhaemoglobin) to form methaemoglobin which in turn converted to cyanmethemoglobin on reaction with cyanide. The intensity of colour formed is proportional to haemoglobin concentration which is compared to known cyanmethaemoglobin standard at 540 nm.

Procedure

Blood was collected from the animals in EDTA coated tubes, 0.02 ml of blood was mixed with 5ml of Drabkin's reagent and kept at room temperature for 5 min. Optical density (OD) was measured against reagent blank. Haemoglobin levels in the sample was calculated using the formula,

$$\text{gm \% of Hb} = \frac{\text{OD of the test x 251 x conc. of Std.}}{\text{OD of Std x 1000}}$$

2.6.4.2 Determination of total count of leukocytes (Cheesbrough and McArthur, 1976)

Principle

Blood sample was diluted in Turk's fluid, acetic acid in the reagent will lyse RBC and the remaining leukocytes will get stained by crystal violet. The number of cells in the large four corner squares of haemocytometer was counted.

Procedure

After mixing 0.38 ml Turks fluid and 0.02 ml whole blood, the vials were kept at room temperature for 2-3 min. The cells were mixed gently and loaded on to the haemocytometer, to count under 10X objective of microscope.

The total WBC counts was determined using the formula,

$$\text{Total WBC/mm}^3 = \frac{\text{No. of cells counted x dilution factor x depth factor}}{\text{Area counted}}$$

where,

Dilution factor = 20

Depth factor = 10mm

Area counted = 4sq.mm

Therefore, Total leukocyte counts/mm3 = N X 50

2.6.4.3 Differential count of leucocytes (Cheesbrough and McArthur, 1976)

Procedure

A thin, even smear of blood sample was made on a clean glass slide and air dried. Slides were stained with few drops of Leishman's stain by pouring over the smear and kept for 3 min. The stain was diluted with distilled water and kept for 10 min, washed under tap water and air dried. Slides were observed under oil immersion with 100X objective to count different cell types by analysing the morphology and a total of 100 cells were counted.

2.6.5. Immunological parameters

2.6.5.1 Sheep RBC collection and preparation (Mehera and Vaidya, 1993)

Sheep blood was collected from Veterinary University, Mannuthy in equal volume of sterile Alsever's solution and stored at 4^{o}C for not more than one week. PBS wash was given three times and the cell pellet was finally resuspended in Hanks balanced salt solution (HBSS). For trypsinization, 4% SRBC and 1% trypsin were mixed in a ratio of 10:1and incubated at 37^{o}C for 30 min. Cells were then washed with PBS and resuspended to get 2% concentration.

2.6.5.2 Anti SRBC antibody production in rabbits (Moudgil and Singh, 1997)

SRBC (2%) suspension was prepared in saline and mixed with Freund's complete adjuvant in a ratio of 1%. This was injected intradermally to young healthy rabbit and after 4 weeks a booster dose was given. Blood was collected

from the rabbit 24 hrs after the booster dose to separate serum, which is heat inactivated (56^{o} C; 30 minutes) and the antibody titre value, was checked by haemagglutination method (Singh *et al*., 1984). Serum was diluted according to the titre value and used for the experiments.

2.6.5.3 Preparation of spleen cells

Animals were sacrificed to collect spleen asceptically without any adherent tissue. Spleen was chopped into small pieces using sterile scissors and teased over a stainless steel mesh in cold PBS or HBSS. Suspension was collected in a centrifuge tube and kept in ice for 2 min to settle the clumps; supernatant was mixed with HBSS and centrifuged. After 3 times washing with HBSS, the cell pellet was re-suspended in RPMI-1640 medium to get required cell number.

2.6.5.4 Preparation of bone marrow cells

All the procedures were done under sterile conditions. Mice were sacrificed by cervical dislocation and femur bone was collected, from both ends the shaft of the femur was removed and using a 26G needle and syringe the bone marrow was flushed out of the cavity by passing a jet of medium with 2% FBS. Collected bone marrow was made into a single cell suspension by repeated pipetting. The cells were pelleted by centrifugation, re-suspended in RPMI-1640 medium and cell number was adjusted to required concentration.

2.6.5.5 Preparation of thymus cells

Thymus was collected from the animals after sacrificing and suspended in HBSS and processed to make single cell suspension in RPMI- 1640 medium with 10% foetal bovine serum.

2.6.5.6 Preparation of peritoneal macrophages

Mice were injected with 0.2ml of 5% sodium caseinate solution i.p. to elicit peritoneal macrophages. After five days animals were sacrificed asceptically, skin was removed and a small slit was made on the peritoneal membrane. After that 5ml PBS or HBSS was injected to the cavity through this slit. The peritoneal cavity was gently prodded and the peritoneal fluid containing

macrophages was aspirated out to wash the cells and resuspended in RPMI-1640 to the desired cell concentrations.

2.6.5.7 Determination of α-naphthyl acetate esterase activity (Bancroft and Cook, 1984)

Principle

The enzyme hydrolyses the substrate to form an invisible primary reaction product. The complex is coupled with the diazonium salt to produce coloured final reaction product.

Procedure

Femur bone was collected from the animals after sacrificing them and the bone marrow cells were flushed out and collected in test tube containing PBS with 10% goat serum. Cells were suspended in minimal volume of PBS with serum and a thin smear of these cells were prepared on a clean glass slides. Smear was air dried and fixed using fixative containing formaldehyde. Reaction solution containing pararosaniline sodium nitrate and α-naphthyl acetate was taken in a coupling jar and slides were dipped in it for 45 min. Slides were then washed and counter stained with haematoxylin for 2 min, again washed and dried. Slides were observed under oil immersion objective of microscope to count yellowish brown coloured α–esterase positive cells.

2.6.5.8 Determination of circulating antibody titre (Singh *et al*., 1984).

Principle

SRBC agglutination by the antiSRBC antibody will result in the formation ‘mat’, whereas the non agglutinated cells will settle in the bottom of the well as a clear ‘button’. The antibody titre value is interpreted as the maximum dilution of anti-sera at which clear agglutination observed.

Procedure

Animals were treated with test materials and then immunized with SRBC. Blood was collected from the animals and serum was separated (antisera) at different

time points. PBS (0.1ml, pH 7.2) was added to each well of 96 well round bottom tissue culture plate, 0.1 ml antisera was added to first well and then it was serially diluted. Trypsinized SRBC (0.1 ml) was added to each well, mixed gently and incubated at room temperature for 3 h. The highest dilution giving clear agglutination was noted.

2.6.5.9 Determination of antibody forming cells (Jerne and Nordin, 1963)

Principle

Spleen cells of SRBC immunised animals will have antiSRBC antibody producing cells. Antibody released by these cells along with complement cause the lysis of SRBC in its vicinity in a semi solid medium, leads to the formation of clear area around the cells (plaque).

Procedure

Agarose (0.5 % at 45^{o}C) 0.5 ml, 0.05 ml SRBC (7%) and 0.05ml spleen cells (8 x10^{6} cells/ml) were mixed in tubes. The mixture was poured over a glass slide, spread in an area of 10cm^{2} and the gel was allowed to solidify. The slides were incubated at 37^{o}C for 1 h with fresh rabbit serum (1:10 diluted with PBS, pH 7.2) as a source for complement. The number of plaques were counted using a colony counter and represented as plaque forming cells/10^{6} spleen cells.

2.6.5.10 Proliferation assay of splenocytes, thymocytes and bone marrow cells (Justo *et al*., 2003)

Principle

Mitogens enhance the cell division in resting lymphocytes and this increased cell division can be indirectly quantified by measuring the thymidine uptake. For this, culture medium was supplemented with radio labelled (tritiated ^{3}H) thymidine and using a beta counter, beta ray emission from the precipitated DNA of the cells was counted.

Procedure

Animals were treated with the test materials and sacrificed to collect spleen, thymus and bone marrow under sterile conditions. Cells were processed in RPMI medium and $5x10^4$ cells were seeded in a 96 well round bottom titre plate. Cells were treated with various mitogens in complete RPMI medium and incubated under standard conditions of 5% CO_2 and 37^oC for 48 h. The mitogens used in this study were PHA (2.5µg/ml), Con-A (10µg/ml), PWM (10µg/ml) and LPS (10µg/ml). Cells were labelled with 1µCi of [^{3}H] thymidine, DNA was precipitated with 10% perchloric acid and the pellets were dissolved in 0.5 ml of 0.5N NaOH. The contents were then transferred to 5 ml scintillation fluid, kept overnight in dark and radioactivity was measured using a Rack Beta fluid scintillation counter as Counts per min (CPM).

2.6.6 Analysis of cell mediated immune response (Kim *et al*., 1980; Gupta and Bhattacharya, 1983**)**

Cell mediated immune response against tumour cells including natural killer cell (NK cell) mediated killing, antibody dependant complement mediated cytotoxicity (ACC), antibody dependant cellular cytotoxicity were determined by ^{51}Cr-release assay (Kim *et al*., 1980).

Principle

Viable cells uptake ^{51}Cr, which can bind to cytoplasmic proteins and when the cell membrane gets damaged ^{51}Cr is released in to the medium.

^{51}Cr labelling of target cells

K-562 (10^6) and SRBC (10^7) were used as the target cells. Cells were washed two times with RPMI- 1640 medium and resuspended in few drops of FCS. To this cell suspension, 100µCi of $Na_2{}^{51}CrO_4$ was added and incubated with shaking at 37^{o}C for 1 h. After washing with medium, cells were again incubated for 1 h in 5ml medium at 4^{o}C. Cells were washed and cell number was adjusted to$1x10^5$ cells/ml.

2.6.6.1 Determination of Natural Killer cell mediated cytotoxicity (Kim *et al*., 1980; Gupta and Bhattacharya, 1983**)**

Equal volume of Spleen cells and labelled target cells (K-562) were mixed in a 96- well round bottom titre plates to get an effector: target ratio of 100:1. Plates were incubated at 37^{o}C for 4 h, centrifuged for 15 min, supernatant (100µl) was collected and radioactivity measured in a gamma counter.

The following control tubes were kept along with each experiment.
Spontaneous release (SR) - wells contained only target cells and medium.
Total release (TR) - wells contained target cells, medium and 0.1 ml of 1N HCl

Calculations

$$\% \text{ Lysis} = \frac{\text{Experimental release- Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

2.6.6.2 Determination of antibody-dependent cellular cytotoxicity (ADCC) (Kim *et al*., 1980; Gupta and Bhattacharya, 1983**)**

Here SRBC is used as the target cells. Spleen cells (effector cells) and SRBC were mixed in equal volume to get effector-target ratio of 100:1. The suspension along with 0.05ml of anti-sera against SRBC was incubated at 37^{o}C for 4h. ^{51}Cr release assay was performed to determine the antibody dependant cellular cytotoxicity.

2.6.6.3 Determination of antibody-dependent complement-mediated cytotoxicity (ACC) (Kuttan *et al*., 1985)

Principle

Complement proteins will interact with the Fc portion of antibodies when they bind to specific antigens. This will leads to the activation of classical complement pathway and target cell lysis.

Procedure

Rabbit serum diluted to 1:10 is used as source of complement and the antiserum collected from the animals were diluted to 1:1, 1:2 and 1:4 dilutions. To 10^6 B16F10 cells, 0.1 ml antiserum and 0.05 ml rabbit serum were added and then the final volume was made up to 2ml and incubated at 37^{o}C for 3h. Cytotoxicity was assessed by trypan blue exclusion method (Kuttan *et al*., 1985; Manu, 2009).

2.6.7 Effect of the test materials on the metastatic progression of B16F-10 melanoma cells

C57BL/6 mice were used for the antimetastatic studies and metastasis was induced by injecting $1x10^6$ B16F10 melanoma cells through the lateral tail vein of the mice and the lung colony formation ability was determined by the method of Fidler *et al*. (1978). On the 21st day animals were sacrificed and lungs were taken out to observe the metastatic colonies. Using this lung tissue biochemical parameters such as lung collagen hydroxyproline, uronic acid and hexosamine were measured along with the serum sialic acid and serum γ-glutamyl transpeptidase levels. Life expectancy of the metastasis bearing animals was calculated along with histopathological examination of the lung tissue.

2.6.7.1 Estimation of protein (Lowry *et al*., 1951)

Principle

Peptide nitrogen reacts with copper ions to form protein copper complex and subsequently, the aromatic aminoacids reduces Phosphomolybdate-Phosphotungstate reagent (Folin Ciocaltaeu reagent to form blue coloured complex.

Reagents

Solution A

Sodium potassium tartrate	-	1ml (2%)
CuSO4	-	1ml (1%)
Na_2CO_3	-	98ml (2% in 0.1N NaOH)

Solution B

Folin's phenol reagent - 1N, diluted 1:1 with distilled water

Procedure

Different concentrations of BSA (150µg, 100µg, 50µg and 25µg) were used as standard to prepare standard curve. For the assay, 20µl sample/standard was made up to 1.2ml with distilled water. Reagent A (6 ml) was added to this and incubated for 10 min at RT. Reagent B (300 µl) was added to this mixture, vortexed and incubated for 30 min at RT. OD was measured at 660 nm.

2.6.7.2 Estimation of Hydroxyproline (Bergman and Loxley, 1970)

Principle

Chloramine T oxidises Hydroxyproline in the sample to form a coloured product which is stabilized by adding isopropanol.

Reagents

1. Oxidant solution

Sodium acetate	- 5.7g
Trisodium citrate	- 3.75g
Citric acid	- 0.55g
Isopropanol	- 38.5ml
Distilled water	- 61.5ml

2. Ehrlisch's reagent

p-dimethyl amino benzaldehyde	- 4.4g
Perchloric acid	- 10.2g (60%)
Isopropanol	- 25ml (Final volume)

3. Chloramine T -1.75g/25ml oxidant solution, freshly prepared

Procedure

One gram lung tissue was taken in a homogenizing tube and homogenate was prepared with 4 ml isotonic saline, to which 6 N HCl was added, sealed and incubated at 110^{o}C for 24 h. Hydrolysate (1 ml) was taken and neutralized with KOH then made up to 5ml with H_2O. Isopropanol (2.5 ml) was added to 0.5 ml sample and then 1ml oxidant solution was added drop by drop by mixing and kept at room temperature for 4 min. To this mixture, 2ml Ehrlisch's reagent was

added and incubated for 20 min in a water bath at 60^0C. The tubes were cooled at room temperature (1 h) and the absorbance was measured at 560nm.

2.6.7.3 Extraction and estimation of Uronic acid (Schiller *et al*. (1961), Bitter and Muir (1962)

Uronic acid was extracted from the lung tissue and estimated the levels by the method of Schiller *et al*. (1961). Lung tissue was processed with 10 mg of crude papain/g dry weight of tissue in 5ml of 0.5M acetate buffer (pH 5.5) at 650C for 24 h. Aliquot of samples containing approximately 5-15 mg uronic acid was taken and levels were estimated by the method of Bitter and Muir (1962).

Reagents

1. Sulphuric acid reagent
   Sodium tetraborate : 0.952g in 100ml of Con.H_2SO_4 (0.025M)
2. Carbazole reagent
   carbazole : 0.125g in 100g absolute alcohol.
3. Acetate Buffer (0.5M)
   Solution A : 0.25M solution of acetic acid
   Solution B : 0.25M solution of sodium acetate
   Mix A and B

Procedure

Sulphuric acid reagent (5 ml) was taken in tubes and kept at 4^oC to cool, to this 1ml of sample or standard glucuronolactone solution containing 5-20mg was layered. Using glass stoppers tubes were closed and the rack was shaken first gently and then vigorously, then incubated in a boiling shaking-water bath for 10 minutes and cooled at room temperature. 0.2ml of carbazole reagent was added and the tubes were shaken and again kept in a boiling water bath for 15 minutes and cooled. The pink colour thus developed was read at 530nm. Uronic acid content of the tissues were expressed as μg/100mg wet weight.

2.6.7.4 Estimation of Hexosamine (Elson and Morgan, 1933)

Hydrolysis of lung tissue (5 mg, lyophilized lung tissue) was performed using 2N HCl (5ml) at 100^{o}C for 6 h. Then the samples were kept for evaporation and the residue obtained was dissolved in a known volume of water.

Principle

The amino sugars (hexosamines) occurs in α or β pyranose form in many structural polysaccharides. Hexosamines have reducing properties with phenyl hydrazine but not osazones, because the C-OH has been replaced by NH_2.

Reagents

1) **2% acetyl acetone**

   2ml of acetyl acetone in 100ml of 0.5M Na_2CO_3

2) **Ehrlisch's reagent**

   p-dimethyl aminobenzaldehyde (PDAB) : 1.33g

   ethanol: conc. HCl (1:1) : 100 ml

3) 95 % Ethanol

Procedure

Freshly prepared 2% acetyl acetone reagent (1 ml) was added to the processed samples (containing approximately 10-15µg hexosamine) and kept in boiling water bath for 15 minutes. The tubes were cooled under tap water, 5ml of 95% ethanol and 1ml of Ehrlisch's reagent were added and after a thorough mixing incubated at RT for 30 min. The purple red colour developed was read at 530nm against water as blank. Standard glucosamine solutions of various concentrations were also treated similarly to get a standard curve. Tissue hexosamine levels were expressed as µg/100mg dry weight.

2.6.7.5 Estimation of protein bound serum sialic acid (Skoza and Mohos, 1976)

Principle

Acid hydrolysis of serum for liberation of sialic acid forms a coloured compound with thiobarbituric acid.

Reagents

1) H_2SO_4	:	0.2N
2) Periodic acid	:	25µM in 62.5 mM H_2SO_4
3) Sodium arsenite	:	0.2% in 0.5M HCl
4) Thiobarbituric acid	:	0.6%
5) Dimethyl sulphoxide		

Procedure

Serum samples 200µl was mixed with equal volume of 0.2N H_2SO_4 and kept 1h in water bath at 80^o C for hydrolysis. 50µl periodic acid (25µM) was added to this hydrolysate and incubated for 30 minutes at 37^oC. 50µl of sodium arsenite was added to this reaction mixture, followed by 100µl of thiobarbituric acid and kept in a boiling water bath for 7.5 min. After heating, 400µl of DMSO was added to intensify the colour and read at 549nm and 532nm.

2.6.7.6 Estimation of γ-glutamyl transpeptidase (Szasz, 1976)

Principle

γ-glutamyl transpeptidase (GGT) catalyses the transfer of γ-glutamyl moiety of a γ- glutamyl donor to a variety of acceptors.

γ-glutamyl-p-nitroanilide + acceptor $\xrightarrow{\text{GGT}}$ γ- glutamyl acceptor + p-nitroaniline

Reagents

L- γ-glutamyl-p-nitroanilide	:	2.5mM
Glycyl glycine	:	20mM
Tris-HCl (pH 8.0)	:	0.05M
NaCl	:	75mM

Procedure

The standard assay mixture contained (1ml) 0.05M Tris HCl, 75mM NaCl, 2.5mM/L- γ-glutamyl-p-nitroanilide and 20mM glycyl glycine along with 25µl sample. The rate of release of p-nitroaniline was measured at a wavelength of 410 nm using a spectrophotometer.

2.6.7.7 Histopathological Analysis

The respective tissue was fixed in 10% neutral formalin and dehydrated in series of alcohol. Cleaned with xylene and tissues were embedded in paraffin wax. Sections of 5- 6μm thickness were taken and mounted on to glass slides and stained with haematoxylin and eosin. Slides were observed under light microscope to visualise the histological changes (Culling, 1976).

2.6.7.8 Determination of the rate of survival

Metastasis was induced to the animals by injecting $1x10^6$ B16F-10 melanoma cells through the lateral tail vein of C57BL/6 mice. Death pattern of the animals was noted and the percentage increase in life span (% ILS) was calculated from the formula,

$$\%ILS = \frac{T- C}{C} \times 100$$

where, 'T' is the number of days the drug treated animals survived and 'C' is the number of days the control animals survived.

2.6.7.9 Tumour cell adhesion assay (Inokuchi *et al*., 1991)

Principle

Transformed cells found to exhibit increased adhesive attachment rates to a variety of homotypic or heterotypic cell substrates, especially metastatic cells shows higher rates of homotypic attachment (Nicolson *et al*., 1978).

Procedure

Flat bottom 96 well titre plates were coated with type I collagen (25μg/well) and B16F-10 melanoma cells ($5x10^3$ cells/well) were seeded on to the wells and incubated for 5 h at 37^oC in 5% CO_2 atmosphere. Spent medium was removed and wells were washed with PBS. Adhering cells were fixed with 5% formaldehyde (15 min) and stained with crystal violet for 10 minutes and cells were counted under inverted microscope.

2.6.7.10 Collagen matrix invasion assay (Albini *et al*., 1987)

Modified Boyden chambers (Blind well chambers) were used to study the collagen matrix invasive property of B16F10 cells by the method of Albini *et al*.

(1987). Serum free DMEM was filled in the lower compartment of the chamber and a polycarbonate filter coated with 25µg Type I collagen was placed over this. After placing the upper chamber, B16F10 cells (10^5 cells/150 µl DMEM) was seeded along with different concentrations of the test material and incubated for 10 h at 37 ^{o} C in 5% CO_2 atmosphere. Cells remained in the upper side of the filter was removed using a cotton swab after 10 h. Filter was then fixed with methanol and stained using crystal violet (3 min). Cells invaded to the lower surface of the filter were counted in 10 fields under the microscope. Percentage inhibition of invasion was determined using the formula,

$$\text{\% Inhibition of invasion} = 100 - \left[\frac{\text{Mean No. of migratory cells in the test}}{\text{Mean No. of migratory cells in the control}} \right] \text{ X } 100$$

2.6.7.11 Tumour cell motility assay

The assay was performed using Boyden chamber as per the same protocol described above. Here the poly carbonate filter was not coated with collagen and the same density of cells were seeded on to the upper-chamber and incubated at 37^{o}C for 24 h. Migrated cells were collected from the lower chamber and counted using a haemocytometer. Results were calculated as,

$$\text{\% Motility} = \frac{\text{Mean No. of migrating cells in test}}{\text{Mean No. of migrating cells in control}} \text{ X } 100$$

2.6.7.12 Gelatin Zymography (Billings *et al*., 1991)

Principle

Metastatic tumour cells liberate protease in to the medium, which was initially resolved on SDS- poly acrylamide gels, which were incorporated with gelatin. If gelatinases are separated on the gel, they will form a clear zone upon incubation in activation buffer. Gels are stained to view these clear zones.

Reagents

1) 0.25M sucrose- 0.01M Tris-HCl buffer (pH 7.4)

Sucrose : 85.87g

Tris-HCl : 1.21g

D.D H_2O : 1000ml (Final volume)

2) 0.1M Tris-HCl, 10mM $CaCl_2$ (pH-8.0)

$CaCl_2$. $2H_2O$: 1.47g

Tris-HCl : 12.1g

D.D H_2O : 1000ml (Final volume)

pH adjusted with Conc.HCl

3) Trypsin solution

Trypsin : 75µg/ml in 0.1M Tris-HCl, with 10mM $CaCl_2$, pH 8.

4) Activation buffer (0.1M Tris-HCl, 10mM CaCl2, pH 7.8)

Tris HCl	:	12.1g
$CaCl_2.2H_2O$	:	1.47g
D.D H_2O	:	1000ml (Final volume)
5) Gelatin (dissolve by heating)	:	180mg/2ml D.W

6) Preparation of gels

a) Resolving Gel : 11% Polyacrylamide gels with 0.1% SDS and 0.6% gelatin

29.2% acrylamide + 0.5% bisacrylamide	:	11 ml
0.1M Tris-HCl, pH 8.8	:	1.2 ml
20% SDS	:	0.15 ml
20% Ammonium per sulphate	:	0.10 ml
Gelatin	:	2 ml
D.D. H_2O	:	6.505 ml
TEMED	:	0.045 ml

Mix and pour at room temperature.

b) 5% Stacking gel

29.2% acrylamide +0.5% bis acrylamide	:	1.67ml
0.1M Tris-HCl, pH 8.8	:	1.75ml
20%SDS	:	0.10ml
20% Ammonium per sulphate	:	0.10ml

D.D. H_2O	:	6.36ml
TEMED	:	0.02ml

Mix and pour above the resolving gel at room temperature.

7) Sample buffer (2X)

Glycerol	:	1ml
1M Tris-HCl, pH 6.8	:	0.25ml
20% SDS	:	1ml
Bromophenol blue (Tracking dye)	:	1.65mg

Made up to 5ml with double distilled water

8) Running buffer

Tris base	:	3g
SDS	:	2g
Glycine	:	14.2g

Made up to 1L with double distilled water

9) 2% Triton X-100

Triton X-100	:	2ml
0.1M Tris HCl, pH 7.8	:	100ml (Final volume)

10) 10mM EDTA solution

EDTA- Na_2	:	372.24mg
0.1M Tris-HCl, pH 7.8	:	1000ml

Procedure

Gelatin Zymography was performed as per the method of Billings et al. (1991) with some modification. B16F-10 tumour cells cultured bottles of about 70% confluency was taken and washed with serum free medium and incubated at 37^{o}C for 24 h in serum free DMEM. After 24 h medium was collected from the bottles, centrifuged supernatant was used as samples for zymographic analysis after determining the protein concentration. Samples with protein concentration of 50 µg were activated with trypsin (75µg/ml, 5µl trypsin solution for 100µg

protein) in the presence and absence of test compounds in 0.1 M Tris-HCl, 10mM CaCl2 buffer (pH-8.0) and incubated for 1 h at room temperature. Equal volume of trypsinised / non-trypsinised samples and 2X sample buffer were mixed and loaded on to 0.1%SDS -11% polyacrylamide gels containing 0.1% gelatin. Electrophoresis was carried out at 4^{o}C with constant current of 2mA/tube until the tracking dye (Bromophenol blue) reached the periphery of the gels. The gels were then washed using 2% Triton X-100 on a shaker at 20-25^{o}C for three times (30 min each), to remove the SDS which may hinder the proteolytic activity. Gels were then rinsed (2h) with activation buffer and finally incubated in the same buffer at 37^{o}C for 18 h. Fixation and staining of the gels were performed using Gelcode Blue stain reagent (2 h) and observed for clear bands (Manu, 2009).

2.6.7.13 Tissue to cDNA synthesis

Isolation of RNA from the tissue (Chomczynski and Mackey, 1995)

Lung tissue (100 mg) was collected from the treated and untreated metastasis bearing animals in aseptic manner, washed with PBS and minced well. All the steps were performed under cold conditions and the glass wares and plastic wares used for RNA isolation were treated with Diethyl pyrocarbonate (DEPC) and autoclaved and dried at 40^{o}C. Trizol reagent (1 ml), was added to the tissue and dispersed the cells by gentle pipetting. After incubating for 5 minutes at room temperature the cell solution was transferred to a centrifuge tube kept in ice. The process was repeated with 250 µL of trizol reagent and the cell suspension was collected in the same tube and then centrifuged at 10, 000 rpm for 10 minutes at 4^{o}C. The supernatant was transferred to another tube, kept at room temperature and to this 300 µl chloroform was added and mixed well for 3 minutes. The milky pink solution formed is again mixed by inverting the tube and centrifuged at 10,000 rpm for 10 min at 4ºC. Aqueous layer formed on the upper phase was withdrawn carefully and added to a fresh vial and 600 µL of ice cold isopropanol was added to it. After mixing this solution the tubes were incubated at room temperature for 10 minutes. Then the tubes were centrifuged at 12,000 rpm for 10 min at 4ºC. The supernatant was discarded; the pellet was dissolved in 70% ethanol (prepared in DEPC-treated water) and kept at room temperature for 10 min. This was again centrifuged at 13,000 rpm for 10 min at

4ºC to discard the supernatant and the pellet obtained was allowed to dry and then dissolved in 50µL of DEPC-treated water and stored at -70ºC. The quality of the RNA samples was checked by agarose gel electrophoresis. RNA was quantified by measuring the absorbance ratios of 260/280 and 260/230.

2.2.10.2 cDNA synthesis from RNA (Sambrook and Russel, 2001)

An aliquot of RNA sample containing 4 µg of RNA was used for cDNA synthesis.

The reaction mixture consists of the following components.

RNase free water	–	4.4 µL
AMV RT buffer	–	1 µL
Oligo (dT)	–	0.6 µL
dNTPs	–	1 µL
25mM $MgCl_2$	-	1 µl
RNA sample	-	1 µL (depending up on the conc. of RNA sample)
Total	=	9 µL

The reaction mixture was incubated at 65ºC for 5 min. This was followed by cooling at room temperature for 5 min. DTT (100 mM, 1 µL) was added, followed by addition of 1 µL of AMV RT. The tubes were kept at 42ºC for 30 min. The prepared cDNA was stored at -20ºC.

2.2.10.3 Amplification of genes using specific primers (MMP-2, MMP-9, TIMP-1, TIMP-2, VEGF, GAPDH)

The master mix was prepared as described below.

Components	**Quantity/sample**	
5X PCR Buffer	–	5 µL
Molecular grade water	-	13 µL
dNTPs	–	1 µL
25mM $MgCl_2$	–	2.5 µL
Forward Primer	–	1 µL
Reverse Primer	–	1 µL
cDNA sample	–	1 µL

Taq Polymerase	–	0.5 µL
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Total	=	25 µL

The master mix was then subjected to amplification in the minicycler with the following reaction profile.

Steps	**Temperature**	**Time**
Step 1	94ºC	4 min
Step 2	94ºC	1 min
Step 3	58.5ºC	30 sec (for GAPDH)
Step 4	72ºC	1 min
Go to step 2 and repeat 40 cycles		
Step 5	72ºC	7 min

Annealing temperature and time duration in Step 3 will vary depending up on the primers used, which is noted as follows.

Gene	**Temperature**	**Time**	**No: of cycles**
MMP-2	57.5 ºC	30 sec	40
MMP-9	65 ºC	30 sec	40
TIMP-1	58 ºC	30 sec	40
TIMP-2	59.5 ºC	30 sec	40
VEGF	61.5 ºC	30 sec	40
Nm-23	60.5 ºC	30 sec	40

Primer sequence and product size of the genes: (Manu, 2009)

Name of the gene with primer sequence	**Product size (bp)**
MMP-2	
Forward 5’-GAGTTGGCAGTGCAATACCT-3’	354
Reverse 5’-GCCGTCCTTCTCAAAGTTGT-3’	
MMP-9	
Forward 5’-AGTTTGGTGTCGCGGAGCAC-3’	327
Reverse 5’-TACATGAGCGCTTCCGGCAC-3’	

TIMP-1

Forward 5’-CTGGCATCCTCTTGTTGCTA-3’	414
Reverse 5’-AGGGATCTCCAGGTGCACAA-3’	

TIMP-2

Forward 5’-AGACGTAGTGATCAGGGCCA-3’	525
Reverse 5’-GTACCACGCGCAAGAACCAT-3’	

VEGF

Forward 5’-TGCTCACTTCCAGAAACACG-3’	453
Reverse 5’-GGAAGGGTAAGCCACTCACA-3’	

GAPDH (Glyceraldehyde 3 phosphate dehydrogenase)

Forward 5’-TGCTGGCGCTGAGTACGTCGT-3’	527
Reverse 5-’GTGGAGGAGTGGGTGTCGCTG-3’	

2.6.8. Gene Expression Studies

Cell to cDNA Synthesis

In order to study the gene expression pattern of the cultured macrophages, reverse transcription PCR method was performed. Cells to cDNATM II kit from Ambion Inc, U.S.A., was used to synthesise cDNA from mammalian cells in culture without isolating mRNA and PCR amplification of specific genes from the cDNA was performed using specific primers according to the manufactures protocol (Manu, 2009).

2.6.9. Detection of PCR products

Amplified sample (10 µl) was loaded on 1.5% agarose gel with 0.5 µg/ml ethidium bromide and electrophoresed at 70V for 2 h in TBE buffer.

Reagents

10 X TBE

Tris-HCl, pH 8.3	-	21.6g
EDTA	-	0.372g
Boric acid	-	11g

Made upto 200 ml using double distilled water.

10X Loading dye

Bromophenol blue - 0.05%

TEB 10X pH 8.3 - 1ml

Glycerol - 100µl

1) Gel tray was cleaned and rinsed with 1 X TBE buffer and the edges were sealed with sealing tape, and the comb was placed in its position.
2) Electrophoresis buffer (1X TBE buffer) was filled in the electrophoresis tank.
3) Agarose powder (1.5 %) was added to 1X TBE buffer and heated in boiling water bath/hot plate to completely dissolve the agarose to form a clear solution.
4) Solution was cooled to 45^{o}C and ethidium bromide was added at a concentration of 0.5µg/ml, mixed by swirling and poured in to the gel trey and allowed to cool for 30-45 minutes and the gel tray placed in the electrophoresis tank after removing the sealing tape.
5) 10 µl of amplified product was mixed with 2µl of 10X gel loading buffer and loaded into the wells.
6) The samples were resolved at 70 V until the dye has migrated up to the ¾th length of the gel.
7) The gels were examined using a gel-documentation system (Manu, 2009).

2.6.10. Biochemical and Anti-oxidant parameters

a) Superoxide scavenging activity (Mc Cord and Fridovich 1969)

Principle

Riboflavin reacts with O_2 in presence of light and an electron donor to produce superoxide radicals. NBT is reduced by these radicals to form blue coloured formazan.

Reagents

Phosphate buffer (0.067 M, pH 7.8)

A) KH_2PO_4 : 0.067M

B) Na_2HPO_4 : 0.067M

NBT	:	1.5 mM in phosphate buffer
Riboflavin	:	0.12 mM
NaCN	:	0.0015% in 0.1M EDTA

Assay system

NBT	:	100 µl
Riboflavin	:	50 µl
EDTA-NaCN	:	200 µl
Sample	:	100 µl
Buffer	:	2550 µl

Take OD at 530 nm, then incubate for 15 min and take OD after illumination. Subtract before illumination OD from after illumination OD.

b) Lipid Peroxidation (Ohkhawa *et al*., 1979)

Principle

Lipid peroxidation is measured by thiobarbituric acid reactive substance (TBARS) method. One molecule of MDA reacts with 2 molecules of 2-thiobarbituric acid to yield a chromophore with absorbance maximum at 560 nm.

Reagents

Acetic acid	: 20%
SDS	: 8%
TBA	: 0.67% in boiled water
Pyridine:Butanol	: 1:15

Assay system

Tissue homogenate/serum sample	:	0.1 ml
SDS	:	0.2 ml
Acetic acid	:	1.5 ml
TBA	:	1.5 ml

Reaction mixture was made up to 1 ml with distilled water and incubated for 1 h at 100^{o}C. The mixture was cooled and 1 ml distilled water was added. To that 5

ml Pyridine:Butanol was added and centrifuged at 3000 rpm for 15 min and OD of organic layer was measured at 560 nm.

c) Glutathione (GSH) (Moron *et al*., 1979)

Principle

Reduced glutathione reacts with DTNB to form a yellow coloured complex with absorbance maximum at 412 nm.

Reagents

TCA : 25%

TCA : 5%

Phosphate buffer (0.2 M, pH 8)

a) Na_2HPO_4 : 2.8392 g/100 ml

b) NaH_2PO_4 : 2.7598 g/100ml

Mix 94.7 ml A and 5.3 ml B and adjust the pH to 8.

Procedure

To 0.1 ml of tissue homogenate or serum 0.125 ml TCA was added to precipitate proteins. Tubes were cooled in ice for 5 min and the mixture was again diluted with 0.6 ml of 5% TCA. The tubes were centrifuged at 3000 rpm for 5min and 0.1 ml supernatant was taken for GSH estimation. The volume of aliquot was made up to 1 ml with 0.2 M phosphate buffer. DTNB (2 ml) was added to the tubes and intensity of yellow colour formed was read at 412 nm.

d) Glutathione peroxidase (GPx) by the method of Hafeman *et al*., 1974

Principle

GPx degrades H_2O_2 in presence of glutathioine (GSH), there by depleting it. GSH remaining is measured by colour reaction.

Reagents

DTNB : 1 mM

Phosphate buffer (0.1 M, pH 7.0)

A) Na_2HPO_4 : 1.4612 g/100 ml

B) NaH_2PO_4 : 1.3799 g/100 ml

Mix 61 ml A + 39 ml B and adjust the pH

GSH	:	5 mM
Sodium azide (NaN_3)	:	25 mM
Na_2HPO_4	:	0.4 M
H_2O_2	:	1.2 mM
Metaphosphoric acid	:	1.67 %

Assay system

Reagent	Test	Blank
GSH	0.1 ml	0.1 ml
Buffer	2.1 ml	2.1 ml
NaN_3	0.1 ml	0.1 ml
Sample	0.1 ml	------
H_2O_2	0.1 ml	0.1 ml
H_2O	------	0.1 ml

Incubate for 6 min at 37^oC, and then add 1.67 % meta-phosphoric acid and centrifuge at 800 rpm for 15 min. Take 2 ml supernatant from that and add 2 ml Na_2HPO_4 + 1ml DTNB and incubated for 1o min at 37^oC. Absorbance was measured at 412 nm against distilled water.

e) **Alanine amino transferase (ALT/GPT) (Bergmeyer and Bernt, 1974)**

ALT was estimated using kit from Span-Cogent Diagnostics, following the manufactures protocol.

f) Urea (Murray, 1984)

Urea levels in the serum or urine was determined using urea test kit from Span diagnostics (Liquid gold).

g) Creatinine levels (Toro et al., 1975)

Creatinine levels were estimated by Jaffes method using kits from Euro Diagnostic systems.

h) ALP (King and Amstrong, 1965)

ALP levels were diagnosed using the kits purchased from Span diagnostics Ltd., by following the manufactures protocol.

2.7. Statistical Data Analysis

All data were expressed as mean ± standard deviation. Statistical analysis was done by Student's 't' test, One way analysis of variance, Tukey Kramer multiple comparisons test or Dunnets multiple comparisons test using Graphpad InStat version 3.00 for Windows 95, GraphPad Software, San Diego, California, USA.

Chapter 3

Effect of C. orchioides and Curculigoside on the immune system

3.1 INTRODUCTION

Immunomodulators have a foremost role in cancer treatment strategies where, the immune-stimulatory therapy is now being recognised as an alternative to conventional treatment regimens which includes chemotherapy and radiotherapy with a number of side effects including immune deprivation. Previous reports about the humoral immune stimulatory effects of *Curculigo orchioides* (Lakshmi *et al*., 2003, Rathod *et al*., 2010) laid down the basis for the present work. The study was designed to analyze the effects of test materials on specific and nonspecific immune response in a systematic manner. *C. orchioides* and its derived compound, Curculigoside were tested for their stimulatory effects on humoral as well as cell mediated immune system of the normal and tumour bearing mice.

3.2 MATERIALS AND METHODS

3.2.1. Animals

Dose determination and toxicity analysis of the plant extract and Curculigoside was carried out using Balb/c mice (6-8 weeks old). *In vivo* immunomodulatory activity of the test materials were carried out using male Balb/c and C57Bl/6 mice (4-6 weeks old).

3.2.2. Cell lines

Cell lines used in this study were EL-4 and SRBC.

3.2.3. Chemicals

All the chemicals used in the present study were of analytical grade. Crystal violet, acetic acid, pararosaniline hydrochloride, α-naphthyl acetate, haematoxylin, LPS, PWM, Con-A, PHA, ^{3}H thymidine etc were used in this study.

3.2.4. Toxicity analysis of *C. orchioides* extract and Curculigoside

LD 50

***C. orchioides* methanolic extract**

Male and female Balb/c mice were used for LD 50 studies. Both the male and female animals were divided in to five groups (N=5) and group I animals were kept as untreated control whereas group II, III, IV and V animals were administered intra-peritoneally with a single dose of *C. orchioides* extract at different concentrations of 2, 1, 0.5 and 0.25 g/kg b.wt. respectively. All the animals were observed 14 days for mortality, behavioral change, diarrhea or change in body weight, etc. Based on the observations it became clear that even at a concentration of 2 g/kg b.wt. the extract does not produce any mortality or behavioral change to both male and female Balb/c mice, thus the LD 50 is > 2 g/kg b.wt. But the body weight of the animals was found to be decreased in 2, 1 and 0.5 g/kg b.wt. dose treated groups. Therefore, concentrations 0.25 g/kg b.wt. and below were selected for further analysis.

Curculigoside

An LD 50 study of Curculigoside was performed as described above with single dose intra-peritoneal administration of 0.5, 0.2 and 0.1 g/kg b.wt. of the test material. One female Balb/c mice was died in 0.5 g/kg b.wt. treated group and all the animals showed a decrease in their body weights. This body weight reduction was again observed in 0.2 and 0.1 g/kg b.wt. treated animals also. None of them showed any change in their behavior. Therefore for further analysis lower concentrations were selected.

Subacute toxicity analysis

***C. orchioides* Extract**

Male Balb/c mice were used for the toxicity evaluation and the animals were divided into five groups (N=8). Group I was kept as control and received 1% gum acacia as vehicle, where as group II, III, IV and V animals received 14 doses of different concentrations of *C. orchioides* extract (50, 100, 200 and 250 mg/kg b.wt.) intra-peritoneally at 24 hour interval. All the animals were kept under observation for 14 days to monitor total WBC count, change in body weight,

mortality, behavioural changes, diarrhoea *etc*. All the animals were sacrificed by cervical dislocation after 24 h of the last dose of extract administration. Organs including the liver, spleen, thymus, kidney and lungs were dissected out and weights were recorded. Blood was collected by heart puncture to separate serum and used for the analysis of hepatic and renal function markers. Hepatic function markers namely alkaline phosphatase (ALP) (King and Amstrong, 1980), glutamate pyruvate transaminase (GPT) (Bergmeyer and Bernt, 1980) and renal function markers namely creatinine (Toro and Ackermann, 1975) and urea levels (Murray, 1984) were determined. From these studies, a dose of 50 mg/kg b.wt. and below were selected for *in vivo* experiments (Table 3.1.A).

Curculigoside

A dose determination study of Curculigoside was also performed in the similar way as that of *C. orchioides* extract. Four groups of Balb/c mice were used for the study, (n=8/group) and treated with 14 doses of Curculigoside (10, 20 and 50 mg/kg b.wt) intra-peritoneally to group I, II and III. Group IV animals were kept as vehicle control and administered with 1% gum acacia in PBS. Mortality, behavioral changes and change in b.wt. were observed for 14 days.

All the animals were sacrificed on 15th day of drug administration by cervical dislocation and blood was collected by heart puncture. Serum was separated from the blood and used to estimate various liver function (ALP, GPT) and kidney function markers (creatinine, urea). During sacrifice, lungs, thymus, spleen, liver and kidney were dissected out to record the organ weight. A non-toxic dose of 10 mg/kg b.wt. and below were selected for further *in vivo* experiments (Table 3.1.B).

3.2.5 Effect of *C. orchioides* and Curculigoside on the immune system

3.2.5.1 Effect on the hematological parameters

Balb/c animals were divided into 7 groups (8 animals/group). Group I was kept as untreated control. Group II, III and IV animals received *C. orchioides* extract (i.p.) at a dose of 50, 20 and 10 mg/kg b.wt. respectively, whereas group V,VI and VII were treated with Curculigoside (i.p.) at a dose of 10, 5 and 2 mg/kg b.wt. respectively for five consecutive days. From all the test animals, blood was

collected by tail vein bleeding before the administration of the test material and continued for 30 days on every third day. Total WBC count, differential count and hemoglobin levels were analyzed as described in chapter 2.

3.2.5.2 Effect on lymphoid organ weight and cellularity

Balb/c mice were divided into three groups (n=8). Group I animals were kept without any treatment and group II animals were administered with 20 mg/kg b.wt. *C. orchioides* methanolic extract (i.p.) whereas group III animals were treated with 5 mg/kg b.wt. Curculigoside (i.p.) for 5 consecutive days. After 24 h of last dose of test material administration, body weights of the animals were recorded and then sacrificed by cervical dislocation to collect thymus and spleen.

3.2.5.3 Effect on bone marrow cellularity and number of α-esterase positive cells

Femur bones of the above described animals were collected and bone marrow cellularity was determined by the method of Sredni *et al* (1992). Bone marrow cells were suspended in RPMI medium to make serial dilutions and cell count was determined using haemocytometer. Azo dye coupling method (Bancroft and Cook 1984) was employed to determine the number of α-esterase positive cells in the bone marrow cells as described in chapter 2.

3.2.5.4 Effect on the number of circulating anti SRBC antibodies

Balb/c animals were grouped into three (8 animals/group) and group I animals were maintained as untreated control, groups II and III animals received 5 consecutive doses (i.p) of *C. orchioides* extract and Curculigoside respectively. After the last dose of extract/compound administration all the animals were immunized with SRBC (2.5×10^8 cells/0.1 ml/animals, i.p.) and every third day after immunization, blood was collected from the tail vein to separate serum (up to 30 days) and haemagglutination assay was performed as described in chapter 2.

3.2.5.5 Effect on the number of plaque forming cells in the spleen

The study was designed as described in the above experiment, from the 3rd day of immunization animals from each groups were sacrificed by cervical dislocation

and continued up to 9th day to collect spleen. Spleen cells were processed in RPMI medium to single cell suspension and the number of anti SRBC antibody producing cells (plaque forming cells) was analyzed by Jernes plaque assay (Jerne and Nordin, 1963).

3.2.5.6 Effect on the proliferation of bone marrow cells, splenocytes and thymocytes

Three groups of Balb/c mice were used in this study (n=6). Group II and III animals were received *C. orchioides* extract and Curculigoside for 5 consecutive days (intra-peritoneal administration), whereas group I animals were retained as untreated control. On the 6th day all the animals were sacrificed to collect spleen, thymus and bone marrow in a sterile manner in RPMI medium to make single cell suspension. Rate of proliferation was determined by thymidine (3H-thymidine) incorporation assay in the presence and absence of various mitogens like, Con-A (10 μg/mL), PHA (2.5 μg/mL), LPS (10 μg/mL) and PWM (10 μg/mL) as described in chapter 2.

3.2.5.7. Effect of *C. orchioides* extract and Curculigoside on CTL production and activity

CTLs were produced by alloimmunization and three methods were employed to generate effector cells from the spleen cells of Balb/c mice. Winn's neutralization assay was performed as per the protocol of Kobayashi et al., (1992) to evaluate the CTL activity.

Method I (in vivo)

Balb/c mice (donor) were alloimmunized with spleen cells (2×10^7 cells/ animal) from C57BL/6 mice and the effector spleen cells were collected from Balb/c mice, 7 days after alloimmunization. Donor mice were treated with and without *C. orchioides* or Curculigoside (20 or 5 mg/kg body weight respectively; i.p.) for 5 days after alloimmunization. Control animals were kept without further treatment. Thereafter, Winn's neutralization test was performed. For this, spleen cells from the above animals (effector cells; 1×10^7) and the target cells ($5 \times l0^5$

EL-4 cells) were mixed at an effector:target ratio of 20:1. Then, the cells were incubated for 1h at 37°C in 5% CO_2 atmosphere.

Neutralized cells (0.2 mL) were injected in Balb/c mice and the survival rate of the animals was observed. For this, Balb/c mice (*n* = 8/group) were divided in to six groups (Method I). Group I received EL-4 cells alone (5×10^5/0.1 ml; i.p.). Group II and III animals were administered with *C. orchioides* and Curculigoside (i.p.) respectively along with EL-4 cells. Group IV animals received EL-4 cells incubated with normal spleen cells (0.2 mL) from the Balb/c. Group V and VI animals received EL-4 cells incubated with spleen cells from *C. orchioides* or Curculigoside treated alloimmunized mice respectively. The survival rate of treated animals was compared with that of the animals received tumour cells alone. Increase in survival time directly relates to the increase in CTL activity.

Blood was collected from the above mentioned animals; 2 days after the last dose of *C. orchioides* or Curculigoside administration, and the levels of the interleukin-2 (IL-2) and interferon-γ (IFN-γ) were assayed using an ELISA kit purchased from Pierce Biotechnology, (USA), according to the described protocol by the manufacturer.

Method II (in vitro)

In this methodology, the effector cells were produced by mixed lymphocytes culture (MLC) of spleen cells (responder cells) from *C. orchioides*/Curculigoside treated or untreated Balb/c mice and Mitomycin (50 μg/mL) treated spleen cells (stimulator cells) from C57BL/6 mice for 5 days. Four groups of animals (*n* = 8/group) were used in this method. Group I animals received EL4 cells alone, Group II animals received EL-4 cells co-cultured with effector cells generated using normal spleen cells. Groups III and IV animals received EL-4 cells co-cultured with effector cells formed using spleen cells from C. *orchioides* and Curculigoside treated spleen cells respectively.

Method III (in vitro)

In this method, splenic co-culture was treated with the extract or Curculigoside to stimulate the CTL production *in vitro*. Splenic lymphocytes (2×10^7) from normal

Balb/c mice (responder cells) and mitomycin-C treated spleen cells (effector cells; 1×10^7) from C57BL/6 were co-cultured at 37°C in 5% CO_2 atmosphere for 5 days, in the presence or absence of *C. orchioides* (5µg/ml) or Curculigoside (2 µg/ml). Then, the cells were injected to four groups of Balb/c mice. Group I received EL-4 cells alone, group II received EL-4 cells incubated with normal co-cultured spleen cells. Groups III and IV received EL-4 cells incubated with co-cultured spleen cells in the presence of *C. orchioides* and Curculigoside respectively. All the animals were observed for their survival.

3.3 RESULTS

3.3.1 Effect of *C. orchioides* and Curculigoside on Total WBC count

The total WBC count obtained for *C. orchioides* (50 mg/kg b.wt.) treated animals was 12938 ± 928 cells/mm^3 on the 6^{th} day, whereas extract administration at a dose of 20 mg/kg b.wt. also resulted a significant elevation of total WBC count to 11050 ± 793 when compared to the normal count of 6675 ± 398 on the same day (Figure 3.1.A). Curculigoside treatment also resulted in a significant increase of the total WBC count in Balb/c mice. A higher concentration of Curculigoside, 10 mg/kg b.wt. was also found to be immune stimulatory (11900 ± 527), but not significantly higher than the WBC count of 5 mg/kg b.wt treated group (11850 ± 516; Figure 3.1.B). From these results, minimum concentration with maximum immune stimulatory effect (extract: 20mg/kg b.wt and Curculigoside: 5mg/kg b.wt) was selected for further experiments.

3.3.2 Effect of *C. orchioides* and Curculigoside on the relative weight of lymphoid organs

Administration of *C. orchioides* (20 mg/kg b.wt.) and Curculigoside (5 mg/kg b.wt.) resulted in a significant ($p<0.01$) increase in the weight of thymus (0.19 ± 0.01 and 0.18 ± 0.01 g/100 g body wt.), when compared to control animals. The size and weight of spleen was also enhanced in a significant manner ($p<0.05$) by the administration of Curculigoside and *C. orchioides*, when compared to control animals (Table 3.2).

3.3.3 Effect of *C. orchioides* and Curculigoside on Bone marrow cellularity and the number of α-esterase positive cells

Normal Balb/c animals showed a bone marrow cellularity of 16.3 ± 0.86 and 814.7 ± 42.47 alpha esterase positive cells and both the bone marrow cellularity and the number of differentiating monocytes were enhanced with *C. orchioides* and Curculigoside treatment. The increased number of bone marrow cells was 24.6 ± 1.4 and 23.7 ± 1.8 whereas the number of α-esterase positive cells was 1109 ± 88.6 and 1045 ± 62.4 respectively, for the extract and the compound treated animals (Table 3.3).

3.3.3 Effect of *C. orchioides* and Curculigoside on the circulating antibody titre

Production of antibodies against SRBC was found to be enhanced with *C. orchioides* and Curculigoside treatment when compared to SRBC alone administered animals. Sheep RBC alone treated animals showed a maximum titre value of 64 on the 9^{th} day which was maintained up to 18^{th} day whereas the maximum titre value obtained for the extract administered animals was 1024 on 12^{th} day and it was maintained up to 15^{th} day. Antibody levels of Curculigoside treated animals was at maximum on the 15^{th} day with a titre value of 1024, found to be in a steady level up to 18^{th} day (Figure 3.2).

3.3.4 Effect of *C. orchioides* and Curculigoside on the number of antibody producing cells (Plaque forming cells, PFC)

The number of anti SRBC antibody producing cells in the spleen were maximum for the Curculigoside treated animals on the 6^{th} day of antigenic stimulation ($368/10^6$ spleen cells) whereas the extract treated animals also showed a similar kind of elevation in the number of plaque forming cells ($344/10^6$ spleen cells). The maximum number of PFC obtained for SRBC alone treated animals were 152 $/10^6$ spleen cells on the 6^{th} day itself (Figure 3.3).

3.3.4 Effect of *C. orchioides* and Curculigoside on Lymphocyte Blastogenesis

Effect of *C. orchioides* and Curculigoside on splenocyte blastogenesis is given in Figure 3.4. Extract and compound at a dose of 20 and 5 mg/kg b.wt. respectively, found to exert significant mitogenicity. A strong mitogenic transformation of

quiescent splenic lymphocytes to their blastic nature, resulting in the increased uptake of [^{3}H] thymidine was brought about by *C. orchioides* and Curculigoside. These results were comparable to commonly used mitogens such as Con A, PHA, LPS and PWM. PHA encourages the proliferation of spleenocytes (3233.5 ± 126.2 cpm), which was significantly enhanced by the treatment of the animals with *C. orchioides* extract (5429.8 ± 126.2 cpm) and Curculigoside (5502.6 ± 128.2 cpm), whereas the normal untreated splenocytes showed [^{3}H] thymidine uptake of 1103.8 ± 159.2 cpm. When Con A was employed as the mitogen, the spleen cell proliferation was stimulated to 3287.8 ± 121.7 cpm, which on pre-treatment with the extract again enhanced to 5536.8 ± 91.8 and 5624.8 ± 169.8 cpm on pre-treatment with Curculigoside. A significant synergistic effect on the proliferation of splenocyte was observed with LPS and PWM when used along with Curculigoside and *C. orchioides* extract.

We found that *C. orchioides* and Curculigoside treatment resulted in an increased mitogenic activity of thymocytes in the presence of other mitogens. Proliferation of thymocytes was significantly enhanced from 1079.5±86.3 cpm to 3279.5±138.5 cpm and 3369.3±130.7cpm when the animals were administered with *C. orchioides* extract and Curculigoside respectively. Along with this, addition of mitogens like Con A, PHA and PWM to the thymocytes resulted in a synergistic enhancement of thymocyte proliferation. *C. orchioides* at a dose of 20 mg/kg b.wt. and Curculigoside at a dose of 5 mg/kg b.wt. acted synergistically along with other mitogens resulting in higher [^{3}H] thymidine uptake showing 5455.5 ± 132.7 and 5457.3 ± 134.8 cpm with Con A, 5775 ± 106.3 and 5780 ± 115.2 cpm with PHA and 4165.5 ± 138.5 and 4221.7 ± 141.6 cpm with PWM, respectively (Figure 3.5).

C. orchioides extract and Curculigoside was found to enhance the bone marrow proliferation significantly ($p < 0.01$, Figure 3.6). When compared to the normal animals (1141 ± 88 cpm), the extract alone treated bone marrow cells given a cpm of 1947 ± 133 and 1844 ± 90 cpm for Curculigoside alone treated cells. Upon incubation with various mitogens *C. orchioides* and Curculigoside provided a cumulative effect. Mitogenic potential of Con A (1237± 142 cpm), PHA (3244 ± 143 cpm), PWM (2221 ± 153 cpm) and LPS (3512 ± 105 cpm),was enhanced significantly ($p < 0.05$) in the bone marrow cells of *C. orchioides* extract (Con A -

3134 ± 136 cpm, PHA-4418 ± 137 cpm, PWM - 2930 ± 203 cpm, LPS - 4983 ± 148 cpm) and Curculigoside (Con A, 3065 ± 290 cpm; PHA, 4651 ± 208 cpm; PWM, 3049 ± 90 cpm; LPS, 5085 ± 137 cpm) treated animals.

3.3.6 Effect of *C. orchioides* and Curculigoside on the CTL production (*in vivo*)

From the previous studies it was evident that *C. orchioides* extract and Curculigoside possess potent immune stimulatory effects in healthy condition. This study was designed to check the role of these immune stimulatory effects under conditions of tumour challenge, especially to evaluate the T-cell mediated tumour response enhancement. Both the *in vivo* and *in vitro* methods for the CTL production were performed in the presence and absence of the test materials. The extract and the compound were found to enhance the CTL activity both by the *in vivo* and *in vitro* treatment modalities.

Method I

Effect of *C. orchioides* and Curculigoside on *in vivo* CTL generation is given in Table 3.4. The survival rate of animals in the untreated tumour bearing group (EL4- alone) was 34.5±2.31 days. When these animals treated with 5 doses of *C. orchioides* and Curculigoside the survival days was increased to 45±1.15 and 49.4±1.62 days, respectively, with an 30.4% and 43.2% increase in their life spans whereas animals injected with EL4 cells incubated with normal alloimmunized effector cells the survival rate was only 39.9±1.96 days (%ILS 15.6). But when the animals received EL4 cells incubated with *C. orchioides* and Curculigoside treated alloimmunized spleen cells, the number of survival days was significantly ($P<0.001$) increased to 53.6 ± 2.47 days (%ILS 55.5) and 51±2 (%ILS 47.8) respectively showing the *in vivo* generation of CTL that destroys CTL sensitive thymoma cells (EL4) and decrease mortality due to tumour burden.

3.3.7 Effect of *C. orchioides* or Curculigoside on CTL production (*in vitro*)

Method II

The effect of *C. orchioides* and Curculigoside on *in vitro* generation of CTL by *Method II* (*in vitro*) is given in Table 3.5. Animals treated with EL4 cells alone survived only 34.5 ±2.31 days. The survival days of animals that received EL4

cells incubated with effector cells from co cultured normal spleen cells were 41 ± 2 days with an %ILS 18.9%.When EL4 cells were incubated with *C. orchioides* or Curculigoside treated alloimmunized effector spleen, survival days were increased to 55.4±1.62 (% ILS 60.5) and 52.6 ± 2.13 (%ILS 52.5), respectively. This study confirms the effect of test compound on the generation of CTL.

Method III

In this system also, the survival days of animals treated with EL4 cells alone was 34.5 ± 2.31 days. Whereas the animals that received EL4 cells incubated with co cultured normal spleen cells alone, the survival days slightly increased to 41.4 ± 3.46 with %ILS 20. When the animals were received EL4 cells incubated with co cultured normal spleen in the presence of *C. orchioides* (5 μg/mL) and Curculigoside (2 μg/mL), the survival days of animals were significantly ($P<0.001$) increased to 49.5 ± 2.83 and 50.5 ± 1.86 with an increase in %ILS to 43.5 and 46.4 respectively. In this system, increase in life was higher in Curculigoside treated group compared to the other two systems (Table 3.6). This study also gives further evidence for the generation of CTL by the test compound.

3.3.8 Effect of *C. orchioides* and Curculigoside on cytokine release

Effect of *C. orchioides and* Curculigoside on IL-2 and IFN-γ production by animals carrying EL4 cells incubated with *in vivo* generated effector cells is presented in Figure 3.7 and 3.8 respectively. Administration of *C. orchioides* or Curculigoside significantly increased cytokines which are involved in immunomodulation namely IL-2 and IFN-γ when compared to tumour alone as well as tumour plus normal alloimmunized spleen cells.

3.4. Discussion

Immune system is designed to maintain the tissue homeostasis, providing protection from invading or infectious pathogens and also by eliminating damaged cells (de Visser *et al*., 2006). *C. orchioides* and Curculigoside was found to enhance both the humoral and cellular wings of immune system without causing any adverse effects to the experimental animals. *C. orchioides* is an

edible plant without any toxicity and used as a rejuvenating agent in the Ayurvedic preparations (Asif and Kumar, 2010).

Study on the effect of total WBC count revealed that, the plant extract and Curculigoside can stimulate the non specific immune responses in Balb/c mice. Bone marrow contains the hematopoietic stem cells and is the primary lymphoid organ where the maturation of B cells occurs. The extract and Curculigoside enhanced both the bone marrow cellularity and the number of differentiating monocytes. Thymus is the primary lymphoid organ where T cell development and proliferation occurs. Spleen is the largest secondary lymphoid organ and 30-40% of the spleen cells are T-cells. Both the extract and Curculigoside enhanced the mitogenic potential of T and B cell mitogens in splenocytes and thymocytes along with an increase in the relative weight of thymus and spleen.

At the same time, specific immune responses against sheep RBC was also elevated with the administration of *C. orchioides* and Curculigoside indicating the enhancement of the humoral immune response against antigens. Both antibody levels and number of antibody producing B lymphocytes were elevated with the extract and Curculigoside treatment.

Cell-mediated immunity (CMI) is the chief defence mechanism against tumour development and is mediated by T lymphocytes. Antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes and antigen nonspecific natural killer (NK) cells forms the key players of cell mediated immune response (Herberman *et al*., 1975). Cytotoxic T cells are the key players behind the direct inhibition of tumour growth and also the cytokine mediated lysis of tumour cells (Rachmut *et al*., 2013). EL4- bearing animals are associated with decreased immune responses, especially cell-mediated immune response due to impaired generation of CTL (Sheeja and Kuttan, 2007, Win *et al*., 2002). *Curculigo orchioides* methanolic extract and the Curculigoside enhanced the CTL production and response in the EL-4 bearing animals. Increased number and activity of CTL reduced the tumour burden and delayed the death due to tumour development. Enhancement of CTL activity gives insight to the stimulatory effect of the plant extract and Curculigoside on the cellular wing of immune response.

IL-2 and IFN-γ forms the two major cytokines involved in the T- cell development and maturation. IL-2 is released by T cells during immune response and it is necessary for the growth, proliferation, and differentiation of T cells to become 'effector' T cells (Welte *et al*., 1982). Whereas IFN-γ which is produced predominantly by natural killer (NK) and natural killer T (NKT) cells as part of the innate immune response, and by CD4 Th1 and CD8 cytotoxic T lymphocyte (CTL) once antigen-specific immunity develops (Milstone and Waksman, 1970, Naylor, 1983). Both the extract and Curculigoside enhanced the production of IL-2 and IFN-γ which is giving insight to the enhanced T-cell development and activation on tumour challenge (Figure 3.9).

Thus Curculigoside and *C. orchioides* was found to exhibit profound immune response stimulatory effect in the experimental animals, and being a non toxic plant it is safe to be administered in the humans also. The immunestimulatory effects of these plant products can be utilized in the conventional cancer treatment regimens were the immune deprivation is the major drawback.

Table.3.1.A. Toxicity profile of *C. orchioides* extract

	Control	*C. orchioides* (mg/kg body weight)			
		50	100	200	250
Mortality	None	None	None	None	None
Behavioural change	None	None	None	None	None
B.wt change (g)	+2.02 ±0.8	+2.23±0.62	+2.62±0.82	+2.35±0.45	+2.13±0.63
Organ weight (g/100 g b.wt)					
Liver	5.32±0.97	5.54±0.84	5.13±0.58	5.43±0.85	5.22±0.66
Spleen	0.34±0.08	0.41±0.10	0.44±0.22	0.47±0.53	0.48±0.58
Thymus	0.13±0.002	0.15±0.005	0.15±0.004	0.158±0.005	0.14±0.005
Kidney	1.38±0.2	1.34±0.12	1.36±0.10	1.38±0.2	1.36±0.02
Lungs	0.66±0.08	0.63±0.05	0.63±0.06	0.65±0.08	0.66±0.07
Serum ALP (U/ml)	14.26±2.26	13.87±1.88	13.63±1.78	14.23± 1.02	13.58±1.66
Serum GPT (U/ml)	58.00±4.45	63.00±3.30	59.00±4.00	56.00±2.28	60.00±2.56
Blood urea (mg/dl)	44.80±4.56	45.60±3.3	45.4±5.36	43.5±6.02	45.8±5.60
Serum creatinine (mg/dl)	0.89±0.03	0.87±0.02	0.89±0.03	0.88±0.05	0.89±0.04

Animals were treated with different doses of extract (14 days), observed for 14 days and then sacrificed on 15^{th} day to check the parameters. Values are the mean ± SD. All the treated animals were carefully examined for 14 days for any signs of toxicity (behavioural changes and mortality) none - no toxic symptoms were seen during the observation period.

Table.3.1.B. Toxicity profile of Curculigoside

	Control	Concentrations of Curculigoside (mg/kg b.wt)		
		10	20	50
Mortality	0/8	0/8	0/8	1/8
Behavioural change	None	None	None	None
B.wt change	+2.24 ± 0.7	+2.26 ± 0.62	+1.82 ± 0.66	-0.86 ± 0.38
Organ weight (g/100 g body weight)				
Liver	5.77 ± 0.82	5.28 ±0.89	5.29 ± 0.58	5.92 ± 0.74
Spleen	0.39 ± 0.07	0.48 ± 0.07[a]	0.50 ± 0.06[a]	0.52 ± 0.11[a]
Thymus	0.12 ± 0.002	0.16 ± 0.004[c]	0.16 ± 0.005[c]	0.16 ± 0.006[c]
Kidney	1.18 ± 0.06	1.28 ± 0.05	1.24 ± 0.16	1.34 ± 0.08
Lungs	0.64 ± 0.08	0.63 ± 0.05	0.65 ± 0.05	0.66 ± 0.04
Serum ALP (U/mL)	16.40 ± 1.82	14.72 ± 1.20	16.80 ± 1.92	18.24 ± 1.16
Serum GPT (U/mL)	54.22 ± 3.22	58.00 ± 3.36	61.25 ± 3.62[c]	88.66 ± 2.88[c]
Blood Urea (mg/dL)	46.20 ± 2.24	44.52 ± 1.82	44.40 ± 1.85	43.50 ± 2.21
Serum Creatinine (mg/dL)	0.84 ± 0.04	0.87 ± 0.04	0.85 ± 0.06	0.83 ± 0.05

Animals were treated with different doses of Curculigoside (14 days), observed for 14 days and then sacrificed on 15th day to check the parameters. All data were represented as mean ± S.D. (n=8). Data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's t test. $^{a}p<0.001$, $^{c}p<0.05$ significantly different from control.

Table.3.2. Effect of *C. orchioides* and Curculigoside on lymphoid organ weight

Group	Relative weight (g/100 g b.wt)	
	Thymus	Spleen
Normal control	0.13 ± 0.04	0.380 ± 0.024
C. orchioides (20mg/kg B.wt)	0.19 ± 0.01[b]	0.452 ± 0.038[c]
Curculigoside (5mg/kg B.wt)	0.18 ± 0.01 [b]	0.434 ± 0.052 [c]

Animals were treated with C. *orchioides* (20 mg/kg body weight) or curculigoside (5 mg/kg body weight) for five days and were sacrificed on 6th day to collect the thymus and spleen. Weight of the thymus and spleen were recorded. All data were represented as mean ± S.D. [b]$p<0.01$, [c]$p<0.05$ significantly different from control.

Table 3.3. Effect of *C. orchioides* and Curculigoside on bone marrow cellularity and Number of α-esterase positive cells

Group	Bone marrow cellularity (x10^6)/femur	Number of α-esterase positive cells/4000 cells
Normal control	16.29 ± 0.86	814.7 ± 42.47
C. orchioides (20mg/kg B.wt)	24.6 ± 1.41[b]	1109 ± 88.6 [b]
Curculigoside (5mg/kg B.wt)	23.7 ± 1.8[b]	1045 ± 62.4[b]

Animals were treated with C. *orchioides* (20 mg/kg body weight) or curculigoside (5 mg/kg body weight) for five days and were sacrificed on 6th day to collect bone marrow from the femur bone. Bone marrow cellularity and number of α-esterase positive cells (azo dye coupling method) were determined. All data were represented as mean ± S.D. (n=6). [b]$p<0.01$significantly different from control.

3.4. Effect of the test materials on *in vivo* CTL generation and survival of tumour bearing animals (Method I)

Group	No. of days survived	% ILS	Relative efficacy (compared to EL-4 alone)
EL-4 alone	34.5±2.31	-----	-----
EL4 + normal alloimmunized effector cell	39.9±1.96 [a]	15.6	1.16 X
EL-4+ *C.orchioides*	45.0±1.15 [a d]	30.4	1.3 X
EL-4+ Curculigoside	49.4±1.62 [a d]	43.2	1.43 X
EL-4+ *C.orchioides* – treated alloimmunized spleen cells	53.6±2.47 [a d]	55.5	1.55 X
EL-4+ Curculigoside - treated alloimmunized spleen cells	51.0±2.00 [a d]	47.8	1.48 X

All the animals received EL4 cells (5×10^6 cells/ml) with effector cells produced *in vivo* by different types of treatments with *C. orchioides* extract or Curculigoside. CTL activity was determined by Winn's neutralization assay and animals were observed for survival. All data are expressed as means ± SD. [a]$p<0.001$ compared with EL-4 alone [d]$p<0.001$compared with EL4 + normal alloimmunized effector cell.

Table 3.5. Effect on *in vitro* CTL generation and survival of animals (Method II)

Group	No. of days survived	% ILS	Relative efficacy (compared to EL-4 alone)
EL-4 alone	34.5±2.31	-----	----
EL4 + normal cocultured spleen cells	41.0±2.00[a]	18.9	1.19 X
EL4 + *C.orchioides*-treated cocultured spleen cells	55.4±1.62 [ad]	60.5	1.61 X
EL4 + Curculigoside-treated cocultured cells spleen	52.6±2.13 [ad]	52.5	1.52 X

Effector cells produced *in vitro* conditions from the animals treated with *C. orchioides* extract or Curculigoside. All the animals received EL4 cells (5×10^6 cells/mL) with *in vitro* produced effector cells. Animals were observed for survival. All data are expressed as means ± S.D. [a]$p<0.001$, compared with EL4 alone, [d]$p<0.001$compared with EL4 + normal co-cultured spleen cells.

Table 3.6. Effect on *in vitro* CTL generation and survival of animals (Method III)

Group	No. of days survived	% ILS	Relative efficacy (compared to EL-4 alone)
EL-4 alone	34.5±2.31	-----	-----
EL4 + normal cocultured spleen cells	41.4±3.46[a]	20	1.2 X
EL4 + *C.orchioides*-treated cocultured spleen cells	49.5±2.83[ad]	43.5	1.43 X
EL4 + Curculigoside-treated cocultured cells spleen	50.5±1.86[ad]	46.4	1.46 X

All the animals received EL4 cells (5×10^6 cells/mL) and effector cells produced *in vitro* culture conditions in the presence and absence of *C. orchioides* or Curculigoside for 5 days at 37°C. CTL activity was determined by Winn's neutralization assay and animals were observed for survival. All data are expressed as means ± S.D. [a]$p<0.001$, compared with EL4 alone, [d]$p<0.001$ compared with EL4 + normal cocultured spleen cells.

Figure: 3.1.A. Effect of *C. orchioides* on total WBC count

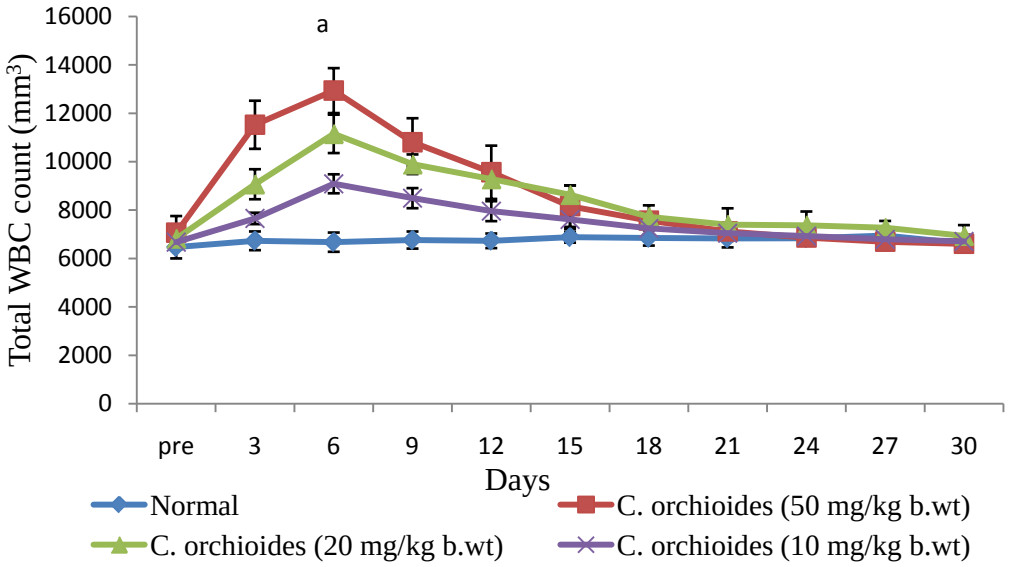
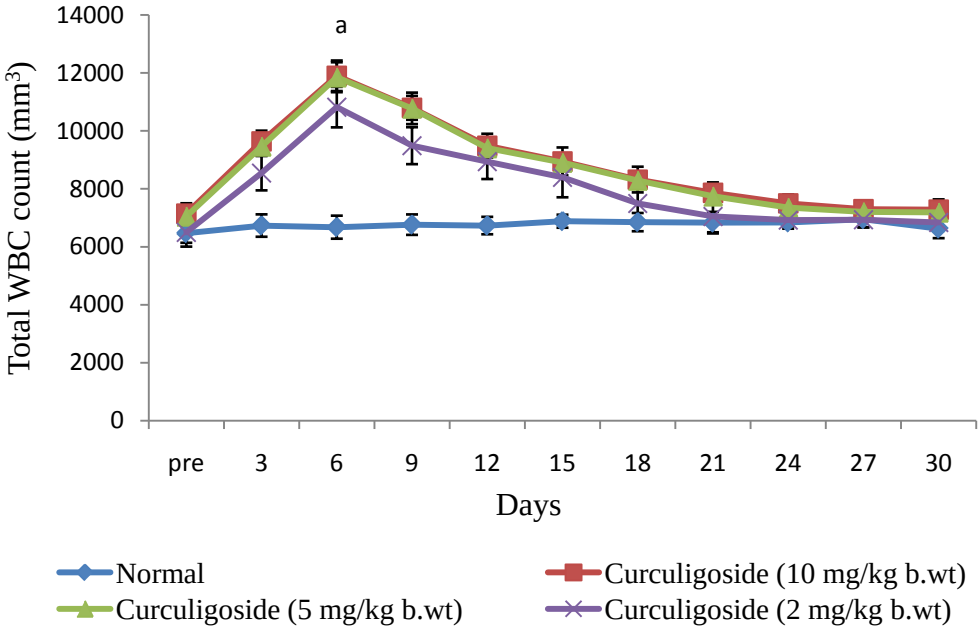
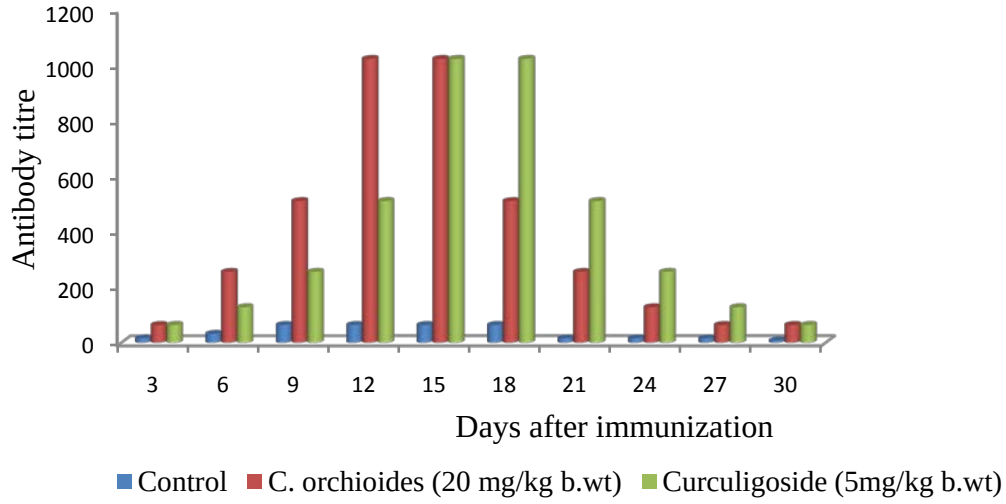


Figure: 3.1.B. Effect of Curculigoside on total WBC count



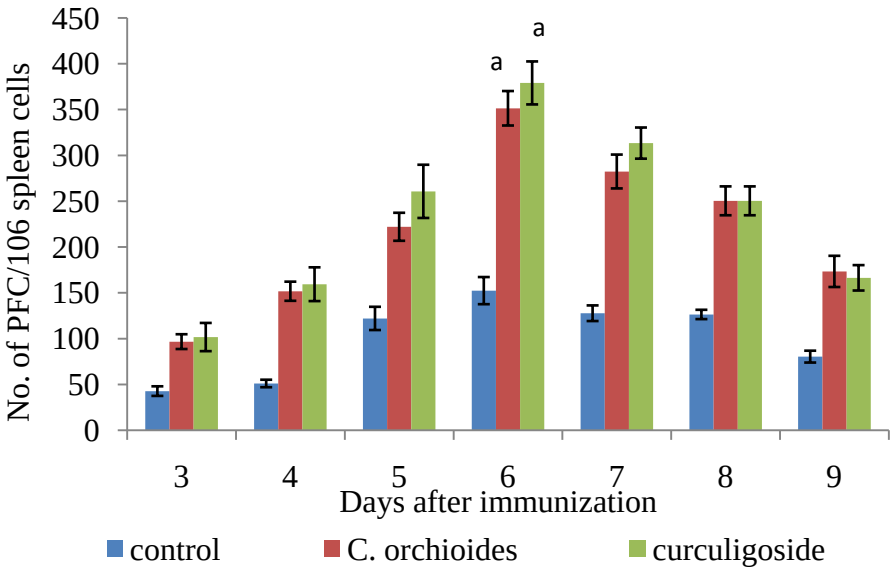
Balb/c mice were treated with or without *C. orchioides* / curculigoside for 5 days and the blood was collected every 3rd day from caudal vein and continued up to 30 days. The blood was mixed with diluting fluid and the total WBC count was determined using haemocytometer. [a]P<0.001, when compared to normal.

Figure 3.2. Effect of *C. orchioides* and Curculigoside on circulating antibody titre



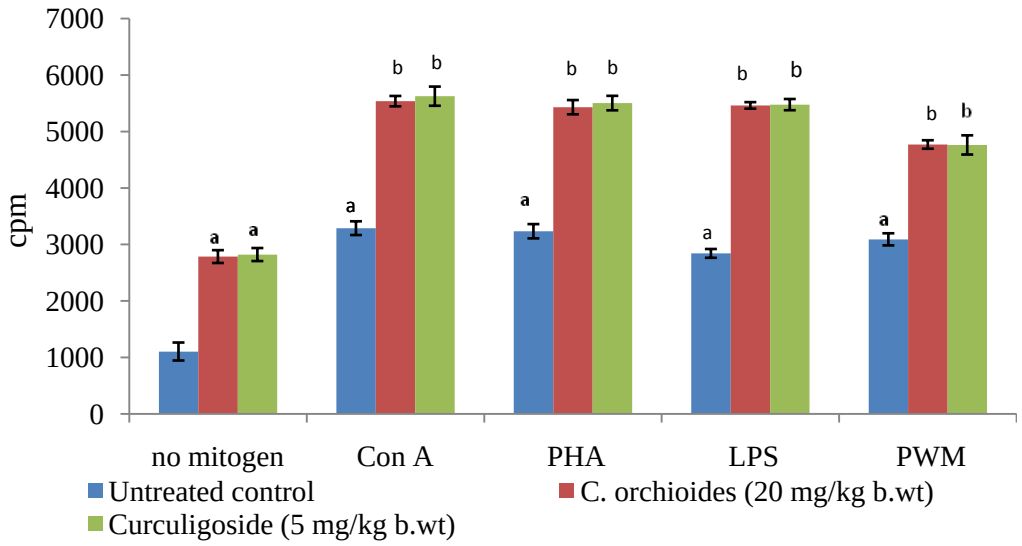
Balb/c mice were treated with or without *C. orchioides* / curculigoside for 5 days and all the animals were immunized with sRBC and the blood was collected from tail vein of all the animals at 3 days interval for 30 days to separate serum and antibody titre was determined using sRBC as antigen.

Figure: 3.3. Effect of *C. orchioides* and Curculigoside on number of plaque forming cells



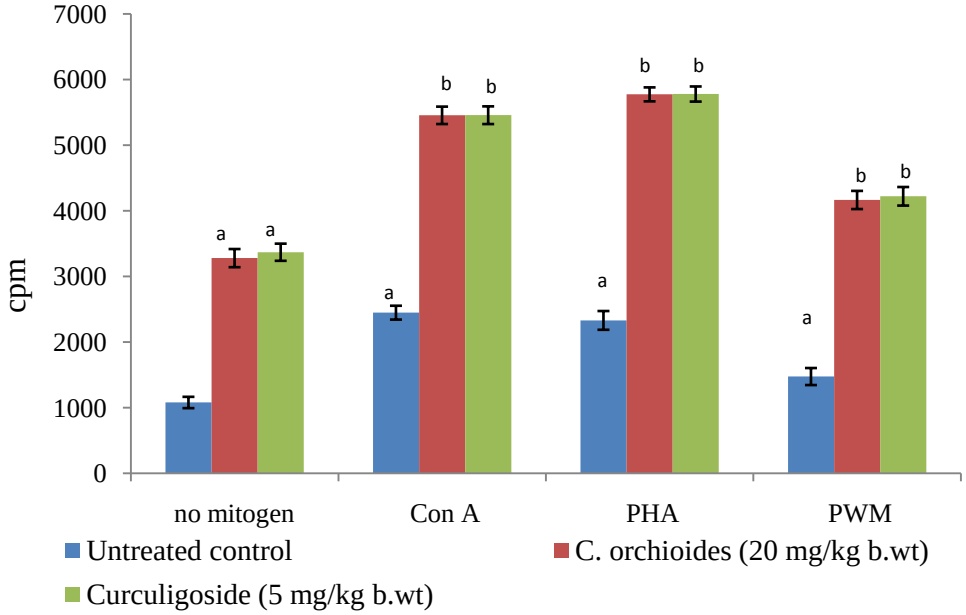
Mice were administered with the test material for 5 days and the animals were immunized with sRBC. Animals were sacrificed at different time points to collect the spleen, spleen cells were processed to determine the number of plaque forming cells by Jerns plaque assay, $^{a}P<0.001$, compared to control.

Figure 3.4. Effect of *C. orchioides* and curculigoside on splenocyte proliferation



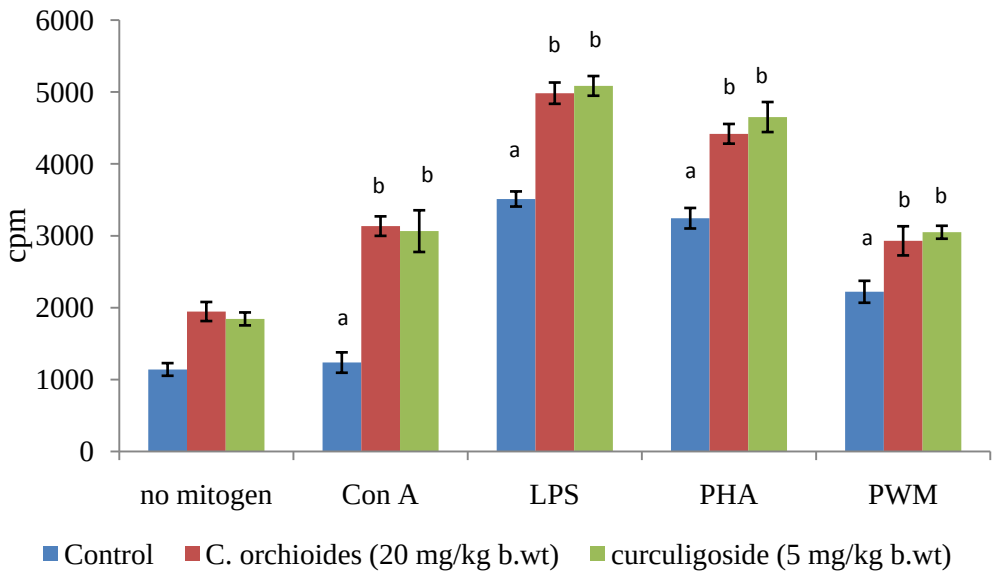
Splenocytes from the test material treated/untreated animals were incubated with different mitogens and the proliferation was determined using thymidine incorporation assay. [a]p<0.01 when compared with normal without mitogen, [b]p<0.01, when compared to normal splenocytes with the respective mitogen.

Figure 3.5. Effect of *C. orchioides* and Curculigoside on thymocyte proliferation



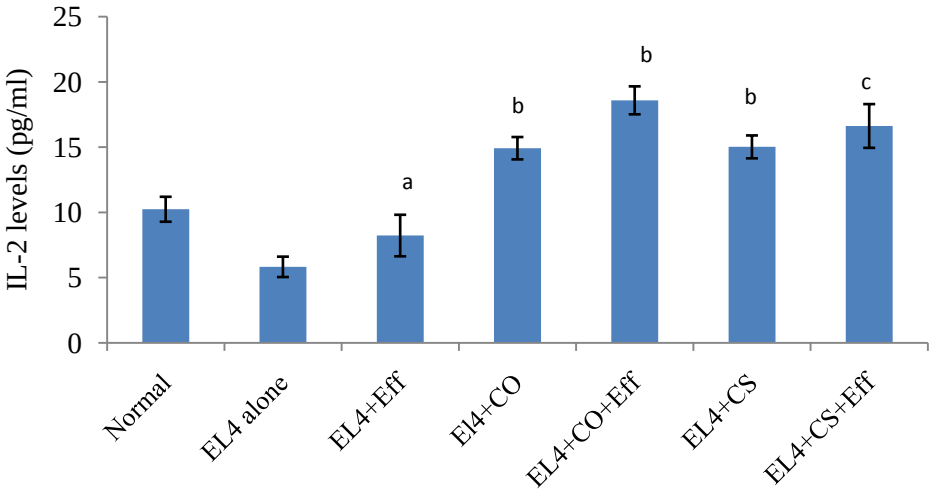
Thymocytes from the test material treated/untreated animals were incubated with different mitogens and the proliferation was determined using thymidine incorporation assay. [a]p<0.01 when compared with normal without mitogen, [b]p<0.01, when compared to normal thymocytes with the respective mitogen.

Figure 3.6. Effect of *C. orchioides* and Curculigoside on bone marrow cell proliferation



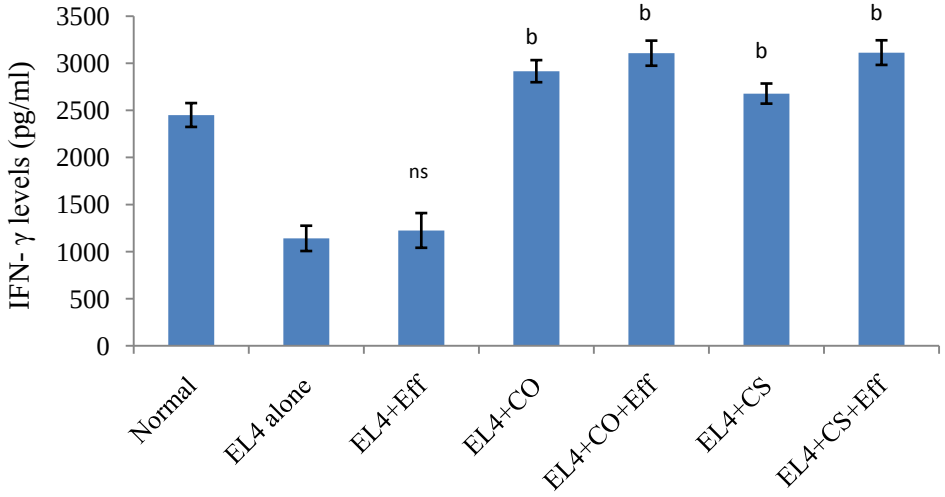
Marrow cells from the test material treated/untreated animals were were incubated with different mitogens and the proliferation was determined using thymidine incorporation assay. [a]$p<0.01$ when compared with normal without mitogen, [b]$p<0.01$, when compared to normal bone marrow cells with the respective mitogen.

Figure 3.7. Effect of *C. orchioides* and Curculigoside on IL-2 levels



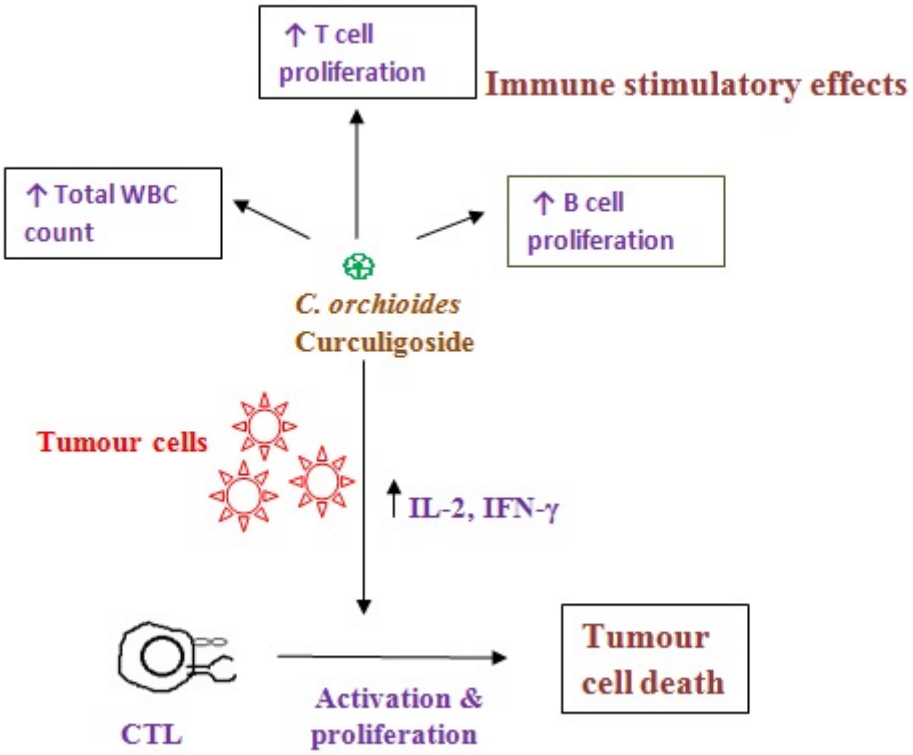
Blood was collected from each animal (Method I) by tail vein bleeding 2 days after the last dose of *C. orchioides* and Curculigoside. Serum IL-2 levels were determined using ELISA kit. Results are expressed as means ± S.D. $^{a}p<0.05$ compared to El-4 alone, $^{b}p<0.001$ compared with EL4 + normal alloimmunized effector cell, $^{c}p<0.01$ compared with EL4 + normal alloimmunized effector cell.

Figure: 3.8. Effect of *C. orchioides* and Curculigoside on IFN- γ levels



Serum IFN-γ levels were determined using ELISA kit. Results are expressed as means ± S.D. $^{c}p<0.001$ compared with EL4 + normal alloimmunized effector cell, ^{ns}p – not significant when compared to El-4 alone.

Figure 3.9. Immune stimulatory effects of *C. orchioides* and Curculigoside



Chapter 4

Studies on the Anti-inflammatory activity of C. orchioides and Curculigoside

4.1. INTRODUCTION

This chapter deals with the studies on anti-inflammatory activity of *C. orchioides* and Curculigoside using acute and chronic inflammatory models. Effect of the test materials on the proinflammatory cytokine profiles were also analyzed during the studies. The mechanism of anti-inflammatory activity was then evaluated using cultured macrophages which were stimulated with LPS. Since cancer is an inflammatory disease, effect of the test materials on solid tumour development was also studied using B16F10 cells in C57BL/6 mice. For ascites tumour model Ehrlischs ascites carcinoma (EAC) cells were injected to Swiss albino mice.

4.2. MATERIALS AND METHODS

4.2.1. Animals

Balb/c, Swiss albino and C57BL/6 mice were used in this study.

4.2.2. Cells

The cell line used for this L929, B16F10 and EAC cells were used in this study.

4.2.3. Chemicals and reagents

Carrageenan, formalin, LPS, highly specific ELISA kits for Interleukin-1β (IL-1β), Interleukin-6 (IL-6), C-reactive protein (CRP), and Tumour necrosis factor-α (TNF-α) were used in this study.

4.2.4. Anti-inflammatory Activity of *C. orchioides* and Curculigoside -Acute Inflammatory Model

Carrageenan Induced inflammation

Anti-inflammatory activity was evaluated by paw oedema method (Langrange *et al*., 1974; Zhou *et al*., 2004). Balb/c mice were treated with or without plant extract (at a dose of 50, 20 and 10 mg/kg b.wt) or Curculigoside (10, 5, 2 mg/kg b.wt) (i.p) for five consecutive days. One group of animals were administered with Diclofenac at a dose of 10 mg/kg b.wt. for 5 days whereas another group of animals received 1% gum acacia and served as vehicle control. One hour, after the last dose of test material administration, paw oedema was induced to all animals by injecting 0.02 ml freshly prepared 1% suspension of carrageenan in

normal saline on sub-plantar region of the right hind paw to induce acute inflammation (Winter *et al*., 1962). The thickness of paw was measured using digital Vernier callipers just before carrageenan injection and continued for 6 hours at 1 hour intervals followed by 24 and 48 hours for all animals.

$$\% \text{ inhibition of paw thicknes} = \frac{(tC_n - tC_0) - (tT_n - tT_0)}{(tC_n - tC_0)} \times 100$$

Where, tC_n=paw thickness at particular time point of control animal; tC_0=paw thickness before induction; tT_n=paw thickness at particular time point of treated animal; and tT_0=paw thickness before induction.

4.2.5. Anti-inflammatory Activity of *C. orchioides* and Curculigoside - Chronic Inflammatory Model

Formalin-induced Model

Balb/c mice (eight animals/ group) were treated with the test materials as described above and chronic inflammation was induced by sub-plantar injection of freshly prepared 0.02 ml of 2% formalin in sterile water on the right hind paw of all animals (Chau, 1989). The paw thickness was measured using digital vernier callipers before and after formalin injection, continued up to 6 days. The percentage inhibition was calculated using the formula given earlier.

4.2.6. LPS induced model

a) Determination of the Effect of *C. orchioides* and Curculigoside on Serum Proinflammatory Cytokines and NO in LPS Treated Animals

Balb/C animals were used for this study (four groups, n=8). Sodium casienate (5%) was administered to the animals intra peritoneally (0.1ml) to elicit macrophages. Group I animals were kept as untreated control whereas, group II and III animals were treated with *C. orchioides* (20 mg/kg b.wt) and Curculigoside (5 mg/kg b.wt) respectively and then received a single dose of LPS, 2 hr after the last dose test material. Group IV animals served as LPS alone control (250 μg/animal. i.p) and after 6^{th} hr, all the animals were sacrificed. Blood

was collected by heart puncture and used for the estimation of NO (Green *et al*., 1982), CRP, and TNF-α.

b) Bioassay of Tumour Necrosis Factor-α

L929 (lung fibroblast) cells are sensitive to TNF- α and based on this, the bioassay was performed to study the activity of the test material on the inhibition of TNF- α production (Tomkins *et al*., 1992). Macrophages were collected from the peritoneal cavity of the above said animals, plated to each well (2×10^5 cells/well) of 96-well titre plate and incubated for 2 hr at 37°C in RPMI medium. Non-adherent cells were removed after incubation and fresh medium was added with the test materials at different concentrations and again incubated for 24 hr at 37°C in CO_2 atmosphere. The plates were centrifuged after incubation and the medium (100 µl) from each well was added to L929 cells (5×10^3 cells) cultured in 96-well titre plate in MEM medium. Plates were incubated for 48 hr and the cell density was assessed by MTT assay (Cole, 1986).

c) Determination of the Effect of *C. orchioides* and Curculigoside on the Production of Proinflammatory Cytokines by LPS-stimulated Macrophages

Macrophages were elicited by injecting 5% sodium caseinate intraperitoneally in Balb/c mice. Macrophages were washed with PBS and resuspended in RPMI-1640 with 10% FCS. The cells were plated in 96-well culture plates and incubated for 2 h at 37°C, in a 5% CO_2 atmosphere. After incubation, non adherent cells were removed and the adherent macrophages were incubated (2×10^6 cells/well) in complete medium (RPMI- 1640, 10% FCS, 100 µg/ml streptomycin and penicillin, 2 mM glutamine). Macrophages were cultured with or without LPS (5 µg/ml) in the presence and absence of *C. orchioides* (5, 2.5, 1 µg/ml) or Curculigoside (2, 1 and 0.5 µg/ml) for 24 h at 37°C in 5% CO_2 atmosphere. After 24 h, the plates were centrifuged and the supernatant was used for the estimation of TNF-α, IL-1β, and IL-6 according to the kit manufactures protocol. Cell lysate was prepared and gene expression studies were carried out with this macrophage by reverse transcription PCR using specific primers to amplify the proinflammatory cytokine genes.

d) Determination of the Effect of *C. orchioides* and Curculigoside on gene expression pattern and the Production of NO by LPS-stimulated Macrophages

Macrophages were cultured as above with or without LPS (5 μg/ml) in the presence and absence of *C. orchioides* extract (5, 2.5, 1 μg/ml) / Curculigoside (2, 1, 0.5 μg/ml) for 24 h at 37°C in 5% CO_2 atmosphere. After 24 h, the plates were centrifuged and the supernatant was used for the estimation NO production by the Griess reagent method (Green *et al*., 1982).

Macrophages were used for cDNA preparation using cell to cDNA kit from Ambion USA, according to the given protocol by the manufacturer. Using specific primers, amplification was performed and bands were visualised on agarose gel under UV light.

4.2.7. Antitumour studies

a) Determination of the effect of *C. orchioides* and Curculigoside on solid tumour development

Solid tumour was induced by injecting B16F10 cells (1 x 10^6cells/animal) subcutaneously to the right hind limbs of 8 groups (8 animals/group) of C57BL/6 mice. Group I was kept as untreated tumour control and Group II were treated with 10 mg/kg b.wt. Cyclophosphamide. Group III, IV, V was treated with 10 consecutive doses of *C. orchioides* extract at concentrations of 50mg/kg b.wt., 20 mg/kg b.wt. and 10 mg/kg b.wt. respectively. Group VI, VII and VIII animals were treated with Curculigoside 10, 5 and 2 mg/kg b.wt. respectively. Treatment was started on 6^{th} day of tumour induction. The radii of developing tumours were measured using vernier calipers at 7 days intervals for one month and tumour volume was calculated using the formula $V = 4/3\ \pi r_1^2\ r_2$, where 'r_1' and 'r_2' represent the major and minor diameter, respectively (Atia and Weiss, 1966).

b) Determination of the effect of *C. orchioides* and Curculigoside on the survival of ascites tumour bearing animals

Seven groups (8 animals/group) of Swiss albino mice were induced ascites tumour by injecting 1 x 10^6 cells/animal to the peritoneal cavity. Group I: EAC

alone (Control) Group II, III and IV: EAC + *C. orchioides* treated with different doses (50, 20 and 10 mg/kg b.wt.). Group V, VI, VII animals were administered EAC + Curculigoside (10, 5 and 2 mg/kg b.wt., respectively) for 10 consecutive days. The death pattern of the animals due to tumour burden was noted and the percentage of increase in lifespan was calculated using the formula,

[(T-C)/T] x 100, where 'T' and 'C' represent the number of days that treated and control animals survived, respectively.

4.3. RESULTS

4.3.1. Effect of *C. orchioides* and Curculigoside on Carrageenan induced Inflammation

In carrageenan induced inflammation, control animals showed a maximum paw thickness of 0.43 cm at 3rd hr after carrageenan administration. Animals treated with alcoholic extract of *C. orchioides* showed a significant reduction in paw thickness during the same time point (0.34 cm) and was found to be normalized after six hrs of carrageenan injection, where as in control animals the normal value was attained only at 24th hr after carrageenan injection. The percentage inhibition in the extract treated group was 42% (Fig. 4.1). Curculigoside (5 mg/kg b.wt) was also found to exhibit significant anti-inflammatory potential with a percentage inhibition of 53 %.

4.3.2. Effect of *C. orchioides* and Curculigoside on Formalin induced Inflammation

The *C. orchioides* extract treated animals showed a significant reduction in paw thickness (44%) after the administration of formalin compared to the control group. A peak value of 0.36 cm of the inflamed paw was obtained on 3rd day in control animals, where as the extract treated animals showed 0.30 cm thickness of the paw on the same day (Figure. 4.2). Curculigoside showed 38 % inhibition in paw oedema formation where as the standard drug diclofenac showed 50% inhibition.

4.3.3. Effect of *C. orchioides* and Curculigoside on NO and Proinflammatory Cytokines levels in LPS Administered Animals

C. orchioides also found to be effectively inhibiting the serum TNF-α, CRP and NO levels in LPS treated animals (Table 4.1). The normal levels of TNF-α and C-reactive protein (CRP) levels (23 ± 2.2 and 537.7 ± 11.2 pg/ml, respectively) were increased by the treatment with LPS (533.5 ± 13.5 and 3910.5 ± 83.1pg/ml, respectively) which was then significantly ($P < 0.001$) reduced to 145.2 ± 14.9 and 1079.7 ± 80.9 pg/ml, respectively, by the administration of *C. orchioides* (20 mg/kg b.wt.). NO levels were also reduced to 31±2.4 μmoles on extract administration when compared to LPS alone treated animals (81 ± 4.4 μmoles). Curculigoside administration provided a comparable results with a reduced levels of TNF-α, C- reactive protein (CRP) and NO release (126.8 ± 11.4 pg/ml, 1267 ± 117 pg/ml, and 30.5 ± 3.3 μmoles respectively).

4.3.4. Effect of *C. orchioides* and Curculigoside on the Production of Proinflammatory Cytokines and NO by LPS-stimulated Macrophages

The release of proinflammatory cytokines such as TNF- α, IL-1β and IL-6 by cultured macrophages were found to be significantly elevated by LPS treatment. The extract was found to be very effective in down regulating the release of by the LPS stimulated macrophages (Figure 4.3.A, 4.3.B and 4.3.C respectively for TNF- α, IL-1β and IL-6). Similarly, Curculigoside was also found to exhibit good inhibitory potential on pro-inflammatory cytokine release by the activated macrophages.

The nitrite level produced in cell culture supernatant was measured, showing that *C. orchioides* and Curculigoside inhibited nitrate production in a statistically significant manner (Table 4.2). NO level was found to be elevated after 24 h of LPS treatment (75.3 ± 4 μmoles) compared to normal macrophage nitrite level (18.4 ± 0.6 μmoles) and the level was significantly reduced by the treatment with *C. orchioides* at a dose of 5 μg/ml (26 ± 2.5 μmoles) and Curculigoside at a dose of 2 μg/ml (23.3 ± 2.01 μmoles) along with LPS.

4.3.6. Gene expression studies

Results of mRNA expression studies were represented in figure 4.4. TNF- α, IL-1β, IL-6, COX-2 and iNOs gene expressions were found to be decreased with the extract and compound treatment when compared to LPS treated macrophages.

4.3.6. Bioassay of TNF –α

Supernatant from LPS stimulated macrophage culture caused the lysis of L929 cells up to 80%, whereas the supernatant from LPS + *C. orchioides* treated macrophages (35%) and LPS + Curculigoside treated macrophages (31%) resulted in decreased cell death showing its inhibitory effect on the TNF production by the cultured macrophages (Figure 4.5).

4.3.7. Effect of *C. orchioides* and Curculigoside on Solid Tumour Development

There was a significant reduction of tumour volume in *C. orchioides* treated animals (Fig. 4.6). Tumour volume of control animals was 3.22 cm^3 on 30th day while that of *C. orchioides* treated (simultaneous, 20 mg/kg b.wt.) animals was only 2.55 cm^3, on the same day; while it was 2.82 cm^3 for Curculigoside treated animals.

4.3.8. Effect of *C. orchioides* and Curculigoside on the Survival of Ascites Tumour Bearing Animals

Life span of ascites tumour bearing mice treated with methanolic extract of *C. orchioides* was found to be significantly ($p<0.01$) increased (Table 4.3). Control animals survived only 18 days after the tumour induction whereas the extract (50 mg/kg b.wt) and Curculigoside (10 mg/kg b.wt) treated animals were survived up to 26 (relative efficacy 1.44 X compared to tumour control) and 24 (relative efficacy 1.33 X compared to tumour control) days respectively. Whereas the efficacy of extract at a dose of 50 mg/kg b.wt when compared to its next dose (20 mg/kg b.wt) was only 0.04 X; similarly in the case of Curculigoside the dose 10 mg/kg b.wt was found to be only 0.043X times efficient than the lower dose 5 mg/kg b.wt.

4.4. DISCUSSION

Even though the area of synthetic drug development has attained remarkable escalation in recent times, their toxic side effects remain as a major drawback where the natural products are getting more attention due to safety reasons. Here comes the importance of conducting a systematic analysis in order to find out the efficacy of plants against inflammation so as to exploit them as herbal anti-inflammatory agents (Paschapur *et al*., 2009).

It is Rudolph Virchow who first proposed that inflammation can lead to cancer development and progression (Heidland *et al*., 2006). As per today's knowledge more than 25% of human cancers are arise from chronic inflammation and chronic infection besides this, chronic inflammation can lead to pulmonary and cardiovascular diseases, diabetes, and neurological disorders also (Morrison, 2012). It has been reported that in the process of carrageenan-induced acute inflammation is a bi-phasic event, with the early phase of histamine, serotonin, and bradykinin production and late phase of neutrophils accumulation, cyclooxygenase production, release of various pro-inflammatory cytokines and NO (Vinegar *et al*., 1969; Akkol *et al*., 2008; Tanas *et al*., 2010). According to Aggarwal and team, there are several pro-inflammatory gene products that can mediate a critical role in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis (Aggarwal *et al*., 2006).

The results of the present study revealed that, administration of the methanolic extract and Curculigoside inhibited the oedema starting from the first hour and during all phases of inflammation, which is probably by the inhibition of different aspects and chemical mediators like proinflammatory cytokines and nitric oxide of inflammation. In the present study, *C. orchioides* and Curculigoside were found to possess a significant anti-inflammatory activity in both acute and chronic models. The extract and compound was found to be more significant in reducing the levels of TNF-α secretion by the macrophages and the results were confirmed by bioassay using L929 cells, which are very sensitive to TNF-α. Extract and compound treatment decreased the TNF-α release by the LPS stimulated macrophages and thus L929 cells showed less cytotoxicity to the supernatant from LPS + extract/Curculigoside treated macrophages when compared to the

supernatant of LPS alone treated macrophages. *C. orchioides* was also found to inhibit the release of other proinflammatory cytokines like IL-1β and IL-6 and chemical mediators like NO by the macrophages by down regulating the gene expressions (Figure 4.8). The study also indicates that, the anti-inflammatory activity of the plant is mainly due to the presence of Curculigoside. Solid tumour and ascites tumour model studies revealed the antitumour efficacy of the test materials that may be partially provided by the immune stimulatory and anti-inflammatory potentials of the plant.

Table 4.1. Effect of *C. orchioides* and Curculigoside on Serum TNF- α, CRP and NO levels of LPS treated Balb/c mice

Treatment	TNF-α (pg/ml)	CRP (pg/ml)	NO (μmoles)
Normal	23 ± 2.2	537.7 ± 11.2	23.7±1.7
LPS treated	533.5 ± 13.5	3910.5 ± 83.1 [b]	81 ± 4.4 [b]
LPS + *C. orchioides* (20mg/kg b.wt)	145.2±14.9[a]	1079.7 ± 80.9 [a]	31 ± 2.4[a]
LPS +Curculigoside (5 mg/kg b.wt)	126.8 ± 11.4[a]	1267 ± 117[a]	30.5 ± 3.3[a]

All data are mean ± SD. [b] $p<0.001$, when compared to normal animals; [a]$p<0.001$ when compared to LPS alone treated animals. LPS- Lipopolysaccharide.

Table 4.2. Effect of *C. orchioides* and Curculigoside on NO release by LPS stimulated macrophages

Treatment (μg/ml)	NO (μmoles)
M alone	18.4 ± 0.6
M + LPS	75.3 ± 4 [b]
M + LPS + *C. orchioides* (1)	56.8 ± 3.4[a]
M + LPS + *C. orchioides* (2.5)	44 ± 3.2 [a]
M + LPS + *C. orchioides* (5)	26 ± 2.5 [a]
M + LPS + Curculigoside (0.5)	50.2 ± 3.4 [a]
M + LPS + Curculigoside (1)	38.3 ± 1.2 [a]
M + LPS + Curculigoside (2)	23.3 ± 2.01 [a]

[b] $p<0.001$, when compared to macrophage (M) alone; [a]$p<0.001$ when compared to LPS stimulated macrophages. LPS- Lipopolysaccharide.

Table 4.3. Effect of *C. orchioides* and Curculigoside on the Survival of Ascites Tumour Bearing Animals

Treatment	No. of days survived	% increase in life span	Relative efficacy (Compared to tumour control)
Control	18 ± 1.7	---	---
C. orchioides 50 mg/kg b.wt	26 ± 2.3	44	1.44 X
C. orchioides 20 mg/kg b.wt	25± 1.5	39	1.39 X
C. orchioides 10 mg/kg b.wt	21 ± 1.8	17	1.17 X
Curculigoside 10 mg/kg b.wt	24 ± 3.2	33	1.33 X
Curculigoside 5 mg/kg b.wt	23± 1.8	28	1.28 X
Curculigoside 2 mg/kg b.wt	21± 1.2	17	1.17 X

One million EAC cells were injected to the peritoneal cavity of the mice to develop ascites tumour and observed for their survival rate.

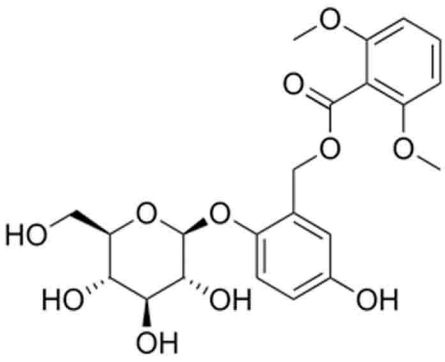
FIGURE 1.1. *CURCULIGO ORCHIOIDES GAERTN.* AND CURCULIGOSIDE

C. ORCHIOIDES PLANT

WITH TUBER

WITH INFLORESCENCE

CURCULIGOSIDE-STRUCTURE



Chapter 5

Analysis of the Anti-metastatic potential of C. orchioides and Curculigoside

5.1. INTRODUCTION

Transition of a normal cell to invasive cancer cell is accompanied by a drastic phenotypic and biochemical changes. These changes include the response to growth factors and signals, cell adhesion pattern, gene expression, motility and invasiveness etc. Various immune cells, adjacent stroma cells as well as chemokines and their receptors also play a vital role in cancer progression. Along with this, the micro-environment, vascularisation and the supply with special cytokines affect the above-mentioned changes (Leber and Efferth, 2009). A number of molecular targets involved in metastatic progression have been discovered till the date to prevent metastasis, but in many patients, by the time of diagnosis, metastasis has already occurred. If this were not the case, surgical excision of primary neoplasms would be curative. Thus metastasis still remains as the major hurdle of cancer treatment (Fidler and Kripke, 2015).

In this part of work B16F10 melanoma cells induced metastatic model was used for the experimental studies on the anti-metastatic effects of *C. orchioides* and Curculigoside. Besides these, the modulatory effects of the test materials on the cell mediated immune responses against the metastatic progression were also analysed. Activity of NK cells, the chief players of anti-tumour immunity and NK mediated antibody dependent cytotoxicity were also determined in this study.

5.2. MATERIALS AND METHODS

5.2.1. Animals

Male C57BL/6 mice (6-8 week old).

5.2.2. Chemicals

Carbazole, p-dimethyl amino benzaldehyde, acetyl acetone, Hydroxyproline, Glucuronic acid lactone, N-acetyl neuraminic acid, L- γ-glutamyl-p-nitroanilide and glycyl glycine are the chemicals used in the study. DMEM with 10% serum was used to culture B16F10 cells. Highly specific quantitative sandwich ELISA kit for mouse IL-2, IFN-γ, TNF-α, IL-1β, IL-6 and VEGF were also used for this study.

5.2.3. Cell lines

B16F-10 melanoma cells, K-562 cells and SRBCs.

5.2.4. Induction of metastasis

B16F10 cells (10^6) were injected through the lateral tail vein to induce lung metastasis (Fidler, 1978).

5.2.5. Determination of the effect of *C. orchioides* and Curculigoside on lung tumour nodule formation and survival rate of tumour-bearing animals

Male C57Bl/6 mice were induced metastasis and were divided in to three groups (16 animals/group). Group I was kept as untreated metastatic tumour bearing control whereas Group II and III were treated with 10 doses of the extract (20 mg/kg b.wt; i.p.) and Curculigoside (5 mg/kg b.wt; i.p.) respectively from the next day of metastasis induction. For prophylactic treatment, test material administration was stated10 days before metastasis induction and for developed treatment modality, test material administration was stated from 7^{th} day of metastasis induction (2 sets of animal groups as described above were used for this). On 21^{st} day, 8 animals from each group were sacrificed, to collect blood and lungs. Lungs were observed for metastatic tumour nodule and then used for the estimation of collagen hydroxyproline (Bergman and Loxley, 1940), hexosamine (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962) contents. A portion of lung was fixed in 10% formaldehyde and used for histochemical analysis. Serum separated from the blood was used for the evaluation of serum sialic acid by the method of Skoza and Mohos, 1976, and γ-glutamyl transpeptidase (GGT) levels by the method of Szasz, 1976. Other group was kept to observe the life expectancy and the percentage increase life expectancy (%ILS) of the treated animals were calculated by the formula, $\%ILS=[T-C/C]\times100$, where T is the number of days survived by the treated animals and C represents the number of days survived by the control animals.

5.2.6. Determination of the effect of *C. orchioides* and Curculigoside on serum cytokines, VEGF and TIMP-1 levels

Experiment was set up in a similar way with 12 animals/group and 6 animals from each group was sacrificed on 7th day and other 6 animals on 21 st day after metastasis induction. Blood was collected from each animal, serum was separated and used for the estimation of various cytokines such as IL-2, IFN-γ, TNF-α, IL-1β, IL-6, VEGF and TIMP-1 was done using specific ELISA kits as per manufacturer's protocol.

5.2.7. Determination of the effect of *C. orchioides* and Curculigoside on gene expression of various pro and antimetastatic genes

Lung tissue was taken from each group, total RNA was isolated and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase. Specific primers were used to amplify MMP-2, MMP-9, VEGF, TIMP-1 and TIMP-2. The amplified products were loaded on a 1.5% agarose gel incorporated with ethidium bromide and electrophoresis was carried out and photographed under ultraviolet light.

5.2.8. Determination of the effect of *C. orchioides* and Curculigoside on cell mediated immune response in metastatic tumour bearing animals

Three groups (36 animals/group) of male C57BL/6 mice were taken and group I animals were kept as metastatic control animals, group II and III animals were treated with 5 consecutive dose of the extract (20 mg/kg b.wt) and Curculigoside (5 mg/kg b.wt). On the fifth day metastasis was induced to all the animals as described above and the animals were sacrificed on various time points. Spleen and blood was collected and processed to analyse NK cell activity, ADCC (by 4 h ^{51}Cr-release assay) and ACC (by trypan blue dye exclusion method) as described in chapter 2.

IN VITRO ANTIMETASTATIC STUDIES

5.2.9. Determination of cell viability by MTT assay

B16F10 cells were seeded in 96-well flat-bottom plates (5000 cells/well) and allowed to adhere for 24h at 37^{o}C with 5% CO_2 atmosphere. Different

concentrations of *C. orchioides* extract (10-100 μg/ml) and Curculigoside (5-100 μg/ml) were added and incubated further for 48h. MTT assay was performed as described in chapter 2.

5.2.10. Tumour cell adhesion assay

Type I collagen was coated on 96 well flat bottom titre plates and the assay was performed as described in chapter 2 by adding *C. orchioides* (10, 5 and 2 μg/ml) and Curculigoside (5, 2 and 1 μg/ml). Each experiment was done in triplicate.

5.2.11. Collagen matrix invasion and motility assay

Tumour cell invasion and motility assay was carried out as described in chapter 2. B16F-10 cells were seeded in modified Boyden Chamber for the assay and various concentrations of the test materials were used as described above.

5.2.12. Gelatin zymography

Experiment was performed according to the method of Billing *et al*., as described in chapter 2. Supernatant containing the proteases from the extract (10, 5 and 2 μg/ml) and Curculigoside (5, 2 and 1 μg/ml) treated and untreated melanoma cells were subjected to zymographic analysis (with or without trypsin activation) after determining the protein concentration.

5.3 RESULTS

5.3.1. Effect of *C. orchioides* and Curculigoside on lung tumour nodule formation and survival rate of tumour-bearing animals

B16F10 metastasis derived lung tumour nodule formation was found to be reduced upon *C. orchioides* and Curculigoside treatment in a significant manner when compared to the untreated metastatic tumour bearing control animals (Figure 5.1). Lungs of the control animals were filled with massive number of tumour nodules and assigned an arbitrary number of 250 (Liotta, 1986). Lifespan of the tumour bearing animals was also enhanced upon the treatment with the extract (51%) and compound (43%) which is represented on table 5.1. Simultaneous administration of the test materials provided better results, but

prophylactic administration also found to be significant in increasing the life expectancy.

5.3.2. Effect of *C. orchioides* and Curculigoside on lung collagen hydroxyproline content

Lung collagen hydroxyproline content of metastatic control animals were highly elevated (23.07 ± 2.38) when compared to the normal animals. Treatment with extract and Curculigoside reduced the hydroxyproline contents of the metastasis bearing lung to 15.03 ± 1.87 and 17.47 ± 2.4 respectively. Data is represented in Table 5.2.

5.3.3. Effect of *C. orchioides* and Curculigoside on lung uronic acid level

Effect of *C. orchioides* and Curculigoside on lung uronic acid levels is shown in Table 5.2. The elevated levels of uronic acid in the metastatic tumour bearing control animals was found to be 301.2 ± 21.3 μg/100mg tissue, which was significantly reduced to 199.3 ± 28.35 μg/100mg tissue and 235.9 ± 18.22 μg/100mg tissue respectively in the extract and compound treated animals.

5.3.4. Effect of *C. orchioides* and Curculigoside on lung hexosamine content

Along with the metastatic progression, lung hexosamine levels were also found to be elevated significantly (3.44 ± 0.48). On treatment with *C. orchioides* and Curculigoside these elevated levels were put down significantly to 1.91 ± 0.2 and 2.83 ± 0.23 respectively. Results are represented on table 5.2.

5.3.5 Effect of *C. orchioides* and Curculigoside on serum sialic acid and GGT levels

Effect of *C. orchioides* and Curculigoside on serum sialic acid and GGT levels are represented in table 5.3. Metastatic control animals showed a drastic elevation of serum sialic acid and GGT levels (137.5 ± 14.68 and 119 ± 10.83 respectively). For the extract treated group the sialic acid and GGT content in the serum were 93.15 ± 8.36 and 62.63 ± 6.46 whereas in the Curculigoside treated group the levels were 109.52 ± 10.22 and 84.23 ± 9.73 respectively.

5.3.7. Histopathological Analysis of Lungs

Histopathological examination of the metastatic lung tissue showed massive fibrosis and invasive tumour cells with melanin deposition. Alveolar space was reduced due to this fibrosis, reducing the vital capacity of the lungs. Area of necrosis was also present. Administration of *C. orchioides* and Curculigoside reduced lung fibrosis along with a reduction in tumour nodule formation (Figure 5.1).

5.3.8. Effect of *C. orchioides* and Curculigoside on serum pro-inflammatory cytokine levels of metastasis bearing animals

The levels of proinflammatory cytokines like TNF-α (Figure 5.2.A), IL-1β (Figure 5.2.B) and IL-6 (Figure 5.2.C) were significantly elevated during metastatic condition, upon treatment with *C. orchioides* extract and Curculigoside these levels were found to be decreased.

5.3.9. Effect of Test Materials on TIMP-1 and VEGF Level

TIMP -1 level of metastatic tumour bearing animals were drastically decreased on 7^{th} day (355.1 ± 20.6 pg/ml). Upon treatment with *C. orchioides* and Curculigoside these levels were found to be significantly increased to 501.6±29.1pg/ml and 435.3±27.8 pg/ml respectively on the same day (Figure.5.3.A).

VEGF is secreted by the tumour cells and the levels were found to be increased in metastatic control animals. But there was no inhibitory effect on the VEGF levels of the metastasis bearing animals by the treatment of extract or Curculigoside (Figure.5.3.B).

5.3.10. Effect of Test materials on Gene Expression

Gene expression pattern of various pro and anti-metastatic genes were studied and the results demonstrated that *C. orchioides* and Curculigoside down regulated the expression of MMP-2 and MMP-9 where as the TIMP-1 and 2 expressions were increased by the treatment of the test compounds (Figure 5.4).

5.3.11. Effect of *C. orchioides* and Curculigoside on cell mediated immune response on metastasis bearing animals

a) NK cell activity

Both the extract and compound was found to be effective in enhancing the NK cell mediated tumour defence (Figure 5.5.A). The maximum cell lysis was obtained on 5^{th} day of tumour induction in the extract (46%) and compound (42%) treated animals whereas a delayed response with a maximum cell lysis of 12 % was shown by untreated tumour bearing animals on 9^{th} day.

b) Antibody dependent cellular cytotoxicity (ADCC)

Stimulatory effect of the extract and curculigoside on ADCC was given in figure 5.5.B. Percentage cell lysis was maximum for *C. orchioides* and curculigoside treated animals on 9^{th} day (43% and 39% respectively) and that for the metastatic control animals were 11% on the same day. The maximum tumour cell lysis for the control animals were obtained on 13^{th} day (12%).

c) Antibody dependent complement mediated cytotoxicity

Administration of *C. orchioides* and Curculigoside resulted in a significant enhancement in ACC which is represented in Figure 5.5.C. The maximum cytotoxicity obtained for the extract and compound treated animals were 31% and 27% respectively on 15^{th} day of metastatic induction whereas the control animals showed only 13% cytotoxicity on the same day.

NK cells are large granular lymphocyte which forms the effector cells of innate immune system that can kill a target cell that are not restricted by MHC molecules (Kiessling *et al*., 1975 , Lanier *et al*., 1986). The extract and compound were found to enhance the NK mediated cell lysis, ADCC and ACC in metastatic tumour bearing animals.

5.3.12. Effect of *C. orchioides* and Curculigoside on serum IL-2 and IFN-γ levels

IL-2 and IFN-γ are T_H-1 type cytokines that induce T- cell proliferation and differentiation. Normal levels of IL-2 and IFN-γ was found to be 21.8 ± 2.7 and 2771±139 pg/ml respectively, in healthy C57BL/6 mice. Metastatic induction

with B16F10 melanoma cells decreased these levels to 16.8 ± 2.0 (IL-2) and 2189.5 ±112 pg/ml (IFN- γ) on day 7. Both the extract and curculigoside was found to enhance IL-2 and IFN-γ levels (Figure 5.6.A and B).Curculigoside administration enhanced the serum IL-2 and IFN- γ levels significantly ($P<0.001$) in the experimental animals to 26 ± 2.6 and 2618 ± 127 pg/ml respectively, where as in the extract treated animals the levels were 28 ± 1.8 and 2814 ± 91.2 for IL-2 and IFN- γ on the day 7.

5.3.13. Effect of Test Compounds on the Anti-Metastatic Activity (*in vitro)*

a) Cell Viability by MTT Assay

Both the extract and compound was found to be less cytotoxic towards B16F10 melanoma cells in culture. *C. orchioides* extract treatment at a dose of 100 µg/ml produced only 17% cytotoxicity where as the same concentration of Curculigoside exerted only 11% cytotoxicity towards B16F10 cells (Table 5.4).

b) Effect of Test Compounds on Invasion, migration and adhesion of B16F-10 Melanoma Cells

Boyden chambers with collagen coated polycarbonate membranes are used to evaluate the invasive potential of the metastatic cells. The untreated control cells showed the maximum invasive property, which was reduced upon the extract and compound treatment (Figure 5.7). The percentage inhibition on migration of the *C. orchioides* extract (10 µg/ml) treated cells was 52% and that for Curculigoside treatment it was 48 % (Table 5.5).

Tumour cell movement through the pores on polycarbonate membrane was analysed and for this the collagen coating step was avoided. Extract treatment reduced the motility of B16F10 cells by 39% and for Curculigoside the percentage inhibition on motility was 31%.

Adhesion to collagen coated substratum was also inhibited by *C. orchioides* extract (28%) and Curculigoside (25%).

c) Effect of test materials on MMP (gelatinases) production

Inhibitory effect on the MMP production by B16F10 melanoma cells was confirmed by gelatin zymography analysis. Since MMP-2 and 9 are gelatinases, they degrade gelatin and forms clear zones on the polyacrylamide gel containing gelatin during electrophoretic separation. Two clear zones were formed at 72 kDa and 92 kDa position in the gel loaded with trypsin-activated conditioned medium (CM) of B16F-10 melanoma cells which corresponds to MMP-2 and MMP-9 respectively. CM from extract (10 µg/ml and 5 µg/ml) and Curculigoside (5 µg/ml and 2 µg/ml) treated B16F10 cells did not produced any clear zones on the gel. When the CM from untreated B16F10 cells were incubated with EDTA and then run on the gelatin containing gel, no such clear areas were produced, indicating that metalloproteinases are responsible factors for this degradation (Figure 5.8).

DISCUSSION

In the present study we have evaluated the effect of *C. orchioides* and Curculigoside on the metastatic progression of tumour cells in animal model. The first part of study was to analyse the inhibitory effect on the lung tumour nodule formation due to metastatic tumour progression. Both the extract and Curculigoside treated animals showed a significant decrease in the number of tumour nodules when compared to the untreated control animals. In the same way the treatment also increased the life span of metastasis bearing animals. Simultaneous administration of the test materials provided the maximum inhibition showing the hindering effect on the metastatic process.

Lung metastasis is also associated with massive collagen deposition in the lung alveoli. From the estimation of lung collagen hydroxy proline it is become evident that the extract and compound reduced the collagen deposition in the lungs. Similarly the levels of glycosamino glycans like uronic acid and hexosamine concentrations were also significantly lessened by the treatment. The result correlates the inhibition of extra cellular matrix degradation since collagen and glycosaminoglycans are main components of ECM. According to Venkatesan and team higher levels of glycosaminoglycans is seen associated with lung fibrosis. Prohydroxy proline is the inactive form of hydoxy proline and its conversion is

facilitated by glucuronic acid lactone, thus the elevated levels of hydoxyproline and glucuronic acid are considered as a markers of fibrotic lung (Venkatesan *et al*., 1998). Histopathological examination of the lungs also revealed the reduction in lung fibrosis upon treatment with *C. orchioides* extract and Curculigoside when compared to the untreated metastatic control animals. Gamma-GT is a cellular proliferation marker which gets elevated on tumour progression. Similarly, high levels of sialic acid content in serum also imply the tumour metastasis. Serum levels of γ-GT and sialic acid were also brought down by *C. orchioides* and Curculigoside treatment, again evidencing the interference on metastatic progression.

Degradation of ECM is an essential aspect for tumour cell invasion which is executed mainly by MMPs. In order to study the mechanism of action expression of various pro and antimetastatic genes in the lung tissue was studied. The results indicated that the extract and compound effectively down regulate MMP-2 and MMP-9 expression at the same tissue increased the expression of TIMP1 and 2, the natural inhibitors of MMPs. The results were again confirmed by performing gelatin zymography. MMP-2 and MMP-9 are gelatinases thus when they are separated by electrophoresis on a polyacrylamide gel containing gelatin, a clear zone will be formed on the position of MMP-2 and 9 bands on trypsin activation. The plant extract and Curculigoside also inhibited the formation of these clear zones, showing their inhibitory effect on MMPs. Along with this, in the *in vitro* system the test materials also found to inhibit the adhesion, motility and invasion of B16F10 melanoma cells.

Proinflammatory cytokine levels of the test animals were also found to be increased with metastatic progression which was also down regulated by the extract and Curculigoside treatment. Besides these the cell mediated immune response in metastasis bearing animals were also stimulated by *C. orchioides* and Curculigoside. NK cell mediated direct cell lysis, NK cell mediated antibody dependant cell lysis and antibody dependant complement mediated cytotoxicity towards the target cell were enhanced by the test material administration. Serum IL-2 and IFN-γ levels were also elevated by the extract and compound administration. IL-2 is essential for NK and T cell proliferation and functioning and IFN-γ is the effector cytokines of NK and T cells.

Thus the inhibition of MMP production, activation of cell mediated immune response and anti-inflammatory effects are the basics for the anti-metastatic effects exerted by the test materials.

Table 5.1. Effect of *C. orchioides* and Curculigoside on lung tumour nodule formation and survival rate of metastatic tumour bearing animals

Treatment	**No. of lung tumour nodules**	**% inhibition on lung colony formation**	**% ILS**
Tumour control	250 [#]	-------	------
C. orchioides (Prophylactically)	142 ± 15.7 [a]	43	39
C. orchioides (Simultaneous)	120 ± 20.1 [a]	52	51
C. orchioides (Developed)	177 ± 13 [a]	29	26
Curculigoside (Prophylactically)	165 ± 11.3 [a]	34	29
Curculigoside (Simultaneous)	134 ± 16.7 [a]	46	43
Curculigoside (Developed)	195 ± 18.5 [a]	22	18

Animals were induced metastasis and treated with/without the test materials. On 21st day animals were sacrificed, the lung was collected and examined. Another set of animals were kept to examine the increase in life expectancy. All data are ± SD. [a]$P<0.01$, when compared to metastatic control.

Table 5.2. Effect of *C. orchioides* and Curculigoside on lung collagen hydroxyproline, lung uronic acid and lung hexosamine levels

Treatment	Collagen Hydroxyproline (µg/mg protein)	Uronic acid (µg/100 mg tissue)	Hexosamine (mg/100 mg tissue)
Normal	1.69 ± 0.17	26.45 ± 2.78	0.53 ± 0.05
Control	23.07 ± 2.38 [a]	301.2 ± 21.30 [a]	3.44 ± 0.48 [a]
C. orchioides (simultaneous)	15.03 ± 1.87[ab]	199.3 ± 28.35 [ab]	1.91 ± 0.20 [ab]
Curculigoside (Simultaneous)	17.47 ± 2.40 [ab]	235.9 ± 18.22 [ac]	2.83 ± 0.23 [ad]

Animals were induced metastasis and treated with/without the test materials. On 21st day animals were sacrificed, the lung was collected to analyse biochemical parameters. All data are ± SD. [a]P<0.001, when compared to normal, [b]P<0.001, [c]P<0.01, [d]P<0.05 when compared to metastatic control.

Table 5.3. Effect of *C. orchioides* and Curculigoside on serum sialic acid and γ-glutamyl transpeptidase (γ-GT) levels of metastatic tumour bearing animals

Treatment	Sialic acid (µg/ml)	Γ-GT (U/L)
Normal	28.16 ± 2.00	4.64 ± 0.60
Control	137.5 ± 14.68 [a]	119.0 ± 10.83 [a]
C. orchioides (Simultaneous)	93.15 ± 8.36 [ab]	62.63 ± 6.46 [ab]
Curculigoside (Simultaneous)	109.52 ± 10.22 [ac]	84.23 ± 9.73 [ab]

Animals were induced metastasis and treated with/without the test materials. On 21st day animals were sacrificed, blood was collected and serum was separated to analyse serum biochemical parameters. All data are ± SD. [a]P<0.001, when compared to normal, [b]P<0.001, when compared to metastatic control.

Table 5.4. Cytotoxicity analysis of the test materials against B16F10 melanoma cells

Concentration	% cytotoxicity	
	C. orchioides	**Curculigoside**
5 µg/ml	0	0
10 µg/ml	3	2
50 µg/ml	10	11
100 µg/ml	17	14

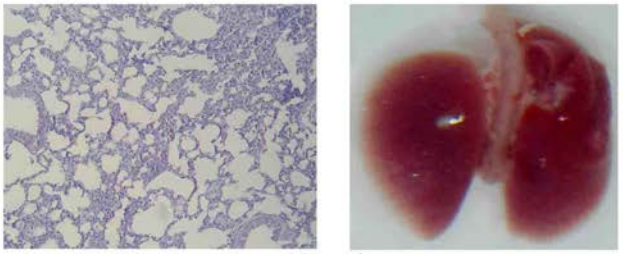
B16F10 cells (5000 cells/well) were seeded and treated with different concentrations of the test materials. Cell viability was determined by MTT assay.

Table 5.5. Effect of *C. orchioides* and Curculigoside on the adhesion, motility and invasion of B16F10 cells

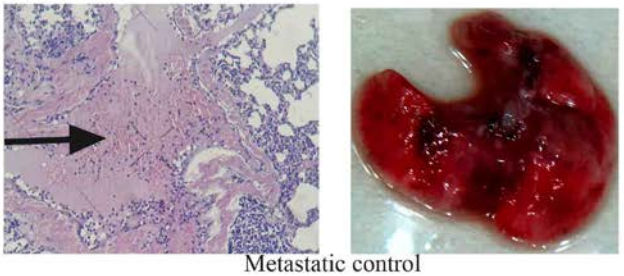
Treatment	% inhibition of Adhesion	% inhibition of motility	% inhibition of invasion
C. orchioides (2 µg/ml)	4	8	13
C. orchioides (5 µg/ml)	12	23	30
C. orchioides (10 µg/ml)	28	39	52
Curculigoside (1 µg/ml)	6	6	12
Curculigoside (2 µg/ml)	13	19	28
Curculigoside (5 µg/ml)	25	31	48

B16F-10 melanoma cells treated with or without test materials were seeded onto collagen type I-coated polycarbonate filters on the upper compartment of the chamber. The lower compartment was filled with DMEM and a nitrocellulose membrane was placed on top. After incubation, filters were removed, fixed, stained and the cells that had migrated in the test and the control were counted.

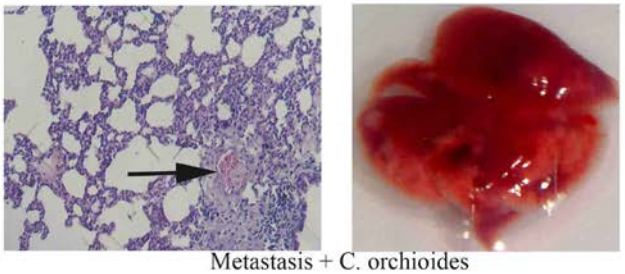
Figure 5.1. Effect of *C. orchioides* and Curculigoside on morphology and histopathology of mestastasis induced C57BL/6 lung tissue



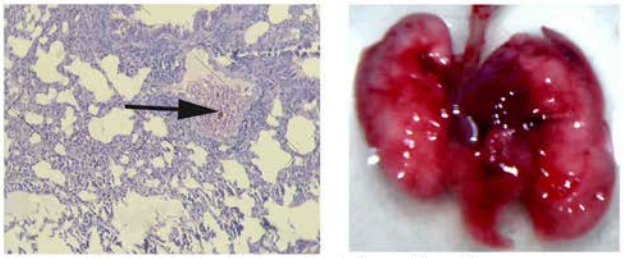
Normal



Metastatic control

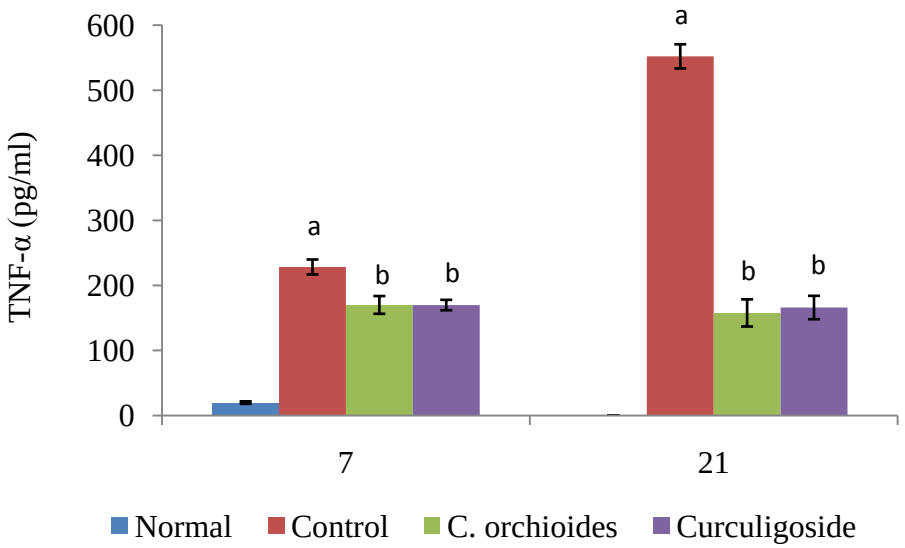


Metastasis + C. orchioides



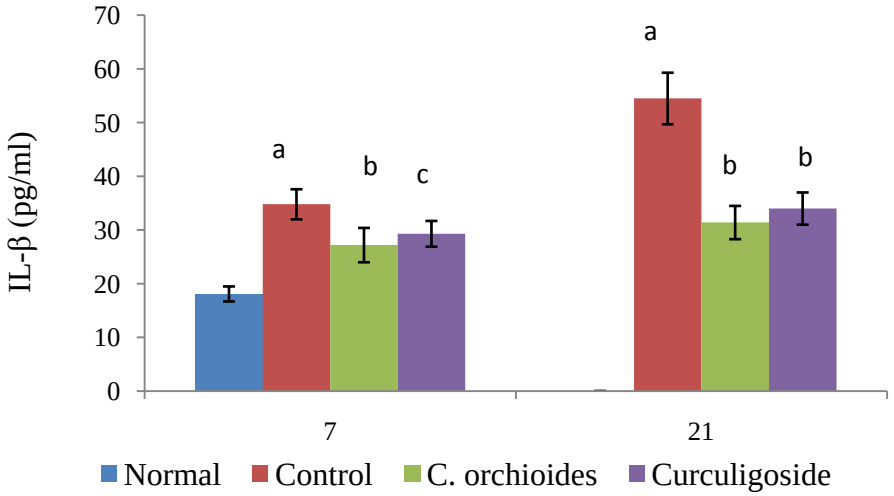
Metastasis + Curculigoside

Figure 5.2.A. Effect of *C. orchioides* and Curculigoside on serum TNF-α levels of metastasis bearing animals



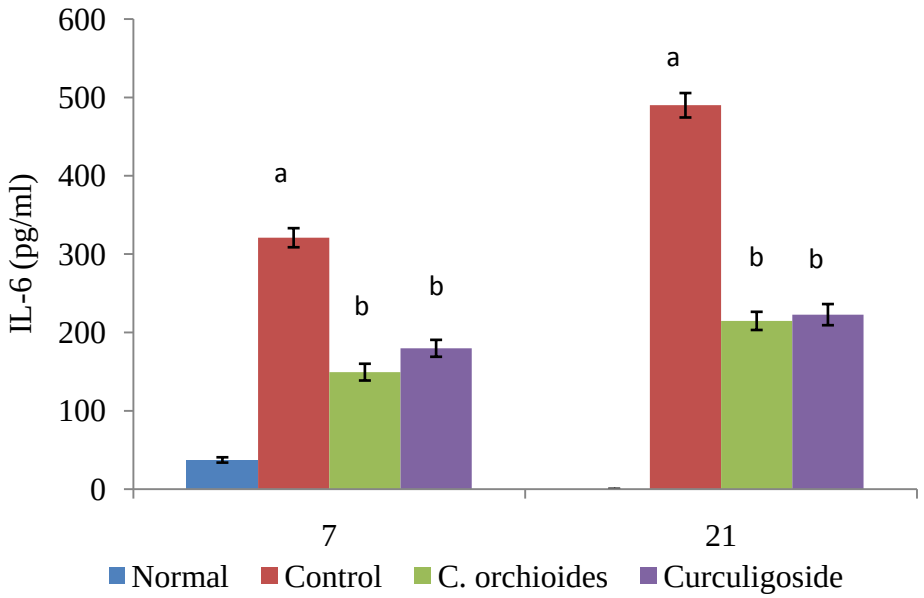
All data are ± SD. [a]P<0.001, when compared to normal. [b]P<0.001, when compared to control.

Figure 5.2.B. Effect of *C. orchioides* and Curculigoside on serum IL-1β levels of metastasis bearing animals



All data are ± SD. [a]P<0.001, when compared to normal. [b]P<0.001, [c]P<0.01when compared to control.

Figure 5.2.C. Effect of *C. orchioides* and Curculigoside on serum IL-6 levels of metastasis bearing animals



All data are ± SD. [a]P<0.001, when compared to normal. [b]P<0.001, when compared to control.

Figure 5.3.A. Effect of the test materials on TIMP-1 Levels

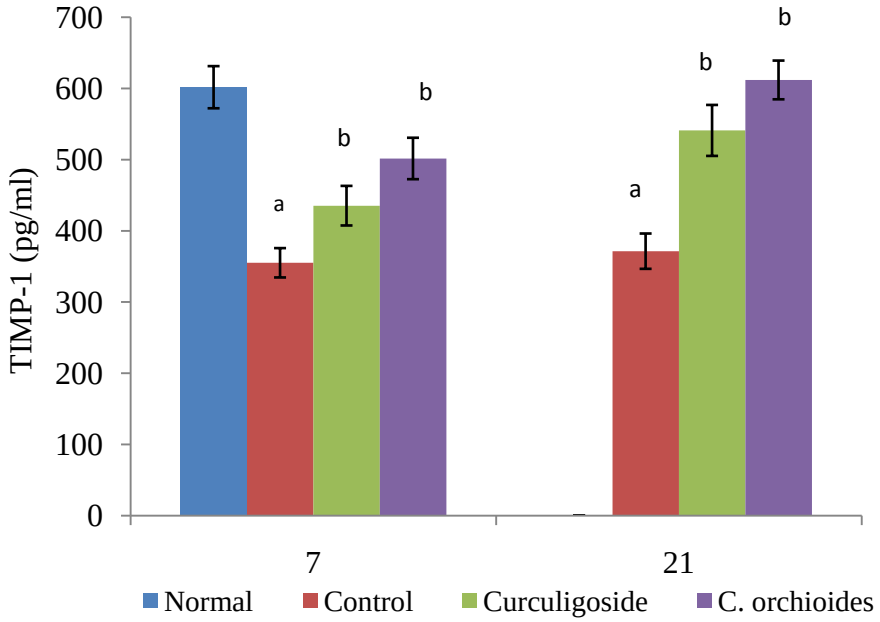
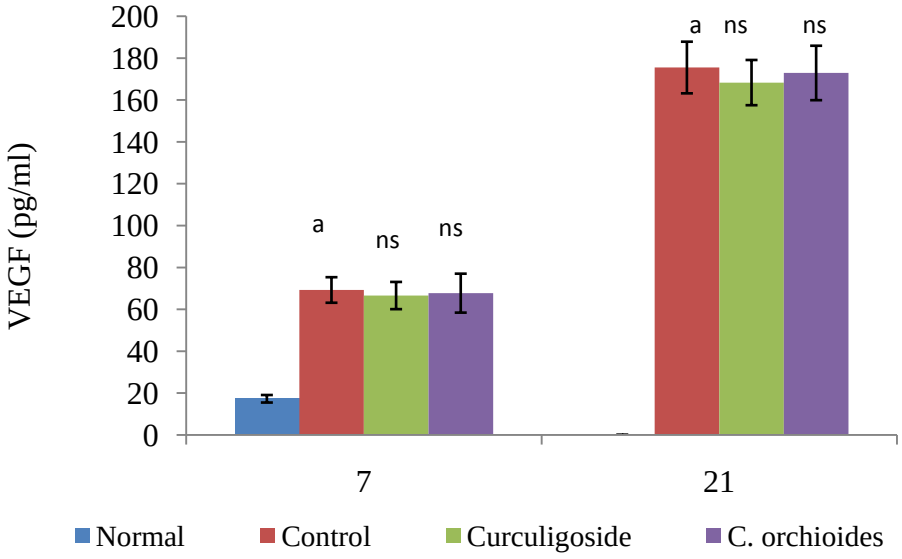
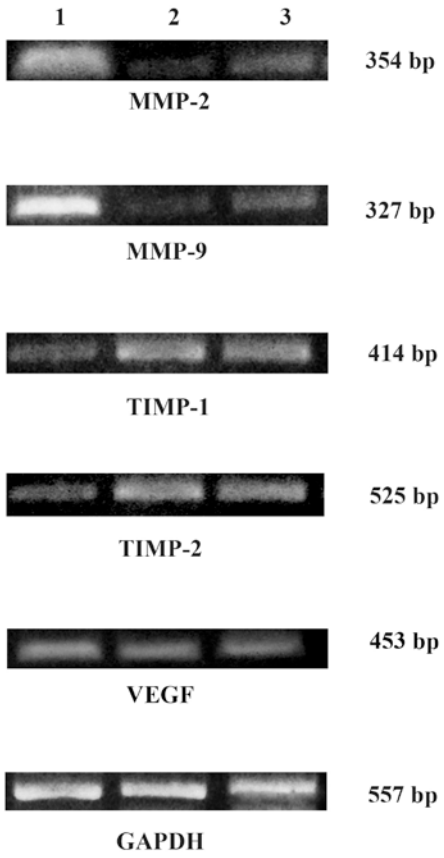


Figure 5.3.B. Effect of test materials on VEGF Levels



All data are ± SD. [a]P<0.001, when compared to normal. [b]P<0.001, when compared to control. [ns]P- non significant , when compared to control.

Figure 5.4. Effect of *C. orchioides* and Curculigoside on gene expression



Lane 1: Metastatic control
Lane 2: Metastasis + C. orchioides
Lane 3: Metastasis + Curculigoside

Figure 5.5.A. Effect of *C. orchioides* and Curculigoside on NK cell activity

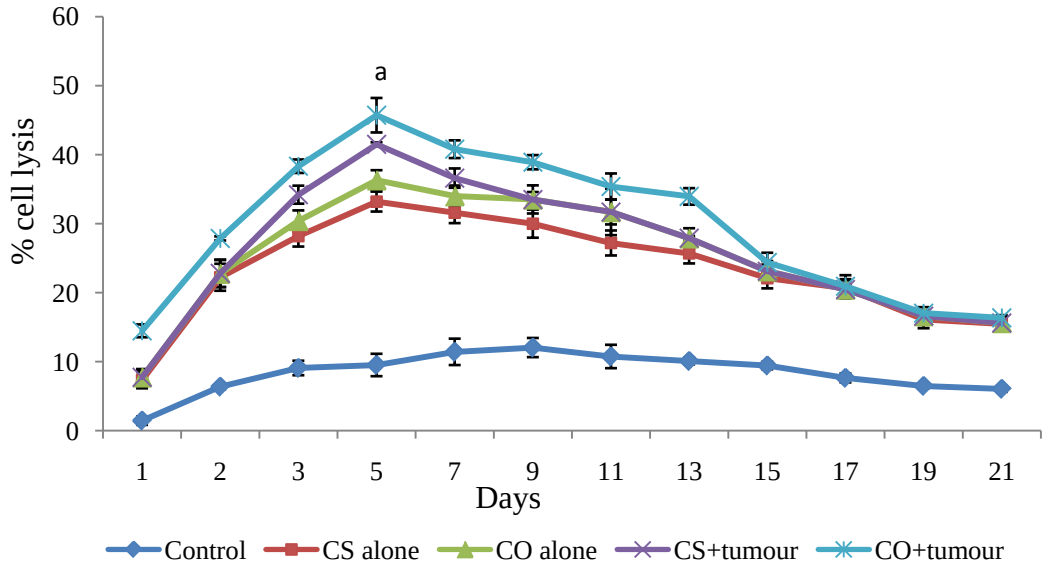
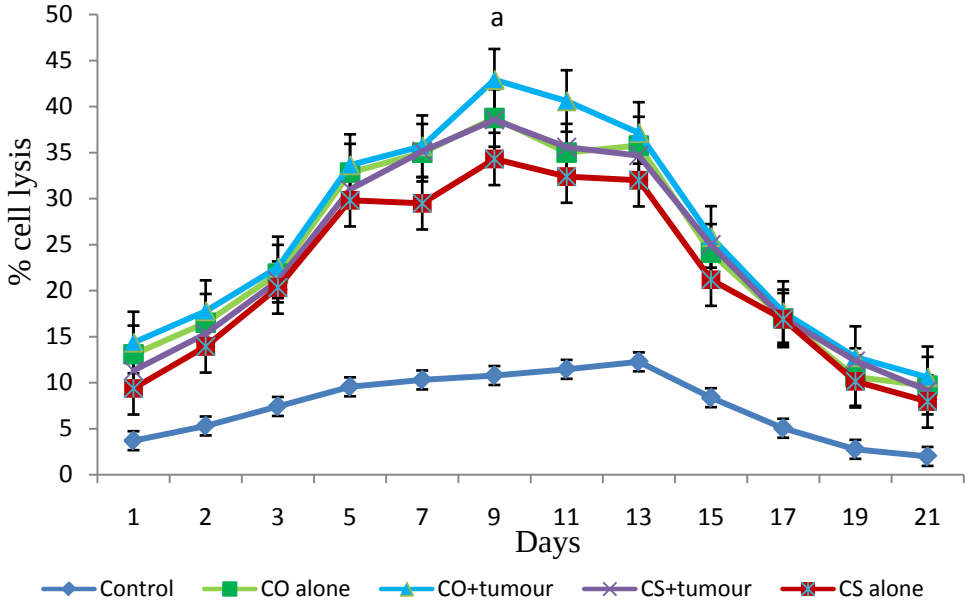
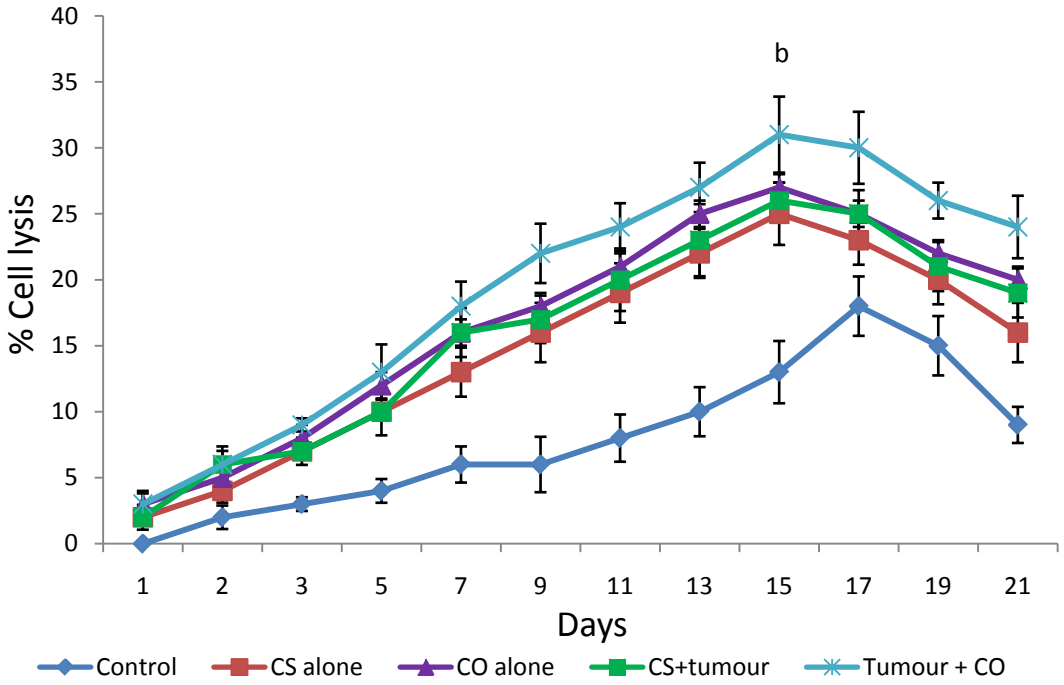


Figure 5.5.B. Effect of *C. orchioides* and Curculigoside on ADCC



Metastasis was induced to the animals and the animals were sacrificed at different time points and collected the spleen as the source effector cells. Chromium release assay was performed to study the NK mediated cell lysis and ADCC. [a]P<0.001, when compared to metastatic control.

Figure 5.5.C. Effect of *C. orchioides* and Curculigoside on ACC



Test material treated or untreated animals were induced metastasis and were sacrificed at different time points to take serum which is used as the source of antibody against tumour antigens. Cell death was determined by trypan blue dye exclusion method. [b]P<0.01, when compared to metastatic control animals.

Figure 5.6.A. **Effect of *C. orchioides* and Curculigoside on IL-2 levels of metastasis induced animals**

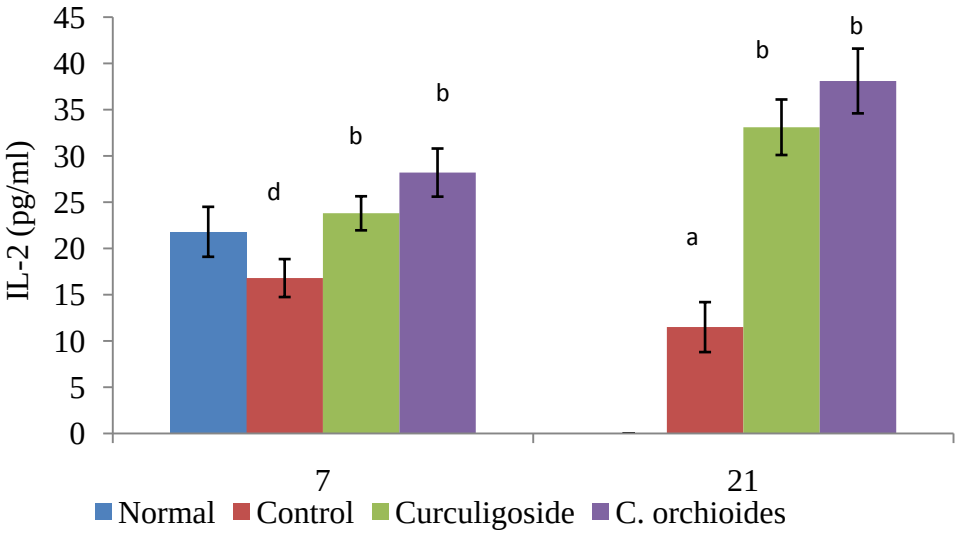
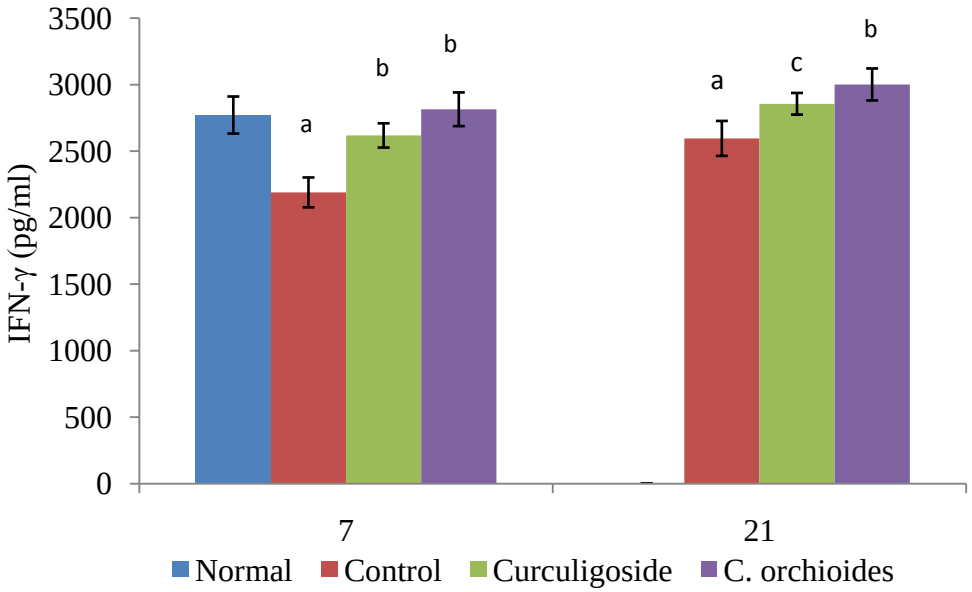
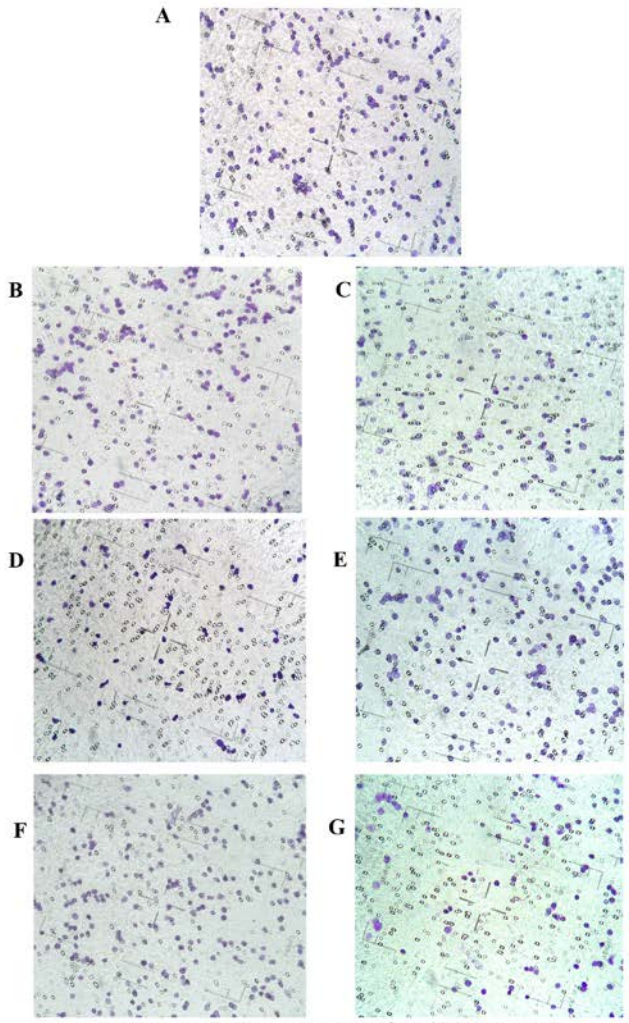


Figure 5.6.B. **Effect of *C. orchioides* and Curculigoside on IFN- γ levels of metastasis induced animals**



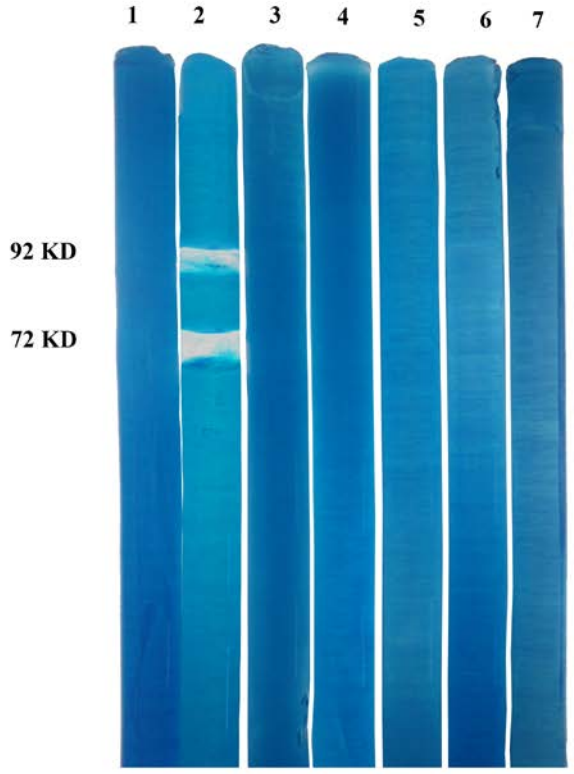
All data are ± SD. [a]P<0.001, [a]P<0.001, when compared to normal. [b]P<0.001, when compared to control. [c]P<0.01, when compared to control.

Figure 5.7. Effect of *C. orchioides* and Curculigoside on tumour cell invasion



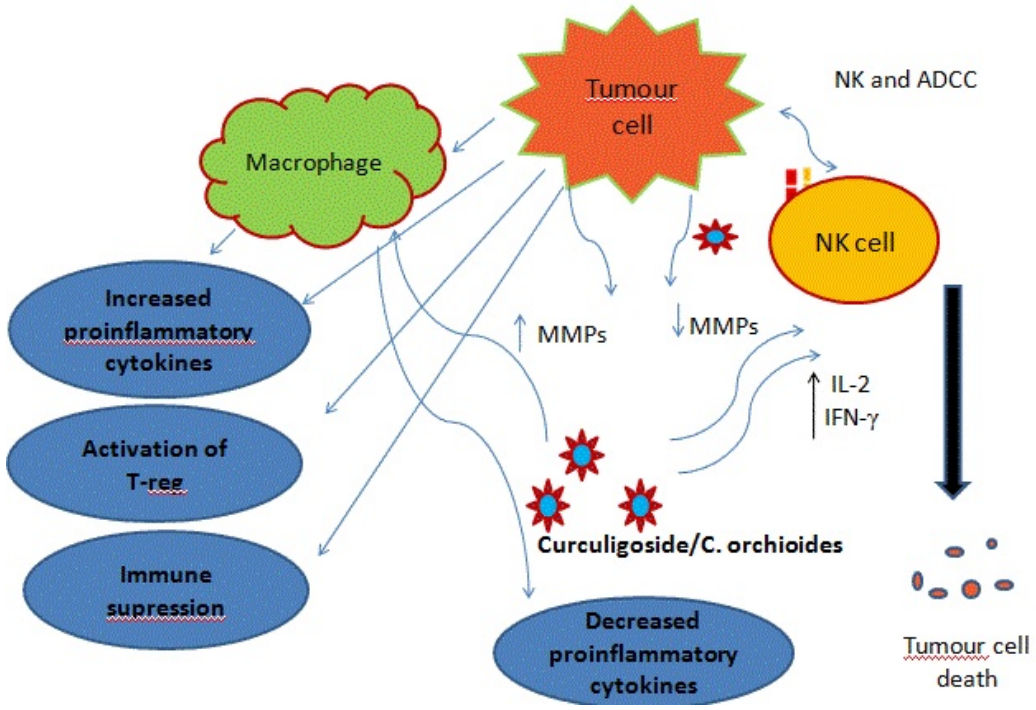
A- B16F10 cells alone; B - B16F10+C. orchioides (2 µg/ml);
C - B16F10+C. orchioides (5 µg/ml); E- B16F10+C. orchioides (10 µg/ml)
E- B16F10+Curculigoside (1 µg/ml): F- F-B16F10+Curculigoside (2 µg/ml)
G- B16F10+Curculigoside (5 µg/ml)

Figure 5.8. Effect of the test materials on MMP production



1. Conditioned medium (CM) without trypsin activation.
2. CM from untreated cells after trypsin activation.
3. CM from pretreated cells (5µg/ml, C. orchioides)+ trypsin
4. CM from pretreated cells (10µg/ml, C. orchioides) + trypsin
5. CM from pretreated cells (2µg/ml Curculigoside) + trypsin
6. CM rom pretreated cells (5µg/ml Curculigoside) + trypsin
7. CM from untreated cells + trypsin + EDTA.

Figure 5.9. Animetastatic activity of *C. orchioides* and Curculigoside



Metastatic tumour cells secrete proinflammatory cytokines, activate T-regulatory cells and also releases matrix metallo-proteinases as an escape mechanism from immune surveillance. Tumour micro environment also activates macrophages to enhance proinflammatory cytokine release. *C. orchioides* or Curculigoside administration helps to decrease the proinflammatory cytokine release and also enhance the release of IL-2 and IFN-Y by macrophages which will stimulate the cell mediated immune response. NK cell activation together with the inhibitory effect on the MMP release by tumour cells brought about the anti-metastatic effects of *C. orchioides* and Curculigoside.

Chapter 6

Ameliorative effects of C. orchioides on cyclophosphamide induced toxicities

6.1. INTRODUCTION

Cyclophosphamide (CPA) is a nitrogen mustard alkylating agent which is widely used to treat a broad spectrum of human cancers and some auto immune disorders. They are mainly used in combination therapy and causes killing of tumour cells and preventing their proliferation (Clovin, 1999). Biotransformation of CPA catalysed by hepatic cytochrome P450 to from 4-Hydroxy cyclophosphamide and its tautomer Aldophosphamide. A small portion of Aldophosphamide under goes a sponataneous elimination reaction to yield phosphoramide mustard and acrolein (causes bladder toxicity). Phosphoramide mustard forms crosslinks both between and within DNA strands at Guanine N-7 position. This reaction is irreversible and leads to cell death. Most of the aldophosphamide is oxidized by the enzyme aldehyde dehydrogenase (ALDH) to make carboxyphophamide (Clovin *et al*., 1976). Cyclophosphamide exhibit dose dependent biological actions like increased cytotoxicity and immunosuppression with increased dose. Immunosupression is caused mainly due to the elimination of T-cells (Berd and Mastrangelo, 1987).

The major side effects of CPA administration are cardiac toxicity, hematopoetic depression, hemorrhagic cystitis, gonodal disfunction, nausea, gastro intestinal toxicity, vomiting, alopecia and renal toxicities etc. (Stavin *et al*., 1975). Most chemotherapeutic drugs cause dose limiting toxicity to bone marrow elements, hence bring about immonosuppression (Cheson, 1999). The use of immunostimulants such as Bacillus Calmette Guarin (BCG), levamisole, cytokines (Lieschke and Burgess, 1992) along with chemotherapy has been found to reduce myelo-suppression and leucopoenia induced by chemotherapeutic agents. Herbal drugs that could enhance the anti tumour activity and reduce the side effects of conventional therapies as well as stimulate immunity will be of great help in improving cancer treatment strategies (Davis and Kuttan. 2000).

In this study we are evaluating the reversal of the immunosupression caused by the CPA administration by *C. orchioides* and its chemoprotective effects against cyclophosphamide induced oxidative damages (Murali and Kuttan, 2015).

6.2 MATERIALS AND METHODS

6.2.1. Animals

Male Balb/c mice and Swiss albino mice (6–8 weeks) weighing 25–28 g were used for these studies.

6.2.3. Chemicals and reagents

Chemicals used in this study includes Cyclophosphamide (Phosmid-500, Neon laborataries Ltd., Mumbai), GSH, 5-5' dithiobis (2-nitrobenzoic acid) (DTNB), Pararosaniline, α-Naphthyl acetate, Glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) analyzing kits (SPAN Diagnostics Ltd).

6.2.4. ELISA kit

Highly specific quantitative 'Sandwich' ELISA kits for mouse GM-CSF, IFN-γ, IL-2 and TNF-α were purchased from Pierce Biotechnology, USA.

6.2.5. Cyclophosphamide administration

Cyclophosphamide (CPA) was administered, at concentrations of 25, 20 and 15 mg/kg *b.wt* intraperitoneally (0.1 ml) for consecutive 10 days.

6.2.6. Reduction of solid tumour volume

In this study male Swiss albino animals were divided into eight groups (8 animals/group). Solid tumour was induced to all the animals by injecting Dalton's lymphoma ascites tumour cells (1 X 10^6 cells/animal), subcutaneously on the right hind limb of mice. Group I was kept as untreated control. Group II, III and IV animals received 10 doses of CPA (25, 20 and 15 mg/kg *b.wt* respectively). Group V, VI and VII animals received 10 doses of *C. orchioides* methanolic extract (20 mg/kg *b.wt*) along with CPA at a dose of 25mg/kg *b.wt*, 20mg/kg *b.wt* and 15mg/kg *b.wt* respectively. Group VIII animals received *C. orchioides* extract alone for 10 days. All treatments were started 48 hours after the tumour induction and CPA was administered 2 hours after the extract administration. Every third day starting from the seventh day of tumour inoculation, developing tumour radii was measured using Vernier callipers for 30 days and tumour volume was calculated using the formula $V = 4/3\pi r_1^2 r_2$, where $r_1$and r_2 are the radii of the tumour along two direction

6.2.7. Effect on total WBC count

Three groups of male Balb/c mice (4-6 weeks old; 8mice/group) were used in this study. Group I animals were treated with 10 doses of CPA (25mg/kg *b.wt*; ip) on consecutive days. Group II and III animals were treated with 10 doses of *C. orchioides*, 10 mg/kg *b.wt* and 20mg/ kg *b.wt* (ip), respectively along with CPA on consecutive days. Blood was collected from the caudal vein prior to CPA treatment and every third day after and total WBC count (Haemocytometer method) was determined.

6.2.8. Lymphoid organ weight, bone marrow cellularity and Number of α-esterase positive cells

Three groups (24 animals/group) of animals were used in this study and all the animals were treated 10 days with CPA (25 mg/kg *b.wt*) intraperitoneally. Group I animals were kept as untreated CPA control whereas Group II and III were treated with *C. orchioides* extract (10 and 20 mg/kg *b.wt*, i.p., respectively) along with CPA for 10 days. At different time points (48 h, 7th and 11th day) *b.wt* of animals were recorded and eight animals from each group were sacrificed by cervical dislocation. Lymphoid organ (thymus and spleen) weight was taken and expressed as relative organ weight. Bone marrow cells were collected from both femurs and the cell number were determined using heamocytometer (Sredni *et al*., 1992) and expressed as total live cells (X 10^6 cells/ femur). The number of α-esterase positive cells were determined by the azo dye coupling method (Bancroft and Cook, 1984). A smear of bone marrow cells from the above preparation was made on clean glass slides, air-dried, stained with α-naphthyl acetate and pararosaniline hydrochloride, counter stained with haematoxylin and observed using under 100X. The number of α-esterase positive cells were expressed out of 4000 cells.

6.2.9. Effect of *C. orchioides* on Biochemical parameters of animals treated with CPA

Blood was collected by heart puncture from each animals grouped above, immediately after the sacrifice to separate the serum. Liver homogenate was made in ice cold Tris buffer (0.1 M, pH 7.4) and used for the estimation of ALP

(King, 1965), GPT (Bergmeyer and Bernt, 1980), and lipid peroxidation (LPO) (Ohkawa *et al*, 1979). Serum was also used to estimate all the above parameters and also the urea and creatinine levels. In order to estimate the levels of GSH by the method of Moron *et al* (1979) Intestinal mucosa was used.

6.2.10. Cytokines production

Blood was collected from the animals used for the study of haematological parameters on 48 h, and 11th day for separating the serum. Estimation of cytokine levels such as IL-2, IFN-γ, GM-CSF, and TNF-α were performed by ELISA method using specific antibodies, according to the manufacturer's instruction.

6.2.11. Histopathological analysis of the intestinal damage caused by CPA

The jejunal portion of the small intestine from the previous experiment was excised and fixed in 10% formaldehyde. Sections (4μm) were stained with eosin and haematoxylin and observed under light microscope.

6.3. RESULTS

6.3.1. Reduction of solid tumour volume

The effect of *C. orchioides* on solid tumour reduction in animals treated with CPA is shown in Figure 6.1. The administered dose of CPA was found to be effective in controlling tumour growth and the administration of *C. orchioides* along with CPA formed a synergistic effect on tumour growth inhibition. *C. orchioides* administered at a dose of 20 mg/kg *b.wt* to CPA-treated (25mg/kg *b.wt*) animals had the lowest tumour volume among all the other groups. CPA at a dose of 15mg/kg *b.wt* along with the *C. orchioides* extract administration was found to be more effective in reducing the tumour volume than CPA alone at a dose of 25mg/kg *b.wt*.

6.3.2. Effect on total WBC count

CPA treated animals showed a significant decrease in their total WBC count and this decline can be directly correlated with the CPA induced myelosuppression

(Figure 6.2). *C. orchioides* administration could significantly increase the WBC count to 4500 ± 232 cells/mm^3 (20 mg/kg b.wt) and 4200 ± 312 cells/mm^3 (10 mg/kg b.wt) which was reduced drastically to 3200 ± 230 by cells/mm^3 CPA administration. The reduced level of WBC count was observed throughout the period of study which was lifted towards the normal by the administration of *C. orchioides* (20mg/kg b.wt).

6.3.3. Lymphoid organ weight

Table 6.1 shows the relative organ weights of thymus and spleen. CPA treatment drastically reduced the relative weights of thymus and spleen in control animals. Administration of *C. orchioides* at 20 mg/kg b.wt showed a significant enhancement in the weights of thymus and spleen.

6.3.4. No. of bone marrow and α-esterase positive cells

Bone marrow cellularity was highly affected by CPA treatment where the CPA alone treated mice showed a severe reduction in the number of marrow cells (6.3±0.2x10^6 cells/ femur; 48^{th} h) compared to the normal cellularity (16 ± 0.58x10^6 cells/ femur). *C. orchioides* extract treated animals showed a cellularity of 8.6 ± 0.74x10^6 cells/ femur on 48^{th} h and also provided a gradual increase on each succeeding time points (Table 6.2).

Similarly, the number of α-esterase positive cells was also significantly decreased after CPA administration. *C. orchioides* treatment elevated the α-esterase activity of maturing monocytes. On the 11^{th} day the number of α-esterase positive cells of the CPA alone treated animals were 686 ± 95 cells/4000 cells which was found to be increased to 926±93 cells /4000 cells on *C. orchioides* treatment (20 mg/kg *b.wt*).

6.3.5. Cytokine levels

CPA administration resulted in a drastic decrease in the levels of cytokines, IL-2 (8.40±0.96), IFN-γ (654±43.26) and GM-CSF (22.60±3.60), where as the level of the pro-inflammatory cytokine TNF-α was found to be markedly elevated (311.8±56.60). All these changes were reversed by the treatment of *C. orchioides*. IL-2, GM-CSF and IFN-γ levels were elevated on the 2^{nd} day to

16.7±2.56, 30.16±4.50 and 1024±123.76 respectively. TNF-α level was reduced to 155.6±27.90 on the 2nd day. Table 6.3 represents the serum IL-2 and IFN- γ profiles and Table 4 shows the data for GM-CSF and TNF- α.

6.3.6. Reduced GSH, LPO, ALP and GPT levels

After 48 h of CPA administration, the control animals (CPA alone) exhibited a decreased glutathione level in the mucosal lining of intestine (6.88±1.23 nmol/ml) as well as in liver tissue (2.72±0.25 nmol/ml) compared to the normal level of 5.68±0.51 nmol/ml (liver). At the same time, a significant elevation in the GSH levels of both intestinal mucosa (15.82±2.56 nmol/ml) and liver tissue (3.51±0.29nmol/ml) was showed by *C. orchioides* administered animals on 11th day (Figure. 6.3).

Together with this, a significant increase in the serum ALP levels were also found with the CPA administration (16.40±1.80 IU/L - 48 h; 17.82±2.62 IU/L- 7th day) when compared to the normal level of 12.60±1.44 IU/L. However the administration of *C. orchioides* could lower the elevated level of serum ALP by day 7 to 14.60±2.58 IU/L. Similar observations were also obtained in liver ALP levels also. The tissue ALP levels of CPA control animals were 19.70±2.80 IU/L (48 h) and 21.20±3.60 IU/L (7th day) where as a significant reduction in these levels was resulted with *C. orchioides* extract treatment (Figure. 6.4).
Serum GPT levels were also found to be elevated in CPA alone treated group (124.00±12.26 IU/L) was lowered by the treatment with *C. orchioides* (88.00 ± 10.22 IU/L) on day 7, when compared to the normal animals (55.80 ± 3.20 IU/L) (Figure 6.5).

High levels of lipid peroxidation were also exhibited by CPA treated animals with an average of 3.88±0.32 nmol/ml (serum) and 4.12±0.90 nmol/ mg/protein formed /min/mg protein (liver) on 48th h (Figure 6.6). Extract administration also decreased the lipid peroxidation levels in both serum (3.25±0.36 nmol/ml) and liver after 48 hours (Figure. 6.6).

6.3.7. Protective effect of *C. orchioides* against the intestinal damage caused by CPA administration

Histopathological analysis of jejunal section of small intestine showed abnormal crypt architecture and damaged intestinal villi in the case of CPA alone treated animals. Simultaneous administration of *C. orchioides* extract along with CPA was found to reduce the intestinal toxicity induced by CPA. The crypt architecture was maintained and the villi damage was reduced (Fig. 6.7).

6.4. DISCUSSION

Cyclophosphamide is a cytotoxic chemotherapeutic drug that acts as an alkylating agent producing reactive carbonium ions, which reacts with DNA (Krishna *et al*., 1986). Cyclophosphamide has been shown to be inactive *in vitro* but was found to be activated to cytotoxic metabolites by the mixed function oxidase in hepatic microsomes (Richard *et al*., 1984). The high therapeutic index of cyclophosphamide was shown to be due to the metabolite 4-hydroxycyclophosphamide and a phosphoramide derivative. It was shown that free radicals are formed during the activation of cyclophosphamide and produce tissue injury. Phosphoramide mustard is known to cause myelosuppression (Latha and Panikkar., 1999). This study was carried out to focus the on the immunostimulant and antioxidant activities of *C. orchioides* along with its efficacy to lessen the toxic side effects of CPA administration.

In the present study it was found that the administration of *C. orchioides* extract along with CPA could enhance the tumour reducing capacity of CPA. Even a lower concentration of CPA (15 mg/kg *b.wt*) could give the similar results as that of 25 mg/kg *b.wt* of CPA when it was administered along with the *C. orchioides* extract. This implicates the synergistic effect of CPA and *C. orchioides* extract in the reduction of tumour volume. This effect may be partly due to the immunostimulatory activity of the plant that will stimulate the humoral and cellular arms of the host's immune system to prevent primary tumour development and shapes the tumour immunogenicity and also due to the antitumor activity of the extract (Singh and Guptha, 2008; Yokosuka *et al*., 2010) which is very evident when comparing the tumour volume among the CPA alone group and Extract + CPA treated group.

During the study it was also confirmed that myelosuppression caused by CPA is effectively prevented by *C. orchioides*. Administration of *C. orchioides* resulted in the normalisation of WBC count toward the end of the treatment, whereas the regenerative capacity in CPA alone treated control animals was very low and did not regain the normal. In CPA treated group, administration of *C. orchioides* elevated the number of cells in bone marrow, especially the proliferation of differentiating stem cells which is observed from the increase in number of alpha esterase positive cells. This indicates that *C. orchioides* stimulated the haemopoietic system, which is highly sensitive to chemotherapy. This hemoprotective effect can also be attributed to the antioxidant activity of the extract which may protect the bone marrow cells from the free radical induced damages caused by CPA. These protective effects altogether can be credited for the faster recovery of the animals from those side effects caused by CPA.

Glutathione is a natural antioxidant tripeptide present in almost all cell types that helps to maintain redox status, immune responses along with the detoxification of toxic materials. In the case of cancer treatment they are responsible for the detoxification of carcinogens (Balendiran *et al*., 2004). The free radicals generated by the CPA metabolism are scavenged by GSH thus the levels of GSH are found to be reduced in CPA treated animals. Extract administration enhanced the GSH levels in both liver and intestinal tissues.

It has been reported that hydroxyl radicals inhibits alkaline phosphatase activity (Ohyashiki *et al*., 1994) and the free radicals generated during the peroxidation reaction will attack the protein molecules in membranes (Fox, 1975; Sunila, 2014). Elevated levels of hepatic marker enzymes like APL and GPT in the CPA treated animals were also lowered by the extract administration.

The free radicals generated during the metabolism of CPA, can initiate the lipid peroxidation (Releigh *et al*., 1989; Sunila, 2014). Increase in the level of TBARS signifying the enhanced lipid peroxidation in the tissue was also resulted by the administration of CPA in experimental animals. But that was significantly lowered by the *C. orchioides* treatment with *C. orchioides.*

Inhibition of LPO in bio-membranes can be caused by antioxidants (Hendry *et al*., 1982).

Cytokines are proteins or glycoproteins which act as cell signalling molecules and their main function is the modulation of immune responses. Several inflammatory cytokines are induced by oxidant stress and they themselves trigger the release of other cytokines and also lead to increased oxidant stress. Interleukine-2 is a cytokine secreted by macrophages, necessary for the T-cell growth, proliferation and differentiation. The level of IL-2 reduced by the CPA administration was found to elevate by *C. orchioides* administration. Interferon-γ (IFN- γ) is a cytokine that is critical for innate and adaptive immunity against viral and intracellular bacterial infections and also for tumor control. IFN-γ is an important activator of macrophages. GM-CSF (Granulocyte monocyte colony stimulating factor) is another immune stimulating cytokine that functions as a white blood cell growth factor which stimulates the stem cells to produce granulocytes (neutrophils, eosinophils, and basophils) and monocytes. Administration of CPA drastically reduced the levels of IFN- γ and GM-CSF where as the simultaneous administration of the extract was found to enhance the production of these two cytokines. Tumour necrosis factor (TNF-α) is a cytokine involved in systemic inflammation secreted predominantly by macrophages after a variety of stimuli. It is a major pro-inflammatory cytokine; their level was drastically elevated after CPA treatment and was reduced by the administration of *C. orchioides* extract. From this it is clear that immunomodulatory and anti-inflammatory activity of the plant is responsible for these effects and one of the mechanisms of action is the regulation of the production of cytokines by the immune cells.

The small intestine has been reported to have a remarkable capacity for repair (Hendry *et al*., 1982; Sunila, 2014). In the present study CPA administered animals exhibited a delayed recovery response that might be resulted from the increased stem cell death whereas the rate of recovery was faster in *C. orchioides* treated group.

Cyclophosphamide dosages can be minimized when it is administered in combination with *C. orchioides* extract which will produce synergistic effect and reduces the tumour development. The results also indicate that administration of alcoholic extract of *C. orchioides* helped to reverse the myelo-supression, the major side effect of CPA. Even though the antioxidant properties of *C. orchioides* are well documented these effects did not interfere with the tumour reducing capability of Cyclophosphamide. Thus it can be concluded that the chemoprotective efficacy of *C. orchioides* is mainly attributed to its immune stimulatory, anti-inflammatory and anti-oxidant potentials.

Table. 6.1. Effect of *C. orchioides* on the relative organ weight of spleen and thymus of CPA administered animals

Group	Weight of spleen (g/kg body weight)			Weight of thymus (g/kg body weight)		
	2nd day	7th day	11th day	2nd day	7th day	11th day
Normal	0.41±0.07			0.11±0.06		
CPA control	0.37±0.09	0.35±0.08	0.32±0.63	0.095±0.002	0.092±0.001	0.089±0.001
CPA+ CO-10	0.39±0.08	0.36±0.06	0.34±0.04	0.099±0.003	0.096±0.002	0.094±0.003
CPA+ CO-20	0.40±0.09	0.38±0.08	0.35±0.05	0.10±0.008	0.098±0.005	0.095±0.005

Eight animals were killed from each group at each time points and the relative organ weights of thymus and spleen were determined. Values are the mean ± SD; CO-10 *C. orchioides* (10 mg/kg b.wt), CO-20 *C. orchioides* (20 mg/kg b.wt)

Table. 6.2. Effect of *C. orchioides* on bone marrow cellularity and Number of α-esterase positive cells of CPA administered animals

a) Bone marrow cellularity (No./femur) x 10^6

Group	2nd day	7th day	11th day
Normal	16 ±0.6		
CPA control	6.3±0.2[a]	9.0±0.5 [a]	11.6±0.9 [a]
CPA+ CO-10	7.1±1.3	11.31±1	14.3±0.9
CPA+ CO-20	8.6±1.1[af]	13.7±1[be]	15.9±2.6[e]

b) No. of α -esterase positive cells (number/4,000 cells)

Group	2nd day	7th day	11th day
Normal	828±61.23		
CPA control	292±19.8 [a]	425±34 [a]	683±94 [b]
CPA+ CO-10	506±25.8	686±75	830±65
CPA+ CO-20	625±85.9 [ae]	701±66[ce]	926±93[e]

Eight mice from every group were sacrificed at different time points to collect the bone marrow. Cell number was determined using counting chamber and out of these the number of α-esterase positive cells was determined by azo dye coupling method. Values are the mean ± SD, [a]p <0.001, [b]p <0.01, [c]p <0.05 significantly different from normal, [e]p <0.001, [f]p <0.01, significantly different from control.

Table. 6.3. Effect of *C. orchioides on* TNF- α and GM-CSF levels of CPA administered animals

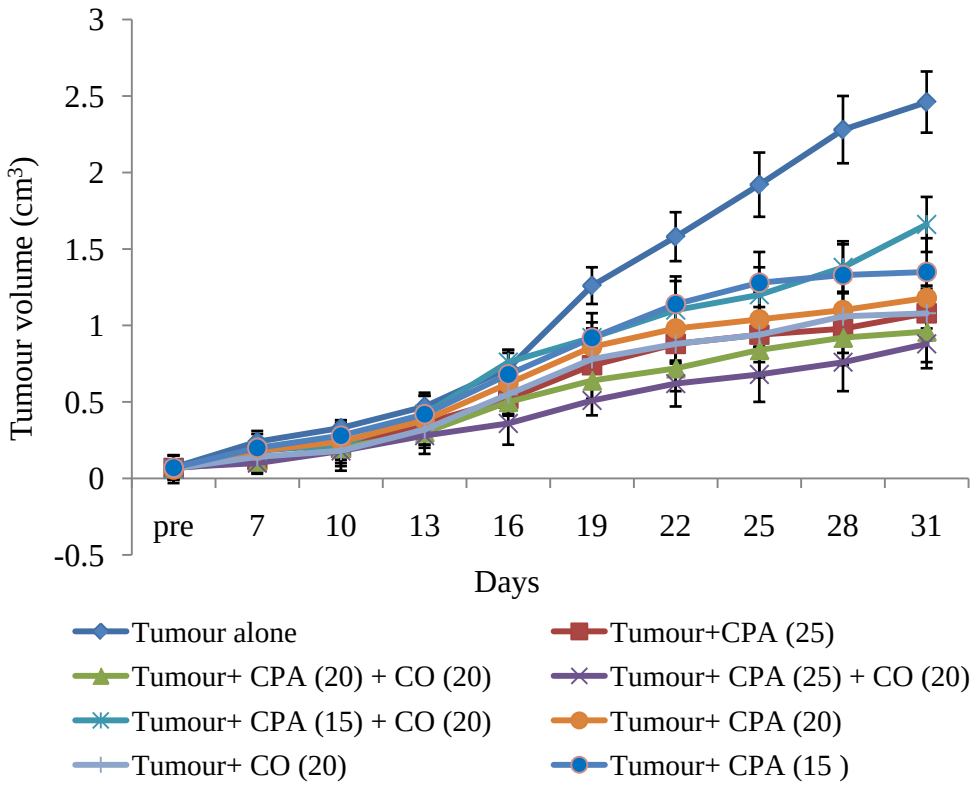
Group	TNF- α (pg/ml)		GM-CSF (pg/ml)	
	48 h	11th day	48 hr	11th day
Normal	30.5 ± 4.89		38.5 ± 4.7	
CPA control	311.8 ± 56.6 [a]	284.8 ± 35.9 [a]	22.6±3.6 [a]	28.30±5.3 [b]
CPA + CO-10	187.5 ± 38.2[ae]	136.2 ± 35.9[ae]	27.3±3.4[a]	31.26±4.8
CPA + CO-20	155.6 ± 27.9[ae]	118.7 ± 22.2[ae]	30.16±4.5[cg]	36.72±6.3[cg]

Table. 6.4. Effect of *C. orchioides* on IL-2 and IFN- γ levels of CPA administered animals

Group	IL-2 (pg/ml)		IFN- γ (pg/ml)	
	48 h	11th day	48 hr	11th day
Normal	16.4 ± 1.6		1585 ± 113.6	
CPA control	8.4 ± 0.96 [a]	14.2 ± 1.3	654 ± 43.3[a]	825.0 ± 76.7 [a]
CPA + CO (10)	12.3 ± 1.7[bg]	18.4 ± 2.4[f]	792 ± 29.9 [ag]	945.8 ± 54.7 [a]
CPA + CO (20)	16.7 ± 2.6[e]	20.6 ± 3.5[e]	1024± 123.8 [ae]	1468 ±116.3[e]

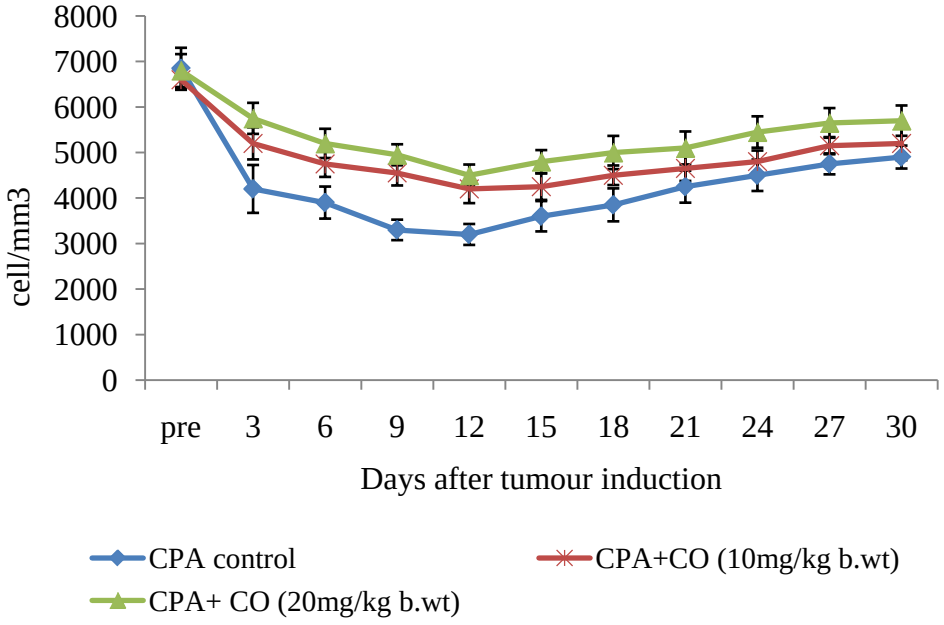
Animals were sacrificed at different time points, blood was collected, serum was separated and the levels of TNF-α, GM-CSF, IL-2 and IFN-γ were estimated using sandwich ELISA , specific for the murine cytokines according to the manufactures protocol. Values are the mean ± SD, [a]p <0.001, [b]p <0.01, [c]p <0.05 significantly different from normal, [e]p <0.001, [f]p <0.01, [g]p <0.05 significantly different from control.

Figure 6.1. Effect of *C. orchioides* on solid tumour volume



All the animals were induced solid tumour by injecting Dalton's lymphoma ascites tumour (DLA) cells (1 X 10^6 cells/animal), subcutaneously on the right hind limb of mice. The radii of developing tumour was measured every third day starting from the seventh day of tumour inoculation for 30 days using vernier callipers.

Figure 6.2. Effect of *C. orchioides* on the total WBC count



Effect of *C. orchioides* extract on total white blood cell count. Mice were given 10 doses of CPA and every 3 days; blood was collected from the caudal vein in a sterile manner, and total white blood cell count was monitored.

Figure 6.3.A. Effect on the Intestinal GSH levels

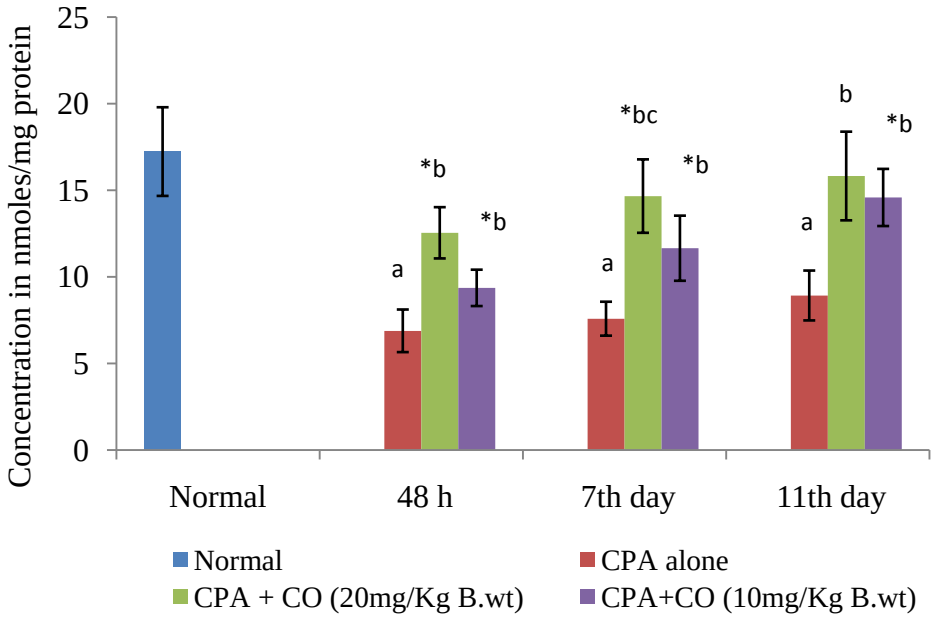
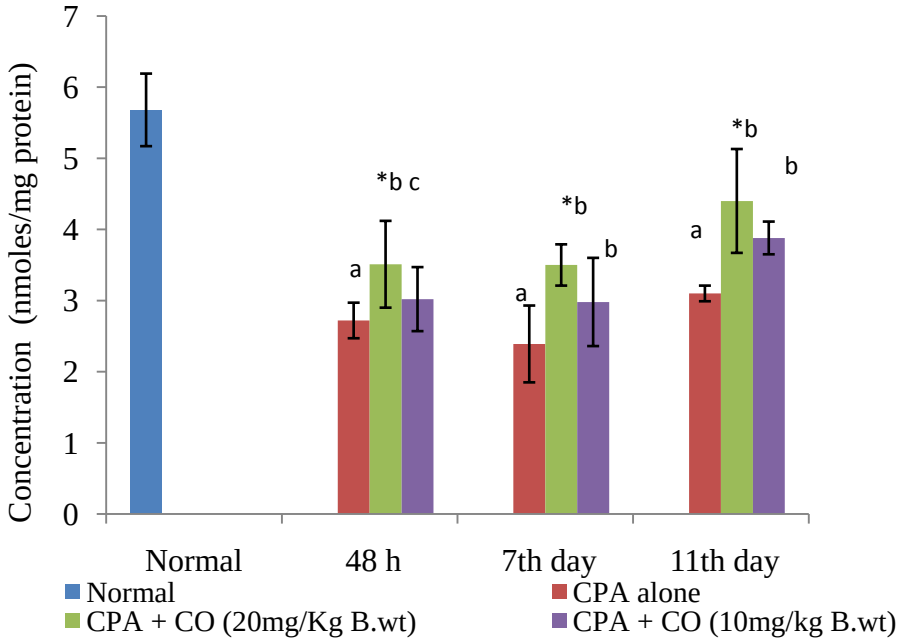


Figure 6.3.B. Effect on the liver GSH levels



Animals were sacrificed at different time points to collect liver and intestinal mucosa and the GSH levels were estimated. Values are the mean ± SD. [a]$p<0.01$, compared to normal. *$p<0.05$ significantly different from control. [b]$p<0.05$ compared to normal. [c]$p<0.05$ significantly different from treated (CPA + *C. orchioides*, 10 mg/kg body weight).

Figure 6.4.A. Effect of *C. orchioides* on Serum ALP levels

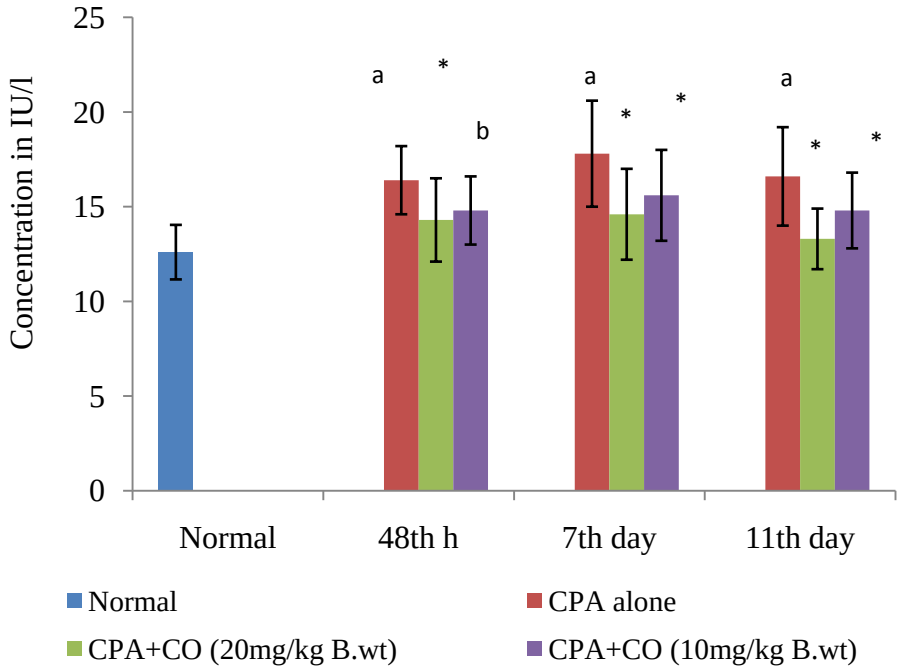
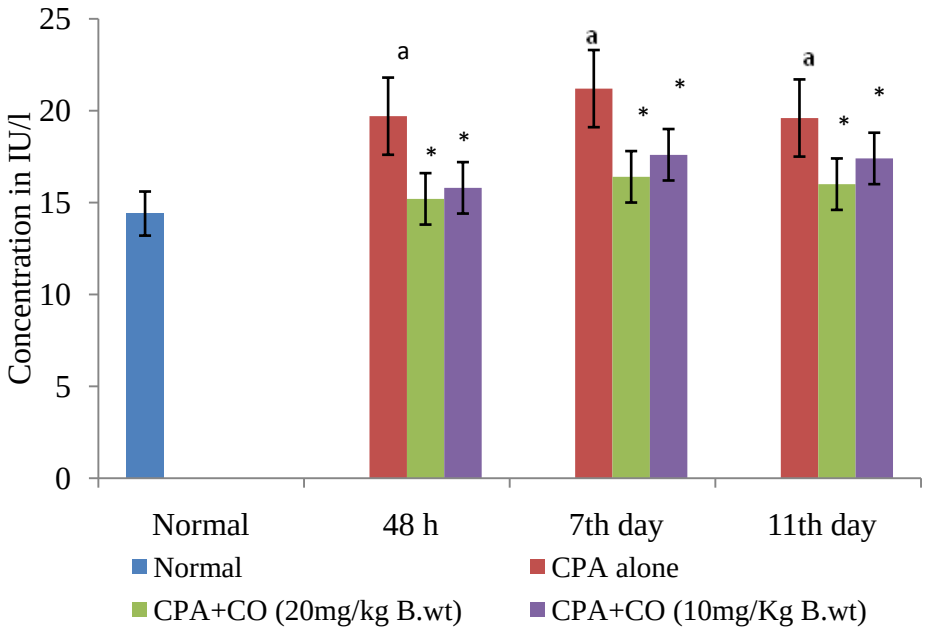


Figure 6.4.B. Effect of *C. orchioides* on liver ALP levels



Animals were sacrificed to collect blood and liver, serum ALP and liver ALP was estimated by the method of King. Values are the mean ± SD. Normal vs. Control: [a]p<0.05, significantly different from normal. Control vs. Treated: *p<0.05 significantly different from control. Normal vs. Treated: [b]p<0.05 significantly different from normal.

Figure 6.5.A. Effect of *C. orchioides* on serum GPT levels

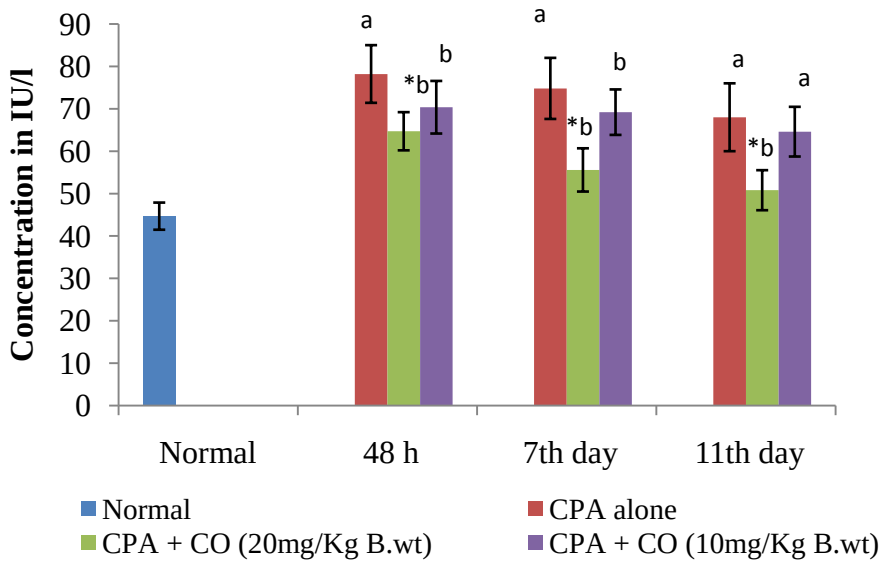
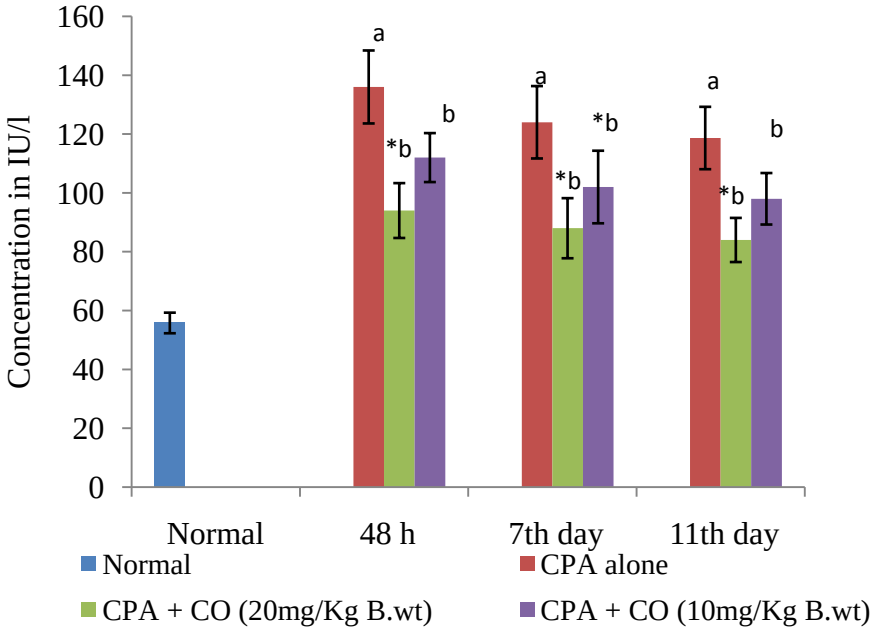


Figure 6.5.B. Effect of *C. orchioides* on liver GPT levels



Values are the mean ± SD. [a]p<0.01, significantly different from normal. Control vs. Treated: *p<0.05 significantly different from control. Normal vs. Treated: [b]P <0.05 significantly different from normal. Treated vs. Treated: [b]p<0.05 significantly different from treated (CPA + *C. orchioides*, 10 mg/kg body weight)

Figure 6.6.A. Effect *C. orchioides* on Serum LPO levels

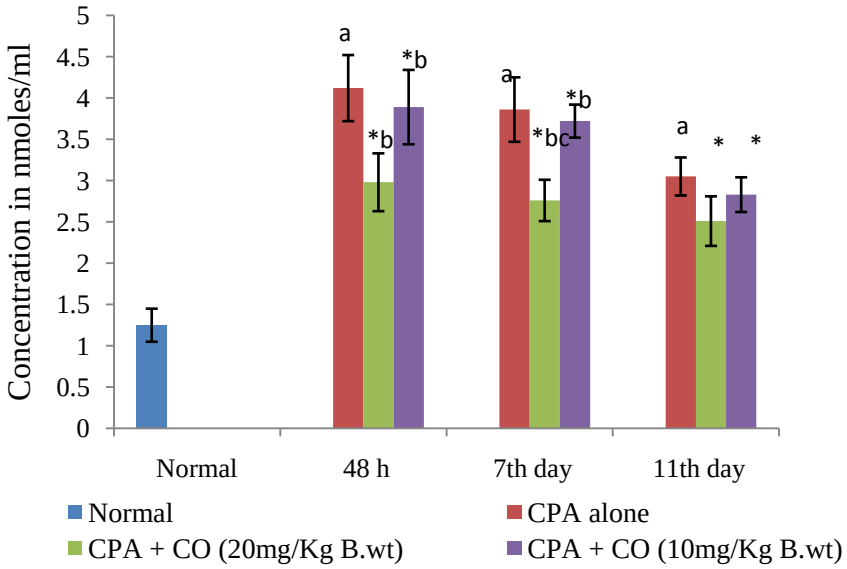
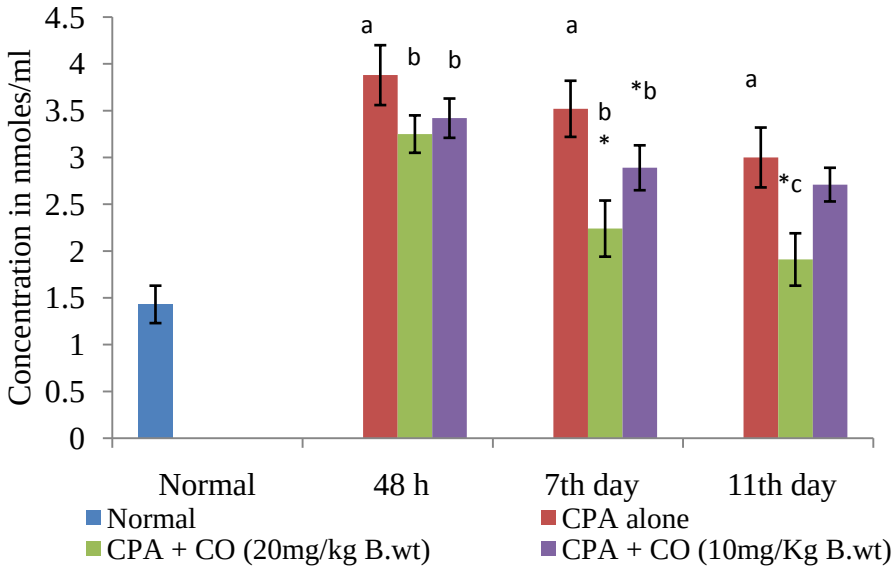
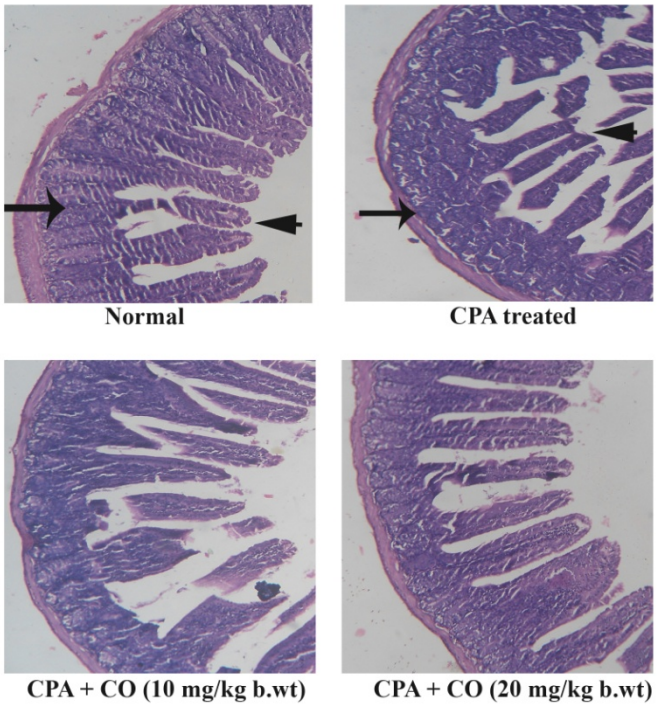


Figure 6.6.B Effect of *C. orchioides* on liver LPO levels



Values are the mean ± SD. Normal vs. Control: [a]p<0.01, significantly different from normal. *p<0.05 significantly different from control. Normal vs. Treated: [b]p<0.01 significantly different from normal. [c]p<0.05 significantly different from normal.

Figure 6.7. Histopathological Analysis of small intestine



Intestinal damages due to CPA administration

Normal :- Arrow : Normal intestinal crypts
Arrow head : Normal villi

CPA treated:- Arrow : Damaged and diffused intestinal crypts
Arrow head : Damaged villi

Chapter 7

Uro and nephroprotective effects of C. orchioides

7.1. INTRODUCTION

Bioactivation of Cyclophosphamide (CPA) by hepatic cytochrome P-450 (CYP) enzymes leads to the formation of acrolein, which is responsible for the damage of host bladder epithelium leading to hematuria and hemorrhagic cystitis which forms the major dose-limiting toxicity for the administration of CPA (Brock *et al*., 1979). Besides these, N-dechloroethylation generates an inactive compound (dechloroethyl-CPA) and chloroacetaldehyde, which is both nephrotoxic and neurotoxic (Yu *et al*., 1999). Supplementation of agents that can provide protection against the toxic side effects of antineoplastic drugs without affecting their chemotherapeutic efficacy will be of great promise as adjuvants in chemotherapeutic treatment regimens.

Mesna (2-mercaptoethanesulfonate) is an organo-sulfur compound used to lessen the prevalence of haemorrhagic cystitis and haematuria occurred as the side-effects of the administration of antineoplastic drugs like CPA. Sulfhydryl group present in Mesna can act as an antioxidant and detoxifies the urotoxic metabolite acrolien (Mashiach *et al*., 2001). The free sulfhydryl (thiol) groups of mesna combine directly with a double bond of acrolein and with other urotoxic 4-hydroxyoxazaphosphorine metabolites (4 hydroxycyclophosphamide) to form stable nontoxic compounds (Hensley *et al*., 1999).

A number of research works suggest that antioxidant supplementation can influence the response to chemotherapy as well as the development of adverse side effects that result from treatment with antineoplastic agents (Jalali *et al*., 2011). This study was designed based upon the former study about the hepato and hemo-protective effects of the extract against CPA induced toxicities (Murali and Kuttan 2015). In this study ameliorative effect of the methanolic extract of *C. orchioides* on the urotoxicity and nephrotoxicity induced by the administration of CPA was analyzed (Murali and Kuttan, 2016).

7.2. METHODS

7.2.1. Animals

Swiss Albino mice (25–28 g, male)

7.2.2. Chemicals and reagents

Thiobarbituric acid (TBA), Hydrogen peroxide (H_2O_2), N-butanol, ascorbic acid, pyridine, EDTA, Nitroblue tetrazolium (NBT), Glutathione (GSH), 5-5-dithiobis-2-nitrobenzoicacid (DTNB) (Ellman's reagent), Riboflavin, Sodium 2-mercaptoethane sulphoxide (MESNA), total protein, creatinine and urea analysing kits (SPAN Diagnostic Ltd). Enzyme-linked immunosorbent assay kit: Highly specific quantitative sandwich enzyme-linked immunosorbent assay kits for mouse IL-2, IFN-γ and TNF-α were used

7.2.3. Effect of *C. orchioides* on CPA-induced nephro toxicity

Swiss Albino mice (24 animals/group) were divided into four groups and group I was kept as normal treated with normal saline ip for 5 days. Other three groups of animals were treated with a single acute dose of CPA (1.5 mmol/kg body wt). Group II animals were kept as CPA alone control. Group III animals were treated with *C. orchioides* extract (20 mg/kg body wt) for 5 days prior to CPA administration. Group IV animals were treated with a single dose of MESNA (150 mg/kg b.wt.). Eight animals from each group were anaesthetized using haloethane and then sacrificed by cervical dislocation, at 4^{th}, 24^{th} and 48^{th} h time points after CPA administration. B.wt.s of the animals were recorded prior to the drug administration and at the time of killing, the weights of bladder and kidney were also recorded. Urine was collected in a water-jacketed, chilled chamber ($4^{O}C$) before killing the animals and centrifuged (2,000g, 5 min) (Wood *et al*., 2001). Blood was collected by heart puncture, and serum separated. Serum and urine were used for estimating total protein contents (Biuret and Dumas method) (Verley *et al*., 1980), serum creatinine (alkaline picrate method), serum and urea nitrogen by diacetylmonoxime (DAM) reagents (Wybenga *et al*., 1971).

7.2.4. Morphological examination

The urinary bladder and kidney were excised from each animal immediately after killing, washed thoroughly in phosphate buffer (pH 7.4) and the entire surrounding connective tissues were removed. Three different persons performed morphological analysis of urinary bladder and kidney by observing the inflammation, colouration and size of the bladder.

7.2.5. Biochemical Analysis

Kidney and bladder homogenates were prepared using ice-cold PBS (50 mmol/L, pH 7), after thorough washing with cold isotonic saline. This homogenate was used for the following biochemical investigations.

7.2.6. Lipid peroxidation and GSH levels

Lipid peroxidation in kidney homogenate was estimated by the method of Ohkhawa (Ohkhawa *et al*., 1979). Measurement of GSH in kidney and bladder homogenate was done according to the method of Moron *et al*. (1979). GSH was measured by its reaction with DTNB (5-5-dithiobis 2-nitrobenzoic acid) (Ellman's reaction) to give a compound that absorbs at 412 nm.

7.2.7. Anti-oxidant profile

The kidney homogenate was centrifuged at 5,000g for 10 min at $4^{o}C$; after removal of the cell debris, the supernatant was used for different assays. Superoxide dismutase (SOD) was performed by NBT reduction method of Mc Cord and Fridovich, (1969) and glutathione peroxidase (GPx) by the method of Hafeman *et al*.(1974) based on the degradation of H_2O_2 in the presence of GSH where as catalase (CAT) activity was determined from the rate of decomposition of H_2O_2 at 240 nm following the addition of tissue homogenate (Aebi, 1974).

7.2.8. Histopathological analysis

Urinary bladder as well as kidney was fixed using 10% formaldehyde and tissue dehydration was performed by several treatments in different concentrations of alcohol, the dehydrated tissue was embedded in paraffin wax. Sections were cut and stained with haematoxylin and eosin and histopathological analysis was carried out.

7.3. RESULT

7.3.1. Effect of *C. orchioides* on urinary bladder and kidney morphology

Noticeable difference between the control and treated groups were observed upon the morphological analysis of urinary bladder. After 4 h of CPA treatment, bladder of CPA control animals was inflamed with evident red colouration due to haemorrhage, whereas the CPA along with *C. orchioides*-treated group showed only a slight inflammation but normal colouration. Severe bladder haemorrhage and colouration was observed in the control group, on 24th h of treatment whereas the *C. orchioides* treated group showed normal bladder morphology. Even after 48 h the urinary bladder of CPA alone-treated group were severely inflamed whereas those of the CPA along with *C. orchioides* treated group were similar to that of normal (Table 7.1). No considerable changes were observed in kidney morphology.

7.3.2. Effect of *C. orchioides* on b.wt. loss after CPA administration

B.wt.s of CPA alone administered animals were found to be decreasing due to its toxic effects at all the three time points and maximum decrease was observed at 48 h (-2.00 g), whereas in CPA along with *C. orchioides* -treated group, there was a gradual increase in b.wt. at three time points compared with control animals (Table 7.2).

7.3.4. Effect of *C. orchioides* on relative organ weight

In all time points relative bladder weight was found to be increasing in the case of CPA-alone treated group indicating inflammation of bladder by CPA and the maximum weight was observed at 4th hour (0.26 ± 0.08 g/100 g body wt). The values are very high when compared with normal value of (0.081±0.016 g/100 g body wt). Administration of MESNA along with CPA maintained the relative bladder weight almost similar to that of the normal one (0.078 ±0.009 g/100 g body wt body wt.) at 4th hour. In CPA along with *C. orchioides* treated animals also, the relative weight of bladder was decreased at 4 h (0.13±0.013 g/ 100 g body wt), 24 h (0.098±0.006 g/100 g body wt) and 48 h (0.083±0.002 g/100 g body wt) (Table 7.3) showing its uroprotective role. Relative weight of kidney did not show much variation in all groups (data not shown).

7.3.5. Effect of *C. orchioides* on serum creatinine level

Increased serum creatinine level was shown by CPA-alone treated group at all the three time points, 4 h (1.16 ± 0.062 mg/dL), 24 h (0.78 ± 0.031 mg/dL) and 48 h (0.52 ± 0.02 mg/dL). A significant decrease in the creatinine levels was resulted by the administration of the plant extract at 4 h (0.45 ± 0.04 mg/dL), 24 h (0.42 ± 0.02 mg/dL) and reached near to normal level (0.37 ± 0.014 mg/dL) at 48 h (0.38 ± 0.03 mg/dL), revealing the nephro-protective role of *C. orchioides* (Figure. 7.1) on CPA induced damage.

7.3.6. Effect of *C. orchioides* on blood urea nitrogen (BUN) and urine nitrogen

Figure 7.2.A and 7.2.B represents urea nitrogen levels in blood and urine respectively. CPA-alone-treated animals showed a significant elevation in the levels urea and creatinine which are the kidney function markers when compared with that of normal animals, indicating renal damage. This increased level was found to be decreased significantly by *C. orchioides* extract treatment. Increase in BUN level (126.8 ± 8.65 mg/dl (4 h) has been observed in CPA-alone-treated group compared with the normal control (32.2 ± 4.0 mg/dl), Analysis of the blood of the CPA along with *C. orchioides* treated groups of animals showed that the extract pre-treatment attenuated the level to 62.6 ± 4.3; 46.6 ± 5.6 and 36. 2± 2.5 mg/dl at 4, 24, and 48 h, respectively (Figure 3). The mesna-treated group retained a normal level of blood urea N_2 level at these time points. Urine urea nitrogen levels were also enhanced significantly by CPA administration to 27.33±0.69 mg/dl on 4^{th} h which was slightly decreased to 24.98±1.58 mg/dl on 48^{th} h whereas on treatment with Mesna, the levels (16.78±1.68 mg/dl on 48^{th} h) were brought towards normal (16.52±1.68 mg/dl). *C. orchioides* treatment also reduced the urea nitrogen levels significantly to 17.53±1.32 mg/dl on 48^{th} h showing its renal protective role.

7.3.7. Effect of *C. orchioides* on bladder and kidney GSH levels

Effect of *C. orchioides* on bladder GSH levels after CPA administration is represented in Table 4. CPA treatment reduced the bladder GSH level to 1.12±0.08 nmoles/mg protein on 4^{th} hour from the normal level of 3.01±0.46

nmoles/mg protein, which was slightly increased to 1.75±0.05 nmoles/mg protein on 48th hour. But there was an increase in bladder GSH level in CPA MESNA-treated group at 4 h (3.28 ±0.51 nmoles/mg protein), and 3.66±0.71nmoles/mg protein at 48 h. Pre treatment with *C. orchioides* extract to the CPA administered animals could also increase bladder GSH level which was at 4 h (2.13±0.031 nmoles/mg protein), 24 h (2.50±0.36 nmoles/mg protein) and the level was normalized by 48 h (3.15±0.40 nmoles/mg protein). Kidney GSH levels were also reduced drastically with CPA treatment (1.18±0.09 nmoles/mg protein, on 4th h) when compared with normal level (4.21±0.67 nmoles/mg protein). A significant re-establishment in GSH level was observed in CPA - *C. orchioides* treated animals (2.89±0.33 nmoles/mg protein) when compared with CPA alone-treated group. Even after 48 hrs only an insignificant increase was shown by the CPA alone experimental animals (1.92±0.25 nmoles/mg protein) where as CPA-*C. orchioides* group reached close to the normal GSH levels by 48h (3.97±0.42 nmoles/mg protein), showing renal protective role of *C. orchioides* (Table 7.4).

7.3.8. Effect of *C. orchioides* on lipid peroxidation

Lipid peroxidation in CPA-treated animals was significantly ($P<0.01$) increased compared with normal animals at 4 h (2.60 ± 0.24 moles/mg protein), 24 h (2.1 ± 0.17 moles/mg protein) and 48 h (2.0 ± 0.18 moles/mg protein) (Fig. 7.3), whereas treatment with *C. orchioides* along with CPA could significantly decrease lipid peroxidation to 1.62 ± 0.08, 1.38 ± 0.15, 1.3 ± 0.11 moles/mg protein at 4, 24 and 48 h, respectively, indicating the cellular level protection of *C. orchioides* from the toxic effect of CPA (Figure 7.3).

7.3.9. Effect of *C. orchioides* on antioxidant status

The activities of renal SOD, CAT and GPx at three time points are represented in Figure 7.4, 7.5, and 7.6. The renal antioxidant status, such as SOD, CAT and GPx activities are significantly ($P<0.01$) decreased in the CPA-alone-treated group of animals compared with the normal ones. Administration of *C. orchioides* was found to significantly elevate the lowered activities of these antioxidant enzymes thereby scavenging the free radicals produced as a result of CPA insult.

7.3.10. Histopathological Analysis

Figure 7.7 shows the histopathological analysis of the urinary bladder, and the bladder morphology of CPA alone administered animals showed denudation of bladder mucosa with inflamed bladder walls. Haemorrhage of mucosal and sub-mucosal region was also observed with diffuse infiltrate of lymphocytes, plasma cells and polymorphs. On 48 hrs after CPA administration the bladder wall showed oedema as well as haemorrhage and extensive necrosis of the mucosal layer. Whereas in the case of CPA + *C. orchioides* treated animals the urinary bladder wall and epithelium was with minimal damage and mild oedema in the submucosal region even after 48 hours and the morphology was almost similar to the normal urinary bladder.

Morphological analysis of kidney (Figure 7.8) revealed the damages of renal tubules with irregular Bowmans capsule in CPA alone administered animals. Renal tubules showed vacualation of lining epithelial cells and the interstitial tissue was with haemorrhage, foci of necrosis and diffused infiltration by lymphocytes and plasma cells. But in the case of CPA+ *C. orchioides* administered animals the renal tissue appears with normal glomeruli and interstitial tissue with mild haemorrhage and a few inflammatory cells. Thus the histopathological examination of kidney and urinary bladder accentuate the protective effects of the plant extract over the damages caused by CPA administration.

7.4. DISCUSSION

Cyclophosphamide is a major antineoplastic agent and immune suppressor with a main drawback of the side effects caused by its toxic metabolites. A large portion of these metabolites are excreted through urine and during the excretion process they reacts with the urothelium of the bladder and reduce the urine retention capacity of the bladder. The deleterious effect of CPA on the bladder include mucosal oedema, haemorrhage, ulceration, sub-endothelial telangiectasia and, in severe cases, fibrosis of the bladder (Davis and Kuttan, 2000; Bishel, 1979; Javadpour and Burakat, 1973). A strategy to diminish the

side-effects of anticancer drugs with the preservation of its chemotherapeutic efficacy is required. *Curculigo orchioides* extract was found to be effective in ameliorating CPA induced side effects.

Administration of CPA and many other anticancer drugs leads to depression of hepatic mixed function oxidase system due to bioreactive molecule formation during the hepatic activation process (Donelli and Gerratini, 1971). Acrolein, the by-product of CPA activation is responsible for its dose limiting urotoxic and nephrotoxic side effects. Acrolein reacts with the free and tissue bound thiols (Girtoo *et al*., 1981) and leads to the depression in the hepatic glutathione and microsomal enzyme activities, thus resulting in kidney and bladder toxicities. Our previous studies revealed that *C. orchioides* is effective in ameliorating the hepatic and intestinal toxicities induced by the administration of CPA without interfering its chemotherapeutic efficacy (Murali and Kuttan, 2015). The results obtained from the present study point out a possibility of blocking acrolein-related nephro and uro toxicities mediated by the antioxidant and anti-inflammatory properties of *C. orchioides*.

Creatinine and GSH levels along with the urine volume are the specific markers to identify renal malfunction. Oxidative stress generated by the free radicals formed due to the administration of CPA and further by its toxic metabolic by-product, acrolein, are together responsible for the pathological mechanism of these changes in renal parameters (Atessahin *et al*., 2003). During our studies, decreased urine volume and significantly elevated levels of serum creatinine were observed in CPA administered animals which is associated with renal dysfunction. Blood urea nitrogen (BUN) level was also found to be elevated in the case of CPA administered animals. Elevated BUN levels and creatinine concentrations are markers for the kidney damage and nephrotoxicity (Cagler *et al*., 2002). *C. orchioides* whole plant extract, when administered along with CPA, could significantly decrease the serum creatinine and BUN levels near to normal level.

Histopathological studies of kidney also indicated glomerular necrosis and haemorrhage to renal tubules whereas the urinary bladder showed oedema with haemorrhagic cystitis and necrosis. Back flow of urine from bladder to the renal tubules due to the decreased storage space for urine within the inflamed bladder may be the reason for these impairments. Treatment with the extract minimized the structural damages occurred to the bladder and kidney by CPA administration along with a reduction in inflammation and the protective effects may be due to the down regulation of proinflammatory cytokines by *C. ochioides* extract.

Glutathione peroxidase (Gpx) is the enzyme participating in the elimination of reactive intermediates by reducing hydroperoxides and thus involves in the repair of radical-caused biological damage. *C. orchioides* administration was found to enhance the Gpx levels in kidney which was significantly reduced by CPA administration. Present study discloses the efficacy of *C. orchioides* extract in scavenging superoxide radicals generated inside the body during the normal metabolism and most importantly in the presence of xenobiotics. Moreover administration of *C. orchioides* significantly increased the catalase and glutathione levels in kidney and urinary bladder. Lipid peroxidation refers to the reaction of oxidative deterioration of polyunsaturated lipids. The extract could inhibit the process of lipid peroxidation thus reduced the free radical toxicity which strongly support its chemoprotective activity.

Morphological, histopathological, biochemical and enzyme analysis showed that *C. orchioides* could alleviate the severe urotoxicity and nephrotoxicity induced by Cyclophosphamide. The present study reveals the protective role of *C. orchioides* extract against Cyclophosphamide induced multiple organ toxicity and at the same time, the extract does not hinder the antitumour efficacy of Cyclophophamide which was revealed from our previous studies (Murali and Kuttan, 2015). Thus *C. orchioides*, which is a nontoxic, immunostimulating and antioxidant plant, can be used as a natural adjuvant with cyclophosphamide to inhibit tumour development.

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Table 1 : Morphological examination of urinary bladder after CPA administration

Group	4h	24h	48h
CPA alone	Red, inflamed bladder with haemorrhage	Inflamed bladder with deep red colouration and severe haemorrhage	Severe inflammation, haemorrhage and dark colour
CPA+ CO	Slight inflammation and light colouration	No inflammation and colour	Appears normal
CPA+ MESNA	No inflammation and colouration	Bladder with normal appearance	Appears normal

All the animals were treated with CPA single dose (1.5 mmol/kg body wt). One group was treated with 5 doses of *C. orchioides* (20mg/kg b.wt.) and other group with MESNA (150 mg/kg b.wt.) along with CPA.

Table 7.2. Effect of *C. orchioides* treatment on change in body weight (in g)

Group	4h	24h	48h
Normal			+1.22±0.068
CPA alone	-0.62±0.16[a]	-1.38±0.25 [a]	-2.00±0.72 [a]
CPA+ CO	+0.06±0.007[b]	+0.40±0.06 [b]	+0.55±0.03 [b]
CPA+ MESNA	+0.08±0.01 [b]	+0.42±0.04 [b]	+0.67±0.04 [b]

All data are represented as mean ± SD. Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test [a] $P<0.05$ significantly different from normal [b]$P<0.05$ significantly different from that of CPA alone.

Table 7.3. Effect of *C. orchioides* treatment on relative bladder weight (g/100g body wt.)

Group	4h	24h	48h
Normal			0.081±0.016
CPA alone	0.26±0.08[a]	0.17±0.025[a]	0.14±0.01
CPA+CO	0.13±0.013[b]	0.098±0.006[b]	0.083±0.002
CPA+ MESNA	0.078 ±0.009[b]	0.084±0.001[b]	0.082±0.002

All data are represented as mean ± SD. Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test
[a] $P<0.01$ significantly different from normal
[b] $P<0.01$ significantly different from that of CPA alone.

Table 7.4. Effect of *C. orchioides* treatment on bladder GSH levels (nmol/mg protein)

Group	4h	24h	48h
Normal			3.01±0.46
CPA alone	1.12±0.08[a]	1.25±0.05[a]	1.75±0.05[a]
CPA+CO	2.13±0.031[b]	2.50±0.36[b]	3.15±0.40[b]
CPA+ MESNA	3.28 ±0.51[b]	3.51±0.66[b]	3.66±0.71[b]

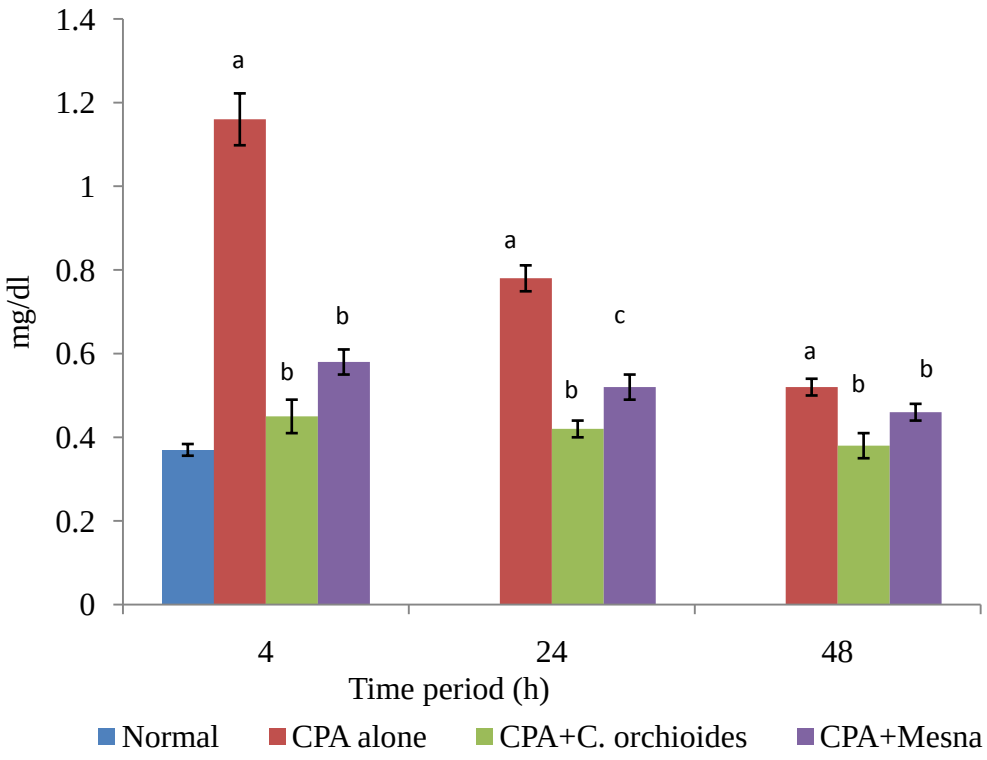
All data are represented as mean ± SD. Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test
[a] P<0.01 significantly different from normal
[b] P<0.01 significantly different from that of CPA alone

Table 7.5. Effect of *C. orchioides* treatment on Kidney GSH levels (nmol/mg protein)

Group	4h	24h	48h
Normal			4.21±0.67
CPA alone	1.18±0.09[a]	1.26±0.16[a]	1.92±0.25[c]
CPA+CO	2.89±0.33[b]	3.21±0.65 [b]	3.97±0.42 [b]
CPA+ MESNA	3.66 ±0.36[b]	3.86±0.48[b]	4.26±0.82[c]

All data are represented as mean ± SD. Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer test
[a] P<0.01 significantly different from normal
[b] P<0.01 significantly different from that of CPA alone
[c] P<0.001 significantly different from that of CPA alone

Figure 7.1. Effect of *C. orchioides* on serum creatinine level



Animals were sacrificed at different time points, blood was collected and serum was separated to estimate the creatinine level. The values are expressed as means ± SD. [a]P<0.01 significantly different from normal. [b]P<0.01 significantly differs from that of CPA alone, [c]P<0.05 significantly differs from that of CPA alone.

Figure 7.2.A. Effect of *C. orchioides* on BUN levels

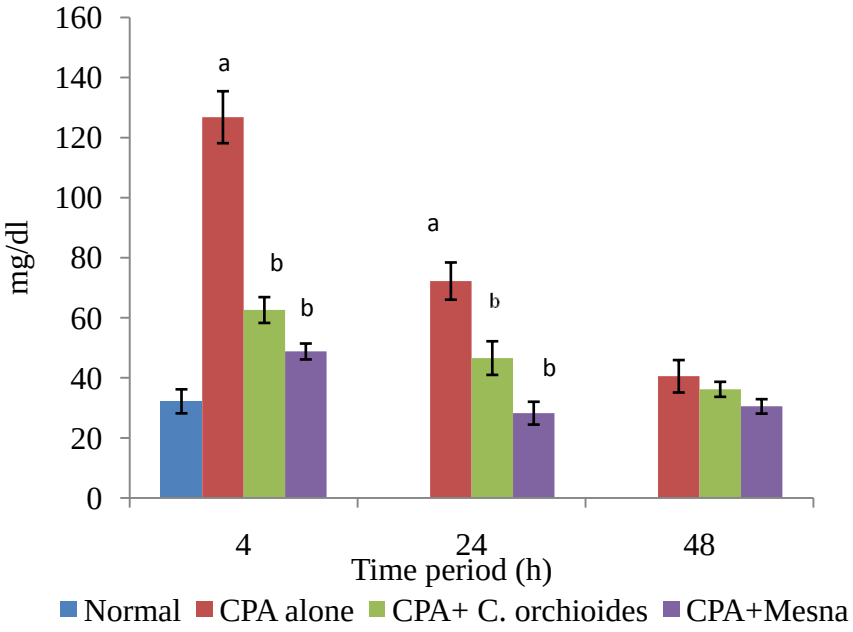
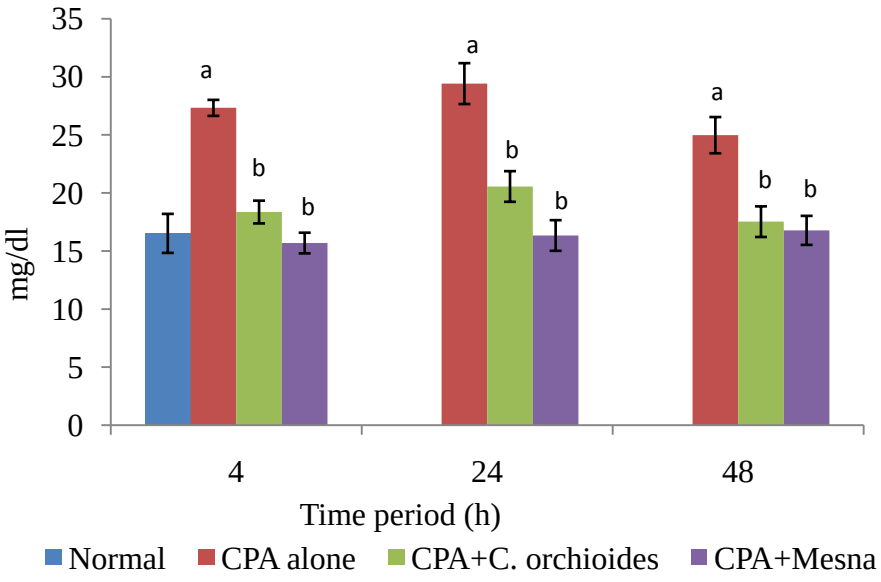


Figure 7.2.B. Effect of *C. orchioides* on Urine urea nitrogen levels



Animals were sacrificed at different time points to collect the blood and BUN was estimated by diacetylmonoxime method. Urine was collected in cooled chamber before killing the animals and used to estimate urine urea nitrogen levels. [a]P<0.01 significantly different from normal. [b]P<0.01 significantly differs from that of CPA alone

Figure 7.3. Effect of *C. orchioides* on kidney lipid peroxidation

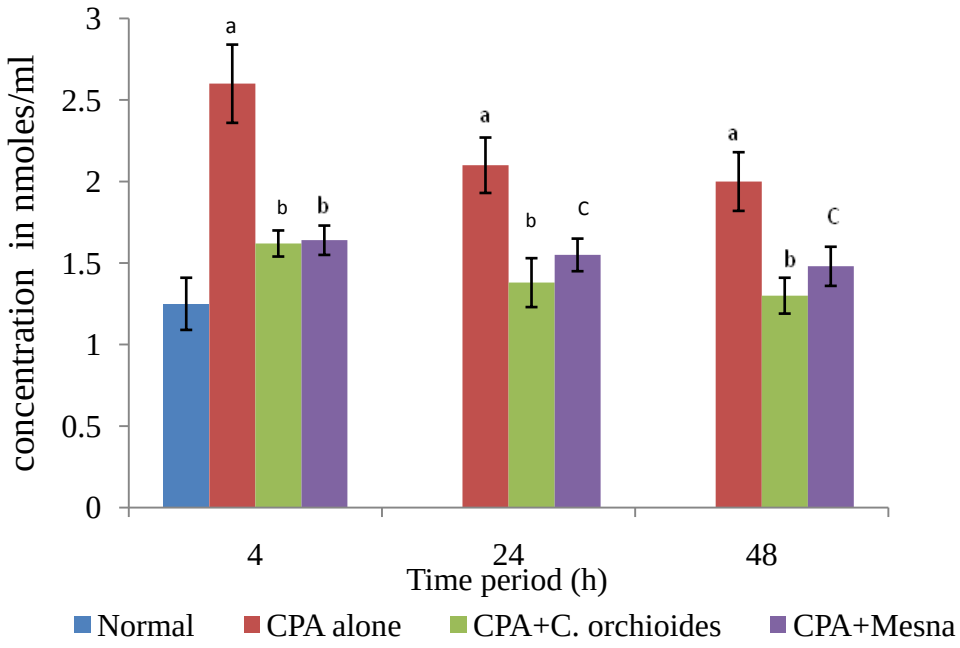
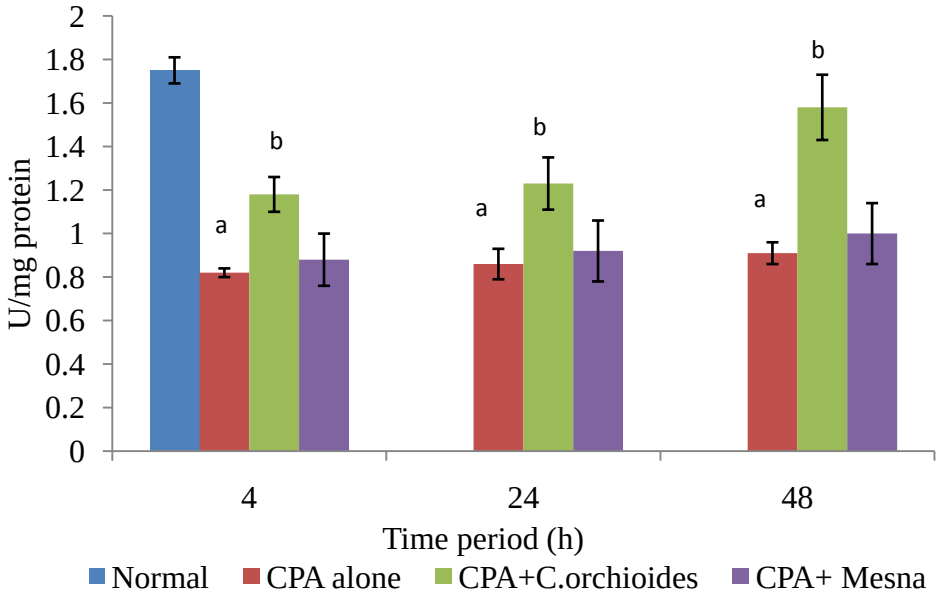


Figure 7.4. Effect of *C. orchioides* on kidney SOD



All the values are mean ± SD, [a]P<0.01 significantly different from normal. [b]P<0.01, significantly differs from that of CPA alone, [c]P<0.05, significantly differs from that of CPA alone.

Figure 7.5. Effect of *C. orchioides* on kidney catalase

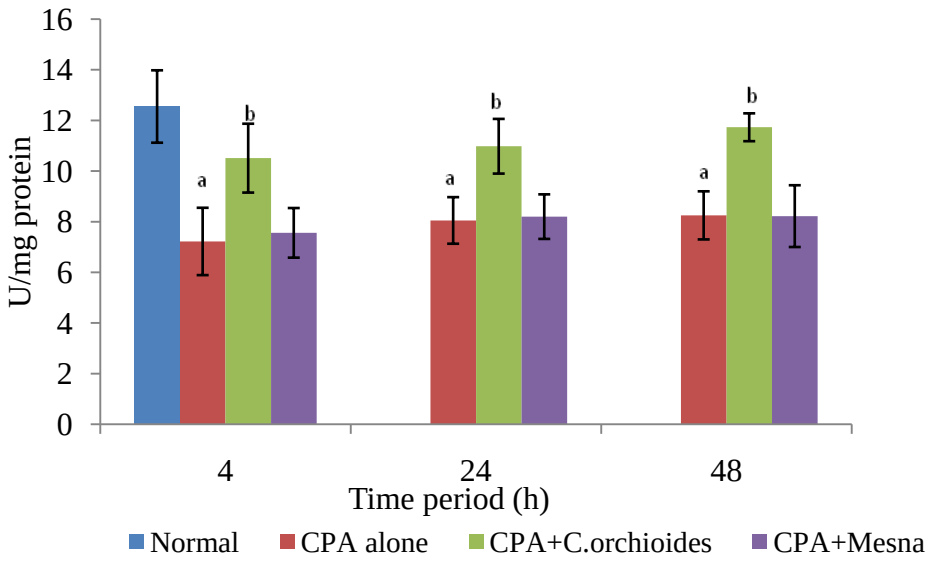
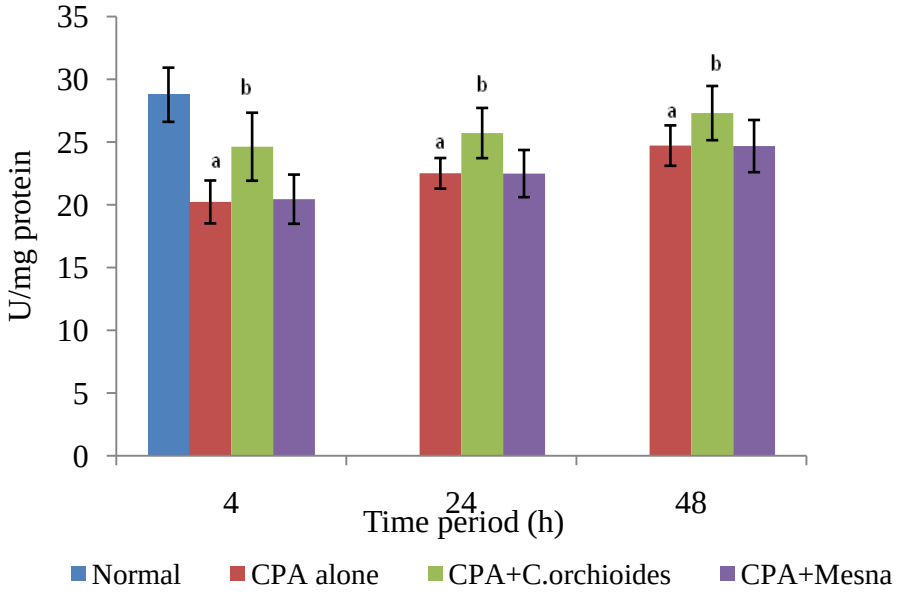
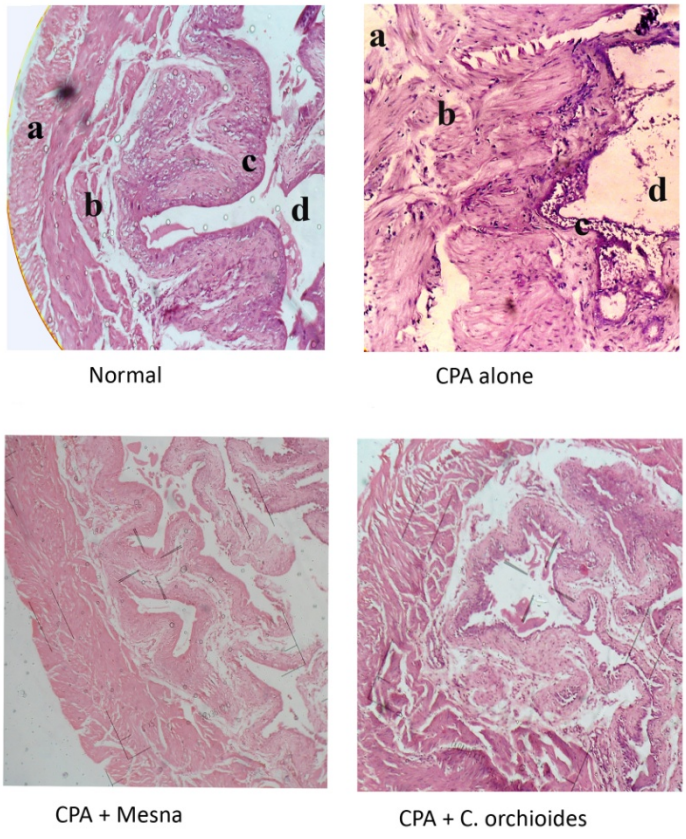


Figure 7.6. Effect of *C. orchioides* on kidney GPx



All the values are mean ± SD, [a]P<0.01 significantly different from normal.
[b]P<0.01 significantly differs from that of CPA alone.

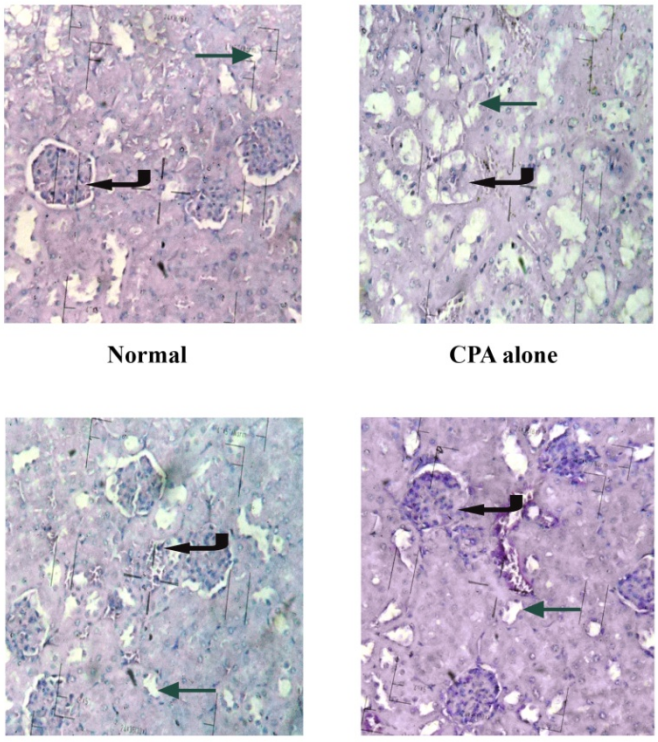
Figure 7.7. Histopathological analysis of the urinary bladder



Normal urinary bladder - normal bladder wall, normal fibrous tissue:a; normal muscularis:b; normal urothelium:c; lumen to collect urine:d

bCPA alone - Haemorrhage to fibrous tissue:a; damaged muscle layer:b denudation of bladder mucosa:c; lumen to collect urine

Figure 7.8. Histopathological analysis of kidney



Normal **CPA alone**

CPA + CO **CPA + Mesna**

Normal kidney - normal glomorulus: bended arrow; normal renal tubule: arrow
CPA alone - irregular Bowmans capsule: bended arrow; renal tubule damage and foci of necrosis: arrow
CPA+ C. orchioides - mild haemorrhage to renal tissues: arrow; normal Bowmans capsule: bended arrow
CPA+Mesna - normal Bowmans capsule: bended arrow and damaged renal tubule: arrow] taken out after 48 hrs. (X400).

Chapter 8

Summary and Conclusion

Curculigo orchioides is a well known Ayurvedic and Chinese medicinal plant reported to have a number of medicinal properties. During our studies we tried to unveil other beneficial properties like the effect on cell mediated immune system during tumour progression, ameliorative effects on the side effects of chemotherapy drug etc. and also to utilize the reported activities to fight against cancer progression.

Plant was collected and 70 % methanolic extract was prepared. According to the literature Curculigoside is the major bioactive component of the plant, thus the compound was purchased. Based on toxicity analysis doses of the extract and Curculigoside were determined for further studies.

The plant is reported to have humoral immune stimulatory effects, so we carried out a detailed study on the immunomodulatory activity of *C. orchioides* and Curculigoside and from these studies the stimulatory effect of the plant as well as Curculigoside on nonspecific, humoral and cell mediated immune system was publicized. Besides the enhancement of nonspecific immune responses, the plant extract and Curculigoside was also found to enhance the antibody production as well as the number of antibody producing B-lymphocytes. The plant itself was found to exhibit mitogenic potential on B and T cell proliferation, along with the stimulation of the mitogenic potentials of various B and T cell mitogens. One of the major findings from our study was the efficacy of the plant material to enhance the cytotoxic T cell response against EL-4 induced tumour development in experimental animals. Levels of IL-2 and IFN-γ that were decreased during tumour development were restored with the test material administration that might be helped to enhance the anti-tumour immunity.

A good immunomodulatory agent should not escort the formation of chronic inflammatory responses, since the pro-inflammatory mediators are reported enhance tumour development and progression. *C. orchioides* and Curculigoside was found to exhibit profound anti-inflammatory effects and these anti-inflammatory effects are brought about by down regulating the proinflammatory cytokines production by activated macrophages. The test materials were found to reduce the nitric oxide production by inhibiting iNOs gene expression, together

with this, the expression of COX-2, a major inflammatory mediator was also down regulated by the plant and Curculigoside.

The next objective of our study was to evaluate the effect of *C. orchioides* methanolic extract and Curculigoside on metastatic progression of B16F10 melanoma cells in C57BL/6 mice through its immune-stimulatory potential. It was found that the test materials are capable to reduce the metastatic lung tumour nodule formation with an increase in average lifespan of the metastasis bearing animals. The test materials were found to inhibit the production of MMP-2 and MMP-1 responsible for the degradation of Type IV collagen. Together with this the test materials also enhanced the expression of TIMP-1 and TIMP-2 the natural inhibitors of MMPs.

NK cells are the part of innate immune system and the major effector cells that drive anti-tumour immune responses. *C. orchioides* extract and Curculigoside enhanced the NK cell activity in metastasis bearing animals. Thus the anti-metastatic potential of the plant and Curculigoside may be attributed to the MMP inhibition, TIMP activation and increased cell mediated immune response brought about by them.

Another important finding was the ability of the plant to alleviate the toxic side effects of the conventional chemotherapeutic agent Cyclophosphamide (CPA), without hindering its anti-tumour efficacy. *C. orchioides* extract when administered along with CPA, they synergistically inhibited the DLA induced solid tumour development in experimental animals. Along with this, via its immune stimulatory potential the plant retained the total WBC count and bone marrow cellularity in CPA administered animals were the immune deprivation is the major drawback of CPA treatment. Besides this, the extract also ameliorated the intestinal and hepato-toxicities induced by the extract administration. Elevated TNF-α levels due to CPA administration was also brought down by *C. orchioides* treatment along with an increased levels of IL-2 and IFN-γ. Renal and bladder toxicities induced by CPA treatment were also alleviated by the extract treatment. Immune stimulatory, Anti-inflammatory and antioxidant potentials of the plant provides the basis of these protective effects.

The conclusions made from this study are listed below.

Conclusions

- *Curculigo orchioides* and Curculigoside were found to be least toxic to the experimental animals and also exhibited good stimulatory effects on the non-specific, humoral and cell mediated immune responses.
- *C. orchioides* and Curculigoside was found to exhibit good anti-inflammatory potential via down regulating the pro-inflammatory cytokine production by the activated macrophages.
- Even if both the plant extract and Curculigoside did not show any significant cytotoxic effect towards B16F10 melanoma cells, exhibited significant anti-metastatic effects in B16F10 melanoma cells induced *in vivo* metastatic model.
- The anti-metastatic effect of the plant was mainly attributed to its Natural Killer (NK) cell stimulatory activity and also due to its inhibitory effects on MMP production.
- Curculigoside was confirmed as the major bio-active component of the plant which is responsible for the above mentioned activities.
- The VEGF levels of the metastasis bearing animals were found to be un-effected with the administration of *C. orchioides* and Curculigoside.
- *C. orchioides* was found to be beneficial in preventing the multiple organ toxicities induced by Cyclophosphamide administration in experimental animals.
- The basement for all these effects was found to be the immune-stimulatory, anti-inflammatory and anti-oxidant effects of the plant.

Future perspectives of the study

- Further analysis has to be performed to find out the signalling pathways and molecular targets affected by *C. orchioides* and Curculigoside to unveil the mechanism of action.
- Drug to drug interactions of *C. orchioides* and cyclophosphamide has to be studied in detail.
- Use of the immune-stimulatory activity of Curculigoside in different aspects other than cancer.

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